Neurosurg Focus 29 (5):E1, 2010

Biochemical and neurochemical sequelae following mild traumatic brain injury: summary of experimental data and clinical implications

Stefano Signoretti, M.D., Ph.D.,[1] Roberto Vagnozzi, M.D.,[2] Barbara Tavazzi, Ph.D.,[3] and Giuseppe Lazzarino, Ph.D.[4]

[1]Division of Neurosurgery, Department of Neurosciences/Head and Neck Surgery, San Camillo Hospital; [2]Department of Neurosciences, University of Rome "Tor Vergata;" [3]Institute of Biochemistry and Clinical Biochemistry, Catholic University of Rome; and [4]Department of Chemical Sciences, Division of Biochemistry and Molecular Biology, University of Catania, Italy

Although numerous studies have been carried out to investigate the pathophysiology of mild traumatic brain injury (mTBI), there are still no standard criteria for the diagnosis and treatment of this peculiar condition. The dominant theory that diffuse axonal injury is the main neuropathological process behind mTBI is being revealed as weak at best or inconclusive, given the current literature and the fact that neuronal injury inherent to mTBI improves, with few lasting clinical sequelae in the vast majority of patients.

Clinical and experimental evidence suggests that such a course, rather than being due to cell death, is based on temporal neuronal dysfunction, the inevitable consequence of complex biochemical and neurochemical cascade mechanisms directly and immediately triggered by the traumatic insult.

This report is an attempt to summarize data from a long series of experiments conducted in the authors' laboratories and published during the past 12 years, together with an extensive analysis of the available literature, focused on understanding the biochemical damage produced by an mTBI.

The overall clinical implications, as well as the metabolic nature of the post-mTBI brain vulnerability, are discussed. Finally, the application of proton MR spectroscopy as a possible tool to monitor the full recovery of brain metabolic functions is emphasized. *(DOI: 10.3171/2010.9.FOCUS10183)*

Key Words • **mild traumatic brain injury** • **N-acetylaspartate** • **concussion** • **brain energy** • **metabolism** • **oxidative stress**

At present, TBI is a major public health concern and a leading cause of disability worldwide.[8] In European countries, the annual incidence of TBI is estimated at between 100[89] and 1967 per 100,000[90] persons, with mild and moderate TBI accounting for 80%–95% and severe TBI accounting for about 5%–20% of all cases. It has been calculated that the ratio of the occurrence of mTBI to that of sTBI is approximately 22:1.[74] In the US, 1.5–8 million people per year suffer from TBI ranging from mild to severe.[8] A proportion of these patients ranging from 75% to 90% is classified as mildly injured.

These wide ranges of annual incidence are probably due to the fact that an unknown proportion of mTBI victims do not seek any medical attention, but it might also be due to the fact that there is still confusion and inconsistency among researchers and organizations in defining and understanding this type of trauma.[15,23]

Mild TBI has, indeed, too many synonyms, including brain concussion, mild head injury, minor head injury, and minimal TBI.[41,49] Even the terms "head" and "brain" have been used interchangeably.

Historically, the most often used system for grading the severity of craniocerebral trauma is the Glasgow Coma Scale (GCS), which permits 120 possible mathematical combinations of eye, verbal, and motor scores, and contains rather crude scoring categories. We all know that different patients with the same GCS score of 15 may not function at the same level.

Given the limitations of the GCS, other parameters,

Abbreviations used in this paper: ADP = adenosine diphosphate; AMP = adenosine monophosphate; ATP = adenosine triphosphate; GCS = Glasgow Coma Scale; GDP = guanosine triphosphate; GMP = guanosine monophosphate; GTP = guanosine triphosphate; HPLC = high-performance liquid chromatography; mTBI = mild TBI; NAA = *N*-acetylaspartate; NAD^+ = nicotinamide adenine dinucleotide; NADH = reduced NAD^+; NADP = NAD^+ phosphate; NADPH = reduced $NADP^+$; RNS = reactive nitrogen species; ROS = reactive oxygen species; SIS = second impact syndrome; sTBI = severe TBI; TBI = traumatic brain injury.

like posttraumatic amnesia and loss of consciousness, have been increasingly scrutinized during the past 10 years. Loss of consciousness was no longer considered a necessary condition for the diagnosis of mTBI, and there was soon a general agreement among experts that the criterion of posttraumatic amnesia must be used with great care because it may be easily under- or overestimated. Therefore, there currently are no objective biological measures to determine the degree of severity of the neuropathology of this condition.

Conversely, the general view that mTBI is a very common entity but should not be considered a very serious injury, leading only to transient disturbances, is mainly supported by the absence of structural brain damage on traditional neuroimaging. If mTBI was as "mild" as we might think, it would be difficult to explain the actual complex management of these patients, which may involve various health care professionals, including family doctors, behavioral psychologists, clinical psychologists, neuropsychologists, neurologists, psychiatrists, neuroophthalmologists, neurosurgeons, physiatrists, nurses, occupational therapists, and physical therapists. Furthermore, long beyond the typical recovery interval of 1 week to 3 months, at least 15% of persons with a history of mTBI continue to see their family physician because of persistent problems.[2,7,31,34,37] Because of such enormous social impact, the number of literature reports addressing many different clinical aspects of mTBI has grown annually during the past 2 decades.

The problem is that to date, due to the formidable challenge of studying this type of cerebral damage in the laboratory, most of the reported experimental data have been obtained in more severe levels of injury, with very little information on the biochemical modifications occurring in mild injury as a function of time after impact. It appears clear that the biochemical and molecular processes triggered by mTBI, where hardly any discernible cell death occurs, are likely to be, at least in part, different from those present following severe injury.

This report is an attempt to recap the results of a series of previously published experiments produced during the past 12 years by a single group of investigators, all focused on understanding the pathophysiology of a "mild" injury to the brain, where apparently there is nothing "obviously" harmful. These findings have been further discussed in light of the most recent findings from other laboratories worldwide.

The "Perfect" Model and the Evidence of "Immediate" Biochemical Damage

For many years, research groups have attempted to provide a clinicopathological correlation to the existence of primary (mechanical) and secondary (delayed, nonmechanical) damage. Two main stages were identified in the development of posttraumatic sequelae: 1) primary damage, occurring at the time of injury, directly responsible for discrete, "focal" anatomical lesions, such as laceration, contusion, and intracranial hemorrhage, and for diffuse axonal injury; and 2) secondary damage, produced by complex processes, initiated at the moment of injury, but which do not present clinically for a period of hours to days. These mainly include damage due to ischemic phenomena, swelling, edema, and alteration of the brain's major endogenous neurochemical mechanisms.

Much of this core of knowledge was obtained by virtue of complicated animal models, such as fluid percussion,[18,51] cortical impact,[17] or the cryogenic focal brain injury model,[40] producing a focal brain contusion[14,47] very similar to those observed in clinical conditions. However, where the basis of these lesions could be determined with a good degree of certainty, it was more difficult to be confident about the nature of the diffuse damage in patients who can present with the widest range of neurological disturbances up to a coma but with minimal or no evidence of intracranial lesions.

A number of clinical and laboratory studies reported over the past 2 decades have now established that the principal mechanism of diffuse brain damage after trauma is due to acceleration/deceleration injury, resulting in unrestricted movements of the head and leading to shear, tensile, and compressive stress. Still, knowledge about changes in cell homeostasis after this type of injury was more difficult to obtain, mainly because of challenges related to modeling the exact circumstances in the laboratory.

In 1994 Marmarou and coworkers[26,48] set up a rodent "closed" head injury model characterized by pronounced diffuse brain damage, the severity of which was modulated by the impact force acting on the skull. Although brain injury was thoroughly characterized in many neuropathological aspects, at that time, there was virtually no information on the biochemical modifications, particularly in the mild injury model. Furthermore, it was not yet clear if trauma per se was responsible for such changes or if they were a consequence of the onset of ischemic-hypoxic phenomena successively occurring following head impact.

The initial efforts from these laboratories were focused on study of the time course of changes of several metabolites representative of the cell energy state (high-energy phosphates, nucleosides, oxypurines, nicotinic coenzymes) and of the occurrence of oxidative stress, a phenomenon defined as an overproduction of ROS from different intra- and extracellular sources[36,71] and leading to a decrease of antioxidant cell defenses with consequent irreversible modification of biologically important macromolecules.[53,91] Reactive oxygen species–mediated damage is mainly characterized by the onset of lipid peroxidation and revealed by measuring tissue malondialdehyde (MDA). For a better understanding of the phenomenon we evaluated the aforementioned parameters from 1 minute to 120 hours after the induction of mTBI both in spontaneously breathing and in mechanically assisted rats.[83]

It was surprising that, starting from 1 minute after trauma, the MDA level progressively increased up to the second hour, when its maximum concentration was recorded. Relatively high levels of this compound, originating from decomposition of peroxidized membrane phospholipids, still persisted at 24 and 48 hours after trauma. It is worth emphasizing that, as previously reported,[45,87] MDA is undetectable under normal conditions. Interestingly, oxidation of ascorbate (the main water-soluble brain antioxidant) paralleled MDA production, although

the minimum ascorbate value was observed 6 hours after trauma, showing a 60% decrease with respect to controls. Comparison of MDA and ascorbate concentrations of spontaneously breathing rats with those of mechanically ventilated rats did not show any statistically significant difference.

Similarly, with respect to values in controls, the levels of ATP and GTP were significantly reduced at 2 hours after cerebral injury, showing their lowest concentrations at 6 hours postinjury. Consequently, their dephosphorylation products showed an opposite trend, with the highest values of ADP, AMP, GDP, and GMP determined after 6 hours. Due to the imbalance between ATP production and ATP consumption, also inosine monophosphate (IMP), oxypurines (hypoxanthine, xanthine, and uric acid), and nucleosides (inosine and adenosine) were subjected to changes as a function of time. Particularly interesting were the 5- and 7-fold increases of xanthine and uric acid, respectively, thus suggesting the activation of xanthine oxidase, which is generally considered one of the major sources of superoxide anion production. Finally, nicotinic coenzymes (NAD^+ + NADH and $NADP^+$ + NADPH) were found to be profoundly affected in the time interval between the 2nd and 24th hours after trauma—that is, for a much longer time than that observed for other energy-linked metabolites. Comparison of all parameters related to energy metabolism of spontaneously breathing and mechanically ventilated animals did not show any statistically significant difference. Therefore, it could be affirmed that no ischemic or hypoxic episodes of any relevance occurred within the monitoring time in our mildly injured rats and that the force acting at the time of impact was directly responsible for triggering energy metabolism imbalance, ROS production, and, consequently, lipid peroxidation.

In this regard, Smith et al.[70] have reported that, in the model of unilateral cortical impact head injury, maximal amounts of hydroxyl radicals (which are the most oxidizing and reactive ROS) are produced within 5 minutes after induction of cerebral damage. They also reported a significant increase of phosphatidylcholine hydroperoxide at the same time point. Once the lipid peroxidation reaction chain is initiated, it propagates spontaneously, as indicated by the maximum level of MDA recorded 2 hours after impact, when no more radicals should be produced. The concomitant significant ascorbate depletion supported the existence of a remarkable oxidative stress following mTBI, which can be explained either with the direct oxidizing action of ROS on ascorbate, or with its utilization in the redox cycling of α-tocopherol (vitamin E), which represents the only membrane-bound lipid-soluble compound capable of breaking the lipid peroxidation reaction chain.[62]

The temporal difference between the onset of oxidative stress and the depression of energy metabolism, both occurring without cerebral conditions of ischemia or hypoxia, was indeed intriguing. In fact, it is highly probable that ROS-mediated lipid peroxidation involves not only the plasma membrane but also the mitochondrial membrane, thereby producing a dysfunction with inhibition of reactions linked to energy production. The slow recovery of the cellular energy metabolism could also be attributed to the drastic decrement of the nicotinic coenzyme pool that would certainly have jeopardized all the oxidoreductive reactions, including those related to the cell energy supply. Several other models of increased oxidative stress have demonstrated that the nicotinic coenzyme pool undergoes a significant depletion, although no valid explanation of this phenomenon had been given.[33,35,56,81] It was demonstrated that NADH and NADPH can be subject to direct ROS attack, which is responsible for the partial irreversible degradation of the coenzymes into ADP-ribose and ADP-ribosephosphate, respectively.[76,77] It could be hypothesized that such ROS-mediated nicotinic coenzyme degradation might be operative immediately after trauma. In rats subjected to this type of mTBI, however, most biochemical parameters returned to almost physiological levels within 1–5 days, thus confirming the "mild" severity level of this experimental model.

The overall evidence from these studies suggested that the traumatic insult, albeit mild, is directly responsible for sudden biochemical changes, triggered immediately, and for the subsequent depression of brain energy metabolism.

This hypothesis was more recently examined by Wu and colleagues[93] in a study concerning synaptic plasticity and cognitive function in the hippocampus following mTBI. The results showed that oxidative stress was clearly implicated in the dysfunction of energy metabolism, and the post-mTBI "energy crisis" compromised synaptic plasticity and cognitive function with subsequent synaptic and cognitive deficits.

N-Acetylaspartate: A Surrogate Marker of Neuronal "Health"

Two fundamental findings brought *N*-acetylaspartate (NAA) to the attention of neuroscientists in general, dramatically accelerating the pace of research into the neurochemistry and neurobiology of this unique molecule.[55] The first of these findings was that NAA is the most prominent compound detectable with proton MR spectroscopy in the human brain, making it one of the most reliable molecular markers for proton MR spectroscopy studies of the brain. The second was the connection to the rare but fatal hereditary genetic disorder known as Canavan disease.

Although the exact role of NAA remained to be established, brain NAA was found to be present in concentrations a hundred-fold higher than NAA in nonnervous system tissue, and it was therefore considered a brain-specific metabolite[54,82] and an in vivo marker of neuron density. Unfortunately, spectroscopic studies have dramatically outnumbered studies into the basic biochemistry of NAA, and this disparity has complicated the interpretation of proton MR spectroscopy results in various diseases due to a lack of basic knowledge of NAA function and metabolism. A decrease in NAA levels has been observed in many neurological diseases that cause neuronal and axonal degeneration such as tumors, epilepsy, dementia, stroke, hypoxia, multiple sclerosis, and many leukoencephalopathies. More generally, any major CNS disease involving either direct neuronal and axonal damage or secondary hypoxic-ischemic or toxic insult will result in abnormalities in the proton spectrum.

The objective of our further research was then to investigate the time course of NAA changes, always starting 1 minute postinjury and continuing for up to 5 days in varying grades of diffuse TBI to verify the hypothesis that reduction of NAA levels was proportional to injury severity, as had been proposed by Garnett et al.[29] in 2000.

By measuring whole-brain NAA via HPLC[79] in 3 different levels of trauma, we demonstrated for the first time that, at 48 hours postinjury, NAA reduction was graded according to the severity of insult, showing spontaneous recovery with lower levels of trauma and irreversible decrease in the others.[69] These data were strongly consistent with previous histological characterization of this type of TBI model.[26] The findings were also consistent with long-term behavioral observation in animals injured using the same model, showing only slight differences from sham-injured animals, with the main differences being present at 1 day postinjury and consistent improvement occurring over time.[6] All these bench observations strongly supported the clinical observations with proton MR spectroscopy with respect to a potential role of NAA in quantifying neuronal damage[12] and predicting neuropsychological outcome after TBI.[27,28]

In the reported study, however, we measured whole-brain NAA using HPLC; thus, especially at the latest time points, it was difficult to delineate whether an absolute percentage reduction represented a uniform reduction within dysfunctional cells or a reduction caused by neuron depletion with normal residual cells. Our finding of recovery in the mildly injured animals implied that at least one process leading to NAA reduction was reversible and not simply due to cell death. Once again, the striking finding was the rapidity of the onset of significant NAA reduction, already detectable after only 1 minute in severe injury, and the spontaneous recovery in mild injury, with a maximum drop observed at 15 hours postinjury, when NAA levels reached 46% of the control value.

The analysis of the temporal course of NAA levels also showed clear differences between the various degrees of TBI, demonstrating that different levels of "physical" injury correlated with different levels and kinetics of "biochemical" damage, reversible in mTBI and irreversible in sTBI. Notwithstanding such robust data and the experimental evidence of a biochemical marker of neuronal distress, a plausible explanation for this clear phenomenon remained controversial.

Energy Metabolism and NAA Synthesis

At that time, the most important link in NAA research worldwide as well as our research project was the initial work by Patel and Clark published in 1979,[63] showing a relationship between NAA synthesis and energy metabolism. The authors found that brain-derived mitochondrial preparations were clearly distinct from those derived from other tissues in their ability to synthesize large amounts of NAA, a phenomenon not detectable with nonneuronal mitochondria. In the same context, Patel and Clark also demonstrated the synthesis and efflux of NAA from mitochondria incubated in the presence of glutamate, malate, and pyruvate. An important finding of this study was that without pyruvate as a source of acetyl-CoA, no NAA efflux was detected.

These data, along with the initial intuition by Tallan in 1957,[75] that in mammals the distribution pattern of NAA closely paralleled the distribution of "respiratory activity," was confirmed by other investigators, but it took many years until a close linear relationship between ATP synthesis and the ability to synthesize NAA was described for the first time.[5] This study showed a biochemical "coupling" between NAA synthesis and energy production in brain mitochondria, a notion soon substantiated by additional reports describing decreases in NAA in a number of conditions of impaired brain energy metabolism in the brain. (For an overview of the data supporting a bioenergetic role for NAA in neurons see Moffet et al. 2007.[55])

In the same set of experiments,[69] together with the measurement of whole-brain NAA concentration, we then assessed ATP concentration and studied its trend course. It was quite surprising to observe that ATP changes literally mirrored the NAA changes. In mTBI, a gradual reduction of ATP levels started within minutes, reaching statistical significance at 2 hours, at which point a net reduction of almost 40% was recorded (relative to the values in controls). The lowest ATP value was found at 6 hours, showing a decrease of 57%. From that time point, there was a spontaneous gradual restoration, completed within 5 days postinjury, after which there was no statistically significant difference between ATP levels in the injured animals and those in control animals.

In contrast to NAA, which was found to decrease comparably in all 3 grades of injury severity during the early phase after trauma and to only show significant intergroup differences beginning at 48 hours after injury, ATP levels showed significant differences at earlier time points, suggesting that the underlying energetic derangement was something like a "preliminary step" necessary for observing NAA decrement.

Although ATP measurement assessed by ^{31}P-MR spectroscopy did not detect posttraumatic fall, several reports documented ATP reduction following TBI always related to mitochondrial dysfunction. Sullivan et al.[72] reported a significant time-dependent alteration in synaptosomal mitochondria, describing an immediate ATP reduction within 10 minutes following cortical contusion injury. Ahmed at al.[1] reported altered mitochondrial membrane potential and a 22%–28% cellular ATP reduction in mixed neuronal and glial cultures that had undergone stretch-induced injury, starting 15 minutes after trauma.

However, although the overall weight of evidence would favor a link between NAA synthesis in neuronal mitochondria and energy metabolism, studies have so far failed to make a direct connection between the synthesis of NAA and that of ATP. Certainly, the relationship between these 2 molecules is more indirect, since NAA synthesis is an energy-requiring process dependent on the availability and the energy of hydrolysis of acetyl-CoA. When acetyl-CoA is used for NAA synthesis instead of entering the citric acid cycle, there is a high energy cost to the cell because that molecule is no longer available to produce reducing equivalents (3 NADH and 1 $FADH_2$) as the fuel for the electron transport chain and subsequent

synthesis of 11 ATP molecules. Only when the ATP deficiency is fully restored does acetyl-CoA become available again to be shifted to the NAA synthesis pathway. Thus, a low NAA concentration can be seen as an indirect marker of metabolic energetic impairment.

A criticism of this interpretation could arise from the observation that ATP reduction might represent a simple metabolic mismatch between energy demand and supply. It is very important then to consider that these metabolic alterations occurred in a trauma model characterized by the constant presence of adequate cerebral blood flow. The marked posttraumatic increase in lactate/glucose ratio and reduced oxygen consumption (VO_2) recently described at 4–6 hours postinjury, with preserved cerebral blood flow,[46] rules out the ischemic etiology and suggests an increase in anaerobic glycolysis for the need to restore ATP, confirming that the mitochondria are dysfunctional.

To better address this issue we performed a supplemental study in which we compared the effects produced by 10 minutes of hypoxia and hypotension, both alone and coupled with traumatic insult.[78] A simultaneous ion-pairing HPLC analysis of MDA, ascorbate, oxypurines (hypoxanthine, xanthine, and uric acid), nucleosides (inosine and adenosine), nicotinic coenzymes, and high-energy phosphates (ATP, ADP, AMP)—these last compounds useful to calculate the energy charge potential (ECP) (ECP = ATP + [1/2 ADP]/[ATP + ADP + AMP)[44]—was performed, showing again a proportional decline with the severity of brain insult. More interestingly, rats subjected to hypoxia and hypotension had minimal ECP values 15 hours after trauma, showing a progressive recovery thereafter; at 120 hours after injury, values were not significantly different from those in sham-injured animals. These findings were of particular interest because the time course resembled that of mTBI. Indeed the entire biochemical derangement of animals subjected to hypoxia and hypotension was similar to that seen in mTBI, simply indicating a reversible type of damage that spontaneously recovers with almost the same kinetics. In contrast, when hypoxia and hypotension were used together with an mTBI model, the metabolic consequences appeared irreversibly catastrophic.

Another important consideration was the net diminution of the nicotinic coenzyme pool (NAD^+ + NADH and $NADP^+$ + NADPH), which clearly plays a pivotal role in the final result of general depression of cell energy metabolism. This depletion jeopardizes either the reducing equivalent supply to mitochondrial oxidative metabolism, or the catalytic activity of dehydrogenase-mediated oxidoreductive reactions. To date, possible mechanisms for this phenomenon are the hydroxyl radical–induced hydrolysis of the N-glycosidic bond of the reduced forms of NAD and NADP and the activation of the enzyme NAD-glycohydrolase.[43] Both mechanisms cause the hydrolysis of nicotinic coenzymes and give rise to the same end products—ADP-ribose(P) and nicotinamide. Independent of the predominant mechanism, the final result is certainly deleterious for the correct functioning of cell metabolism. Finally, the augmentation of poly-ADP ribosylation reactions through the activation of the enzyme poly-ADP ribose polymerase[22,58,61] has been demonstrated to trigger the mechanisms of apoptotic induction.[95]

The fact that NAA, ATP, MDA, ascorbate, oxipurines, nucleosides, and nicotinic coenzymes spontaneously recovered in mTBI and after hypoxia and hypotension alone seemed to suggest that there is a threshold for mitochondrial dysfunction beyond which restoration of neurochemical physiology is prevented.

Mild TBI and the Hypothesis of Postconcussive Brain Vulnerability

With the exception of the reversibility of the modifications induced by mTBI, it was not clear to us what was "mild" about a traumatic event that can have such consequences to the fundamental metabolic and energy states of neuronal cells.

The legitimate and natural objection to this "gloomy" view is that, in spite of everything, animals and patients with mTBI show no or minimal focal neurological problems and all show a radiologically normal brain. In other words, all these biochemical modifications are rather interesting but clinically of negligible utility just because they are all spontaneously and fully reversible.

There was, however, an initial reasonable body of evidence suggesting that the "concussed" brain cells undergo a peculiar state of "vulnerability" for an undefined period of time, during which if they sustain a second, typically nonlethal insult, they suffer irreversible damage and die.[32]

Fascinated by this original hypothesis from Hovda and colleagues at the University of California, Los Angeles, we undertook, as a next step in our research, an analysis of the neurochemical and metabolic effects produced by 2 consecutive concussive mTBIs, the second injury occurring at different intervals from the first, to investigate how the temporal gap between traumatic events could influence the overall severity of injury. We used the same impact acceleration model and applied a new and easily reproducible protocol to simulate a "second impact" condition in rats.[85] Neuronal injury was quantified by HLPC measure of whole-brain NAA concentration with the synchronous assay of whole-brain ATP and ADP concentrations and consequent ATP-to-ADP ratio (an indirect indication of mitochondrial phosphorylating capacity). Animals were randomly assigned to one of the following experimental groups: sham-injured; single mTBI; single sTBI; 3-day interval "double mTBI"; 5-day interval "double mTBI."

We were astonished by the observation of an identical 10% mortality rate in animals subjected to sTBI and animals doubly injured by mTBI with a 3-day trauma interval, whereas no animals died when subjected to a single mTBI or double-impact mTBI with a 5-day interval.

After the second mTBI, delivered 5 days after the first, NAA decreased by 17%, a reduction not significantly different from that observed following a single mTBI. When the second trauma occurred after 3 days, however, the NAA revealed a further 43% reduction when compared with the 5-day interval, a value not statistically different from the dramatic reduction observed in severe injury, in which NAA decreased by 47% versus controls and by a

further 37% versus rats subjected to a single mTBI. These findings were interesting because, at least according to the experimental model adopted, 2 consecutive mTBIs occurring within the shortest interval of time considered for the study (3 days) produced the same biochemical damage observed after sTBI. In particular, neuronal distress, indicated by reduction of NAA levels, doubled when compared with that observed after a single mTBI.

Energetic metabolites showed a very similar trend. In single mTBI, the levels of ATP and ADP and the ATP-to-ADP ratio varied by a value not significantly different from animals in which the second trauma was delivered 5 days after the initial one. In contrast, animals with a second mild injury 3 days after the first exhibited severe energetic imbalance, showing a 50% drop in ATP levels and very low (–70%) ATP-to-ADP ratio, reductions almost identical to those seen after sTBI.

These data provided the experimental demonstration of the exquisitely metabolic nature of the "brain vulnerability" after mTBI, and offered a contribution to the understanding of the complex biochemical damage underlying the clinical scenario of a repeated concussive trauma, sometimes leading to catastrophic brain injury. Most importantly, it was evident that when the second injury took place after a longer interval, recovery of the energetic imbalance was completed and the 2 traumatic insults acted as independent events, the additional injury simply representing a new "mild" event.

Similar data were reported just a few months before these findings by Laurer et al.[42] in a study describing important cumulative effects of 2 episodes of mTBI (24 hours apart) in mice, which led to pronounced histopathological damage compared with animals sustaining only a single trauma. The authors' conclusion was that although the brain was not morphologically damaged after a single concussive insult, its vulnerability to a second concussive impact was dangerously increased.

According to Hovda and colleagues, metabolic alterations can persist for days after concussion, creating no morphological damage, but representing the pathological basis of the brain's vulnerability.[19,30,94] In our study, after a single mTBI, we found a 22% reduction in ATP levels; although neither histological nor behavioral abnormalities have been described with this model, these data confirm that energy recovery was incomplete. The ADP levels did not increase because, despite significant ATP depletion, mitochondria were not yet irreversibly damaged, possessing a sufficient phosphorylating capacity to allow spontaneous full restoration of ATP levels, which was complete after approximately 5 days. A profoundly different situation was observed in sTBI, with a 50% decrease in ATP levels and 35% increase in ADP levels, indicating altered capacity of mitochondria to support the cell energy requirements in terms of ATP synthesis.

When the second mTBI was delivered during the aforementioned restoration period, it caused further mitochondrial malfunctioning leading to the same energetic failure observed in severe injury. It could be concluded that 2 mTBIs that occur too close in temporal proximity simulate the effects of a severe injury, and that one of the biochemical bases of the vulnerable brain is the incomplete overcoming of the initial reversible energetic crisis, triggered by the first insult.

The first striking clinical implication of these experimental data was that the metabolic effects of 2 consecutive concussions occurring within a period of days can be dangerously additive. This information might not be surprising, but similar human data regarding brain metabolites were not available.

Then, the second clinical implication of this notion was again remarkable since it is very difficult to establish how long the above-described period of brain vulnerability will last and the occurrence of a second trauma would be uneventful.

Brain Vulnerability

The high correlation demonstrated between energy metabolism and the ability to synthesize NAA seemed to suggest that the decrease of NAA after mTBI might be considered a reflection of dysfunction in cells that had impaired energy metabolism but were not yet irreversibly damaged. However, a more definite picture of the effect of the time interval between 2 mTBIs, as well as more robust evidence of whether the mitochondrial dysfunction observed was partially or totally reversible, was still lacking. Likewise, the kinetics of the aforementioned period of brain vulnerability were still unclear.

Our next study entailed an extensive screen of markers of mitochondrial-related functions obtained in animals subjected to 2 mTBIs at intervals of 1, 2, 3, 4, or 5 days.[88] Besides measuring NAA values, the levels of adenine nucleotides, oxypurines, nucleosides, free-CoA-SH, acetyl-CoA, malonyl-CoA, propionyl-CoA, and oxidized and reduced nicotinic coenzymes were also determined. Ultimately, the concentrations of the NAA-related compounds, like N-acetylglutamate (NAG) and N-acetylaspartatylglutamate (NAAG), the most concentrated neuropeptide in the human brain, as well as the expression of N-acetylaspartoacylase (ASPA), the enzyme responsible for NAA hydrolysis into aspartate and acetate mainly within oligodendrocytes, were determined.

Overall, the results indicated that a broad interrelated series of metabolites were deeply affected by a second mTBI if it occurred within the brain vulnerability window. The majority of these compounds have the common feature of being related to the mitochondrial activity devoted to the cell energy supply. Levels of NAA, adenine nucleotides, acetyl-CoA, and oxidized nicotinic coenzymes all showed the same pattern of variation with time between impacts, and all showed values similar to controls when the second concussion was delivered 5 days after the first. Of particular note were the variations of acetyl-CoA levels in view of its dual role as a fundamental compound for the reducing equivalent supply in the Krebs cycle activity and as the acetyl group donor in the NAA biosynthetic reaction. This was the first report showing that cerebral acetyl-CoA concentration was remarkably decreased after repeat mTBI. The diminished availability of this compound has a negative consequence on the continuous flow of NADH necessary for the electron transport chain, thus playing an important role in the deeply decreased mito-

chondrial phosphorylating capacity (decrease of the ATP/ADP ratio) and leading to a profound drop in ATP concentration as observed in rats reinjured after 3 days. The almost 50% decrease in NADH recorded in these animals is consistent with this hypothesis and corroborates the concept of extensive involvement of the tricarboxylic acid cycle in the energy state impairment.

A slight but statistically significant decrease in the NAD^+/NADH ratio was observed only when the second mTBI was delivered after 3 days (–20% with respect to controls). Such a phenomenon appears to be mainly attributable to the dramatic decrease in NAD^+ levels. Because brain metabolism is primarily based on glucose utilization, the main site of acetyl-CoA cerebral generation is at the pyruvate dehydrogenase level. According to previous results, pyruvate dehydrogenase activity is markedly inhibited by increased oxidative stress, a pathological condition common in TBI. On the other hand, in the reaction catalyzed by aspartate N-acetyltransferase, the enzyme responsible for NAA biosynthesis, low acetyl-CoA levels should certainly reduce the velocity of NAA production and contribute to the NAA depletion observed after repeat mTBI when the second concussion was delivered 3 days after the first. Therefore, acetyl-CoA availability might represent the phenomenon effectively linking the parallel NAA and ATP changes.

Results of the analysis of mRNA transcript of the *ASPA* gene revealed that there was an effect of the time interval between concussions on *ASPA* gene expression. A progressive increase in the mRNA transcript of the *ASPA* gene was observed, with a maximum 4-fold increase of ASPA expression in animals injured again after 3 days. Animals reinjured past 5 days had values of mRNA for ASPA comparable to those recorded in controls.

From these data it appears that TBI-induced NAA variations may not be simply attributable to a decreased rate of biosynthesis.

In accordance with the knowledge of different compartmentation for NAA biosynthesis (neuronal mitochondria) and degradation (oligodendrocytes), it could be hypothesized that TBI-induced NAA decrease occurs in 2 distinct phases and with 2 different mechanisms. Initially, independently from the severity of injury, a change in mitochondrial permeability[25] causes an increased velocity of NAA outflow from neurons to the extracellular space. Simultaneously, mitochondrial impairment leads the cell to use energy for more "urgent" requirements and, therefore, leads to diminished NAA synthesis. In the case of reversible brain damage such as single mTBIs or repeat mTBIs in which the second impact occurs outside the brain vulnerability "window," recovery of mitochondrial functions takes place with normalization of the rate of NAA efflux and biosynthesis (NAA levels close to controls and no increase in ASPA expression).

In single sTBIs or in repeat mTBIs in which the second impact occurs within the brain vulnerability window, higher amounts of NAA than normal continuously reach oligodendrocytes, which, as an adaptive mechanism, increase the expression of ASPA. This phenomenon, combined with the decreased rate of NAA biosynthesis caused by persistent mitochondrial impairment, is ultimately responsible for the dramatic NAA depletion. Beyond the specific interest in TBI studies, this finding gives an insight into the possible mechanisms of NAA homeostasis, strongly suggesting that NAA concentration within oligodendrocytes regulates the gene expression of ASPA and, in turn, the velocity of its own degradation.

These results were immediately followed by another collaboration study on transcriptomics in which the simultaneous expression of about 30,000 rat genes, whose products are involved in a variety of cellular processes, were studied.[16] Using complementary DNA microarray technology, we reported that following stretch injury to hippocampal slice cultures (as a suitable cell model to induce graded TBI), the expression of 999 genes was altered in mTBI, compared with controls.

The altered genes in mTBI-stretched cells clustered in the "Biological Process" group, which had been shown to be involved in the structural damage of cellular architecture. Most of these genes are involved in signal transducer activity, regulation of transcription, and cell communication. This indicated that even after a mild stretch injury (comparable to a closed-head diffuse mTBI), intense activity involving transcription and signaling exchange is initiated. Additionally, we have found that certain genes involved in the apoptotic process, such as *Vdac1 (voltage-dependent anion-selective channel protein 1), Sh3glb1 (SH3-domain GRB2-like endophilin B1), Phlda1 (leckstrin homology-like domain, family A, member 1), Rock1 (Rho-associated coiled-coil containing protein kinase 1),* and *Eif4g2-predicted (eukaryotic translation initiation factor 4 gamma, 2)* were downregulated. Further, an upregulation was seen in genes involved in the antiapoptotic process, such as *Ccl2 (chemokine [C-C motif] ligand 2), Vegfa (vascular endothelial growth factor A), BIRC3 (baculoviral IAP repeat-containing 3), Tsc22d3 (TSC22 domain family, member 3), Bnip3 (BCL2/adenovirus E1B 19-kD interacting protein 3),* and *Nr4a1 (nuclear receptor subfamily 4, group A, member 1).* The majority of these expression changes were only found following mild stretch injury, indicating that these hippocampal cell cultures have activated protective and repair mechanisms.

The most interesting finding was that more genes were differentially expressed following mild brain injury than following severe injury, further supporting the notion that even following mTBI, in which an absence of radiological and clinical abnormalities is the norm, an invisible complex cellular response is initiated and distinct neuronal dysfunction occurs. This corroborates previous findings that these are "primary" cellular effects not determined by local blood or oxygen delivery or by systemic factors.

Two Often-Neglected Phenomena: Oxidative and Nitrosative Stresses

In the reported works, data showed for the first time that NAAG was also markedly affected by repeat mTBIs. In contrast to the situation with NAA, several biological activities were clearly demonstrated for NAAG either under physiological[92] or pathological conditions.[4,59] With regard to head injury, the beneficial effects connected

to the inhibition of glutamate carboxypeptidase II, also known as N-acetylated α-linked acidic dipeptidase, are of particular interest in models of neuropathies, stroke, and focal TBI.[57,96] Glutamate carboxypeptidase II, which catalyzes the hydrolysis of NAAG to glutamate and NAA, is activated under these pathological conditions, leading to an increase in glutamate release. The NAAG decrease observed in our experiments might contribute to perpetuating glutamate generation. High levels of glutamate are believed to be one of the major contributors to the excessive amounts of intracellular calcium, which is another fundamental mechanism of mitochondrial functional and morphological alteration. An essential point of posttraumatic calcium overloading is the mitochondrial damage due to induced changes of the organelle's membrane permeability, the uncoupling of oxidative phosphorylation, and organelle swelling.[66,97]

As suggested by experiments in which the mitochondrial capacity to catalyze the tetravalent reduction of molecular oxygen through the electron transport chain appears compromised, dysfunctional mitochondria seem to be the main intracellular source of ROS production.[58,60,61,73]

However, in conjunction with oxidative stress, we also studied the importance of another event occurring as a consequence of TBI, a phenomenon known as nitrosative stress. Only more recently the object of substantial investigations, nitrosative stress is defined as an overproduction of reactive nitrogen species (RNS) through the Ca^{2+}-dependent activation of neuronal nitric oxide (NO) synthase and of the increased synthesis of the inducible form of NO synthase, generally taking place in concomitance with oxidative stress.[13] Nitric oxide generation, resulting from elevated neuronal NO synthase and the inducible form of NO synthase activities, can either induce an increase of nitrosylation reaction of various hydroxyl- or sulfhydryl-containing biomolecules (tyrosine, serine, or cysteine residues of proteins, glutathione, and so forth) or react with ROS, giving rise to secondary, highly reactive radicals such as peroxynitrite.[20,21,68]

At this point of the project, it seemed relevant to us to further investigate whether, during the aforementioned window of brain vulnerability, oxidative and nitrosative stresses contributed to the metabolic damage in cerebral tissue following concussion. By assessing different biomarkers reflecting ROS-mediated oxidative stress (MDA, GSH, oxidized glutathione [GSSG], ascorbic acid) as well as indices representative of NO-mediated nitrosative stress (nitrite and nitrate), we studied the effects of repeat mTBI delivered with increasing time intervals.[80]

Consistently, we found that MDA and ascorbic acid in animals in which repeated mTBIs were spaced by 3 and 5 days had values similar to those observed after single sTBI and after single mTBI, respectively. Intermediate time intervals between concussions produced intermediate intensities of oxidative stress. However, much more important was the reported parallelism between oxidative and nitrosative stresses observed in our experiment, strongly reinforcing the concept that the time interval between 2 concussions is a critical factor for transforming the biochemical effects of an mTBI, which are fully reversible, into a metabolic picture of sTBI.

Our data indicated that compounds reflecting NO generation by activated neuronal NO synthase and an induced inducible form of NO synthase (nitrite and nitrate) had maximal concentrations when mTBIs were delivered 3 days apart and minimal concentrations when mTBIs were spaced by 5 days. Animals subjected to mTBIs with the latter interval had approximately normal levels of both compounds, thus confirming that, in this case, mTBIs can be considered as independent events with no cumulative effects. It should be remembered that the concomitant presence of oxidative and nitrosative stresses is highly hazardous because of the risk of generating highly reactive RNS such as peroxynitrite and nitroxyl.

Both these RNSs have damaging effects on biomolecules and greatly contribute to deplete tissue antioxidant defenses.[65] In particular, GSH is a selected target of either peroxynitrite, which transforms GSH into nitrosoglutathione,[38,67] or nitroxyl, which reacts with GSH to give rise to the amidated GSH derivative sulfonamide.[20,21] Therefore, it can reasonably be inferred that in our experiment, the production of RNS through the simultaneous occurrence of oxidative and nitrosative stresses played a significant role in causing the dramatic GSH depletion recorded after repeat mTBIs spaced by 1, 2, 3, or 4 days. Furthermore, our results suggested that, because peroxynitrite is actively scavenged by ascorbic acid,[3,39] this RNS may also have an active role in the depletion of ascorbic acid observed.

The data from this study, as well as those from our previous work on repeat mTBIs, offered again a clear picture of the existence of a temporal window of brain vulnerability, demonstrating that a number of linked molecular events concur to provoke severe biochemical brain damage if a second concussion falls within such a temporal window.

Clinical Implications

Managing mTBI: Does Proton MR Spectroscopy Have a Role?

Since in Western countries a head trauma occurs approximately every 15 seconds, it appears improbable that an emergency physician will not encounter a patient with an mTBI during an emergency department shift.

The fundamental issue of whether to observe, discharge, or obtain imaging studies in each one of these patients is addressed continuously and it is focused essentially on identifying those patients at risk for hemorrhagic complications that would threaten their lives. In fact, dramatic tales regarding patients who "talk and deteriorate," die, or end up with severe permanent disability after a "simple blow to the head" abound; yet such events are very uncommon and frequently are based on no more solid literature than case reports. As a matter of fact, a patient presenting with a GCS score of 15 with no associated risk factor has a chance of intracranial hemorrhage that will require neurosurgical intervention of less than 0.1%.[24]

The real dilemma about mTBI has a completely different perspective. How many individuals sustaining mTBI remain medically unattended and how many of those who present to emergency departments will receive specific advice regarding the possible sequelae of what has just hap-

pened to them? How much is the whole picture confounded by conditions related to age, gender, race, ethnicity, and socioeconomic status? What is the proportion of concussions that are already repeat injuries, so that what the emergency physician is managing is actually a "double mTBI"? How many times are patients asked if they have sustained a prior head injury a few days before? Would the discharge instructions be different in such cases, or should the patient be discharged at all? How much is the risk for subsequent TBIs increased during the week after a TBI? How many times are patients discharged with the strong recommendation to absolutely avoid, for a yet-to-be-defined period, any situation that would raise the chance of suffering another concussion (driving scooters, engaging in sports)? What about infants, toddlers, and other children?

Basic science data collected from the reported bench studies has clarified some aspects of this particular clinical entity, suggesting that, after all, mTBIs are not always as "mild" as the name would suggest, and short-term as well as long-term consequences may very well be overcome simply by understanding the metabolic conditions of the injured brain cells. Data reported in this summary strongly suggest that measuring NAA after an initial concussion and monitoring it until normalization might represent a significant step in quantifying the objective nature of the postconcussive metabolic disturbances. Due to its high concentration within neurons (~ 10 mmol/L brain water), NAA levels are easily demonstrated by proton MR spectroscopy. This technique is based on the ability to localize the MR signal into a specific volume of tissue, thus providing a real-time "image" of the brain neurochemistry.

As recently emphasized,[9] it is profoundly necessary to "biologically" grade the "severity" of mTBI, since absence of clinical signs and symptoms often does not coincide with full cerebral metabolic recovery. In selected cases, monitoring NAA via proton MR spectroscopy until complete normalization might represent a strategy to confirm the total metabolic recovery commonly observed after concussion and to avoid a second mTBI soon afterward that could lead to a more severe injury.

Sports-Related Concussion

The outlined experimental results have implications not only for the clinical sequelae associated with postconcussive syndromes, but also support the current concerns regarding athletes in terms of when they should return to play after sustaining a concussion on the field. As we know, athletes often sustain repetitive head impacts, frequently during a short time course consistent with the timing used in the reported studies.

Clearly, any translation of rodent experimental time frames into human experience is a very complicated matter. But it can be reasonably assumed that the timing of events in rat models would be a lot shorter than similar periods in humans, and it would be realistic to speculate that the periods of postinjury pathophysiological changes last longer in humans. This issue is crucial, because a second concussive injury occurring within a short period (hours, days, or weeks) can be catastrophic,[10,11,50,52,64,] a phenomenon also known as "the second impact syndrome" (SIS).

Unfortunately, many team physicians' decisions are based on limited observations and sideline evaluations and may frequently be made under intense pressure from coaches, fans, sponsors, and other players, who are all eager to see the injured athlete return to play as quickly as possible.

The imprudence of such a strategy was recently demonstrated by a pilot study involving concussed athletes from different sport disciplines. The results clearly showed that after a concussion, despite normal radiological appearance and complete resolution of clinical symptoms, substantial neurochemical abnormalities were present in the injured brain and readily detectable by measurement of NAA using proton MR spectroscopy.[86] As repeatedly observed in the laboratory, NAA decrease in concussed athletes was seen to be a dynamic process, still detectable 15 days after a concussion; its restoration over time appeared to be nonlinear (slow recovery in the first 2-week period, followed by fast recovery until normalization in the next week), and it was profoundly influenced when a second concussive insult occurred during the recovery process, lengthening the NAA normalization curve and causing a significant delay in symptom resolution. Although none of the players who experienced a second concussion in this study suffered from SIS or showed signs of sTBI, all demonstrated a more severe clinical picture, somehow not proportional to the concussive insult.

Most likely, the second concussion occurred when the brain cells were completing the recovery process and, thus, it only produced a limited cumulative effect with a moderate worsening of the expected clinical and biochemical consequences. The severe brain swelling observed in SIS is attributable to the fact that the second insult must take place when the cells are experiencing their maximum distress (oxidative, neurotoxic, mitochondrial, genetic), are still intensely engaged in restoration of their energetic integrity, and therefore are unable to withstand further energetic expenses, thus experiencing uncontrolled swelling.

The use of proton MR spectroscopy to measure NAA levels offers the unique opportunity to detect the actual state of brain metabolism, to have a snapshot of the degree of energetic impairment, and to monitor the eventual recovery curve, in consideration of the much higher risk of recurrent concussions in sports-related activities.

The results of a multicenter clinical trial involving 40 concussed athletes and 30 healthy volunteers have been recently published, revealing that, despite different combinations of field strengths (1.5 or 3.0 T) and modes of spectrum acquisition (single- or multivoxel) among the scanners currently in use in most neuroradiology centers, NAA determination by proton MR spectroscopy represents a quick (15-minute), easy-to-perform, noninvasive tool to accurately measure changes in cerebral biochemical damage occurring in mTBI, the normalization of which should markedly enhance the ability of physicians and trainers to determine when concussed players should be allowed to return to play.[84]

Conclusions

Mild TBI is a relatively "neglected world" from a

research point of view, especially because it is very difficult to accurately reproduce it in laboratories. Trauma is directly responsible for sudden biochemical changes occurring at the time of impact, and the severity of brain insult can be graded by measuring these biochemical modifications—specifically, ROS-mediated damage, energy metabolism depression, alteration of gene expression and ultimately variation of NAA concentration, a surrogate marker of neuron dysfunction.

Within days after injury, this complex biochemical derangement can result in a dangerous state for the brain, generating a situation of metabolic vulnerability, to the point that if another, equally "mild" injury were to occur, the 2 mTBIs would show the biochemical equivalence of an sTBI. The immediate clinical implication derived from the growing body of experimental evidence is that trials are warranted to investigate the application of proton MR spectroscopy for measurement of NAA and for monitoring the full recovery of brain metabolic functions.

Disclosure

The authors report no conflict of interest concerning the materials or methods used in this study or the findings specified in this paper. This study was funded by PRIN 2007JBHZ5F grant from the Italian Ministry of University and Research.

Author contributions to the study and manuscript preparation include the following. Conception and design: Signoretti. Acquisition of data: Tavazzi, Lazzarino. Analysis and interpretation of data: Tavazzi, Lazzarino. Drafting the article: Signoretti. Critically revising the article: Vagnozzi, Lazzarino. Reviewed final version of the manuscript and approved it for submission: Vagnozzi. Study supervision: Vagnozzi.

Acknowledgment

This work is dedicated to the loving memory of Dr. Anthony Marmarou, who recently died. The authors will be forever grateful for having had the honor and privilege of his long and productive collaboration and friendship. His ideas and scientific enthusiasm inspired most of the cited studies, including the present review.

References

1. Ahmed SM, Rzigalinski BA, Willoughby KA, Sitterding HA, Ellis EF: Stretch-induced injury alters mitochondrial membrane potential and cellular ATP in cultured astrocytes and neurons. **J Neurochem 74:**1951–1960, 2000
2. Alexander MP: Mild traumatic brain injury: pathophysiology, natural history, and clinical management. **Neurology 45:** 1253–1260, 1995
3. Bartlett D, Church DF, Bounds PL, Koppenol WH: The kinetics of the oxidation of L-ascorbic acid by peroxy nitrite. **Free Radic Biol Med 18:**85–92, 1995
4. Baslow MH: NAAG peptidase as a therapeutic target: potential for regulating the link between glucose metabolism and cognition. **Drug News Perspect 19:**145–150, 2006
5. Bates TE, Strangward M, Keelan J, Davey GP, Munro PM, Clark JB: Inhibition of N-acetylaspartate production: implications for 1H MRS studies in vivo. **Neuroreport 7:**1397–1400, 1996
6. Beaumont A, Marmarou A, Czigner A, Yamamoto M, Demetriadou K, Shirotani T, et al: The impact-acceleration model of head injury: injury severity predicts motor and cognitive performance after trauma. **Neurol Res 21:**742–754, 1999
7. Bigler ED: Neurobiology and neuropathology underlie the neuropsychological deficits associated with traumatic brain injury. **Arch Clin Neuropsychol 18:**595–627, 2003
8. Bruns J Jr, Hauser WA: The epidemiology of traumatic brain injury: a review. **Epilepsia 44 (Suppl 10):**2–10, 2003
9. Cantu RC: Athletic concussion: current understanding as of 2007. **Neurosurgery 60:**963–964, 2007
10. Cantu RC: Second-impact syndrome. **Clin Sports Med 17:** 37–44, 1998
11. Cantu RC, Voy R: Second impact syndrome: a risk in any contact sport. **Phys Sports Med 23:**27–34, 1995
12. Cecil KM, Hills EC, Sandel ME, Smith DH, McIntosh TK, Mannon LJ, et al: Proton magnetic resonance spectroscopy for detection of axonal injury in the splenium of the corpus callosum of brain-injured patients. **J Neurosurg 88:**795–801, 1998
13. Cherian L, Hlatky R, Robertson CS: Nitric oxide in traumatic brain injury. **Brain Pathol 14:**195–201, 2004
14. Cortez SC, McIntosh TK, Noble LJ: Experimental fluid percussion brain injury: vascular disruption and neuronal and glial alterations. **Brain Res 482:**271–282, 1989
15. De Kruijk JR, Twijnstra A, Leffers P: Diagnostic criteria and differential diagnosis of mild traumatic brain injury. **Brain Inj 15:**99–106, 2001
16. Di Pietro V, Amin D, Pernagallo S, Lazzarino G, Tavazzi B, Vagnozzi R, et al: Transcriptomics of traumatic brain injury: gene expression and molecular pathways of different grades of insult in a rat organotypic hippocampal culture model. **J Neurotrauma 27:**349–359, 2010
17. Dixon CE, Clifton GL, Lighthall JW, Yaghmai AA, Hayes RL: A controlled cortical impact model of traumatic brain injury in the rat. **J Neurosci Methods 39:**253–262, 1991
18. Dixon CE, Lyeth BG, Povlishock JT, Findling RL, Hamm RJ, Marmarou A, et al: A fluid percussion model of experimental brain injury in the rat. **J Neurosurg 67:**110–119, 1987
19. Doberstein CE, Hovda DA, Becker DP: Clinical considerations in the reduction of secondary brain injury. **Ann Emerg Med 22:**993–997, 1993
20. Donzelli S, Espey MG, Thomas DD, Mancardi D, Tocchetti CG, Ridnour LA, et al: Discriminating formation of HNO from other reactive nitrogen oxide species. **Free Radic Biol Med 40:**1056–1066, 2006
21. Donzelli S, Switzer CH, Thomas DD, Ridnour LA, Espey MG, Isenberg JS, et al: The activation of metabolites of nitric oxide synthase by metals is both redox and oxygen dependent: a new feature of nitrogen oxide signaling. **Antioxid Redox Signal 8:**1363–1371, 2006
22. Du L, Zhang X, Han YY, Burke NA, Kochanek PM, Watkins SC, et al: Intra-mitochondrial poly(ADP-ribosylation) contributes to NAD+ depletion and cell death induced by oxidative stress. **J Biol Chem 278:**18426–18433, 2003
23. Esselman PC, Uomoto JM: Classification of the spectrum of mild traumatic brain injury. **Brain Inj 9:**417–424, 1995
24. Fabbri A, Servadei F, Marchesini G, Negro A, Vandelli A: The changing face of mild head injury: temporal trends and patterns in adolescents and adults from 1997 to 2008. **Injury 41:**968–972, 2010
25. Fiskum G: Mitochondrial participation in ischemic and traumatic neural cell death. **J Neurotrauma 17:**843–855, 2000
26. Foda MA, Marmarou A: A new model of diffuse brain injury in rats. Part II: morphological characterization. **J Neurosurg 80:**301–313, 1994
27. Friedman SD, Brooks WM, Jung RE, Chiulli SJ, Sloan JH, Montoya BT, et al: Quantitative proton MRS predicts outcome after traumatic brain injury. **Neurology 52:**1384–1391, 1999
28. Friedman SD, Brooks WM, Jung RE, Hart BL, Yeo RA: Proton MR spectroscopic findings correspond to neuropsychological function in traumatic brain injury. **AJNR Am J Neuroradiol 19:**1879–1885, 1998
29. Garnett MR, Blamire AM, Rajagopalan B, Styles P, Cadoux-

Hudson TA: Evidence for cellular damage in normal-appearing white matter correlates with injury severity in patients following traumatic brain injury: a magnetic resonance spectroscopy study. **Brain 123:**1403–1409, 2000
30. Giza CC, Hovda DA: The neurometabolic cascade of concussion. **J Athl Train 36:**228–235, 2001
31. Gouvier WD, Cubic B, Jones G, Brantley P, Cutlip Q: Postconcussion symptoms and daily stress in normal and head-injured college populations. **Arch Clin Neuropsychol 7:**193–211, 1992
32. Hovda DA, Badie H, Karimi S, Thomas S, Yoshino A, Kawamata T: Concussive brain injury produces a state of vulnerability for intracranial pressure perturbation in the absence of morphological damage, in Avezaat CJ, van Eijndhoven JH, Maas AI, et al (eds): **Intracranial Pressure VIII.** New York: Springer-Verlag, 1983, pp 469–472
33. Humphrey SM, Cartner LA, Holliss DG: Critical early metabolic changes associated with myocardial recovery or failure after total ischaemia in the rat heart. **Basic Res Cardiol 82:**304–316, 1987
34. Ingebrigtsen T, Romner B, Kock-Jensen C: Scandinavian guidelines for initial management of minimal, mild, and moderate head injuries. **J Trauma 48:**760–766, 2000
35. Janero DR, Hreniuk D, Sharif HM, Prout KC: Hydroperoxide-induced oxidative stress alters pyridine nucleotide metabolism in neonatal heart muscle cells. **Am J Physiol 264:**C1401–C1410, 1993
36. Kaminski KA, Bonda TA, Korecki J, Musial WJ: Oxidative stress and neutrophil activation—the two keystones of ischemia/reperfusion injury. **Int J Cardiol 86:**41–59, 2002
37. Kay T, Newman B, Cavallo M, Ezrachi O, Resnick M: Toward a neuropsychological model of functional disability after mild traumatic brain injury. **Neuropsychology 6:**371–384, 1992
38. Kikugawa K, Hiramoto K, Ohkawa T: Effects of oxygen on the reactivity of nitrogen oxide species including peroxynitrite. **Biol Pharm Bull 27:**17–23, 2004
39. Kirsch M, de Groot H: Ascorbate is a potent antioxidant against peroxynitrite-induced oxidation reactions. Evidence that ascorbate acts by re-reducing substrate radicals produced by peroxynitrite. **J Biol Chem 275:**16702–16708, 2000
40. Klatzo I: Presidential address. Neuropathological aspects of brain edema. **J Neuropathol Exp Neurol 26:**1–14, 1967
41. Kushner D: Mild traumatic brain injury: toward understanding manifestations and treatment. **Arch Intern Med 158:**1617–1624, 1998
42. Laurer HL, Bareyre FM, Lee VM, Trojanowski JQ, Longhi L, Hoover R, et al: Mild head injury increasing the brain's vulnerability to a second concussive impact. **J Neurosurg 95:** 859–870, 2001
43. Lautier D, Hoflack JC, Kirkland JB, Poirier D, Poirier GG: The role of poly(ADP-ribose) metabolism in response to active oxygen cytotoxicity. **Biochim Biophys Acta 1221:**215–220, 1994
44. Lazzarino G, Amorini AM, Fazzina G, Vagnozzi R, Signoretti S, Donzelli S, et al: Single-sample preparation for simultaneous cellular redox and energy state determination. **Anal Biochem 322:**51–59, 2003
45. Lazzarino G, Vagnozzi R, Tavazzi B, Pastore FS, Di Pierro D, Siragusa P, et al: MDA, oxypurines, and nucleosides relate to reperfusion in short-term incomplete cerebral ischemia in the rat. **Free Radic Biol Med 13:**489–498, 1992
46. Levasseur JE, Alessandri B, Reinert M, Bullock R, Kontos HA: Fluid percussion injury transiently increases then decreases brain oxygen consumption in the rat. **J Neurotrauma 17:** 101–112, 2000
47. Lighthall JW: Controlled cortical impact: a new experimental brain injury model. **J Neurotrauma 5:**1–15, 1988
48. Marmarou A, Foda MA, van den Brink W, Campbell J, Kita H, Demetriadou K: A new model of diffuse brain injury in rats. Part I: pathophysiology and biomechanics. **J Neurosurg 80:**291–300, 1994
49. McCrea M, Kelly JP, Randolph C, Cisler R, Berger L: Immediate neurocognitive effects of concussion. **Neurosurgery 50:**1032–1042, 2002
50. McCrory P: Does second impact syndrome exist? **Clin J Sport Med 11:**144–149, 2001
51. McIntosh TK, Vink R, Noble L, Yamakami I, Fernyak S, Soares H, et al: Traumatic brain injury in the rat: characterization of a lateral fluid-percussion model. **Neuroscience 28:** 233–244, 1989
52. McQuillen JB, McQuillen EN, Morrow P: Trauma, sport, and malignant cerebral edema. **Am J Forensic Med Pathol 9:** 12–15, 1988
53. Mendez DR, Cherian L, Moore N, Arora T, Liu PK, Robertson CS: Oxidative DNA lesions in a rodent model of traumatic brain injury. **J Trauma 56:**1235–1240, 2004
54. Miyake M, Kakimoto Y, Sorimachi M: A gas chromatographic method for the determination of N-acetyl-L-aspartic acid, N-acetyl-alpha-aspartylglutamic acid and beta-citryl-L-glutamic acid and their distributions in the brain and other organs of various species of animals. **J Neurochem 36:**804–810, 1981
55. Moffett JR, Ross B, Arun P, Madhavarao CN, Namboodiri AM: N-Acetylaspartate in the CNS: from neurodiagnostics to neurobiology. **Prog Neurobiol 81:**89–131, 2007
56. Morgan WA: Pyridine nucleotide hydrolysis and interconversion in rat hepatocytes during oxidative stress. **Biochem Pharmacol 49:**1179–1184, 1995
57. Movsesyan VA, Faden AI: Neuroprotective effects of selective group II mGluR activation in brain trauma and traumatic neuronal injury. **J Neurotrauma 23:**117–127, 2006
58. Nanavaty UB, Pawliczak R, Doniger J, Gladwin MT, Cowan MJ, Logun C, et al: Oxidant-induced cell death in respiratory epithelial cells is due to DNA damage and loss of ATP. **Exp Lung Res 28:**591–607, 2002
59. Neale JH, Olszewski RT, Gehl LM, Wroblewska B, Bzdega T: The neurotransmitter N-acetylaspartylglutamate in models of pain, ALS, diabetic neuropathy, CNS injury and schizophrenia. **Trends Pharmacol Sci 26:**477–484, 2005
60. Nojiri H, Shimizu T, Funakoshi M, Yamaguchi O, Zhou H, Kawakami S, et al: Oxidative stress causes heart failure with impaired mitochondrial respiration. **J Biol Chem 281:**33789–33801, 2006
61. Pacher P, Liaudet L, Mabley J, Komjáti K, Szabó C: Pharmacologic inhibition of poly(adenosine diphosphate-ribose) polymerase may represent a novel therapeutic approach in chronic heart failure. **J Am Coll Cardiol 40:**1006–1016, 2002
62. Palozza P, Moualla S, Krinsky NI: Effects of β-carotene and α-tocopherol on radical-initiated peroxidation of microsomes. **Free Radic Biol Med 13:**127–136, 1992
63. Patel TB, Clark JB: Synthesis of N-acetyl-L-aspartate by rat brain mitochondria and its involvement in mitochondrial/cytosolic carbon transport. **Biochem J 184:**539–546, 1979
64. Saunders RL, Harbaugh RE: The second impact in catastrophic contact-sports head trauma. **JAMA 252:**538–539, 1984
65. Sawa T, Ohshima H: Nitrative DNA damage in inflammation and its possible role in carcinogenesis. **Nitric Oxide 14:**91–100, 2006
66. Schinder AF, Olson EC, Spitzer NC, Montal M: Mitochondrial dysfunction is a primary event in glutamate neurotoxicity. **J Neurosci 16:**6125–6133, 1996
67. Schrammel A, Gorren AC, Schmidt K, Pfeiffer S, Mayer B: S-nitrosation of glutathione by nitric oxide, peroxynitrite, and $^{*}NO/O_2^{*-}$. **Free Radic Biol Med 34:**1078–1088, 2003
68. Sharma HS, Wiklund L, Badgaiyan RD, Mohanty S, Alm P: Intracerebral administration of neuronal nitric oxide synthase antiserum attenuates traumatic brain injury-induced blood-brain barrier permeability, brain edema formation, and sensory motor disturbances in the rat. **Acta Neurochir Suppl 96:**288–294, 2006

69. Signoretti S, Marmarou A, Tavazzi B, Lazzarino G, Beaumont A, Vagnozzi R: N-Acetylaspartate reduction as a measure of injury severity and mitochondrial dysfunction following diffuse traumatic brain injury. **J Neurotrauma 18:**977–991, 2001
70. Smith SL, Andrus PK, Zhang JR, Hall ED: Direct measurement of hydroxyl radicals, lipid peroxidation, and blood-brain barrier disruption following unilateral cortical impact head injury in the rat. **J Neurotrauma 11:**393–404, 1994
71. Solaroglu I, Okutan O, Kaptanoglu E, Beskonakli E, Kilinc K: Increased xanthine oxidase activity after traumatic brain injury in rats. **J Clin Neurosci 12:**273–275, 2005
72. Sullivan PG, Keller JN, Mattson MP, Scheff SW: Traumatic brain injury alters synaptic homeostasis: implications for impaired mitochondrial and transport function. **J Neurotrauma 15:**789–798, 1998
73. Sullivan PG, Springer JE, Hall ED, Scheff SW: Mitochondrial uncoupling as a therapeutic target following neuronal injury. **J Bioenerg Biomembr 36:**353–356, 2004
74. Tagliaferri F, Compagnone C, Korsic M, Servadei F, Kraus J: A systematic review of brain injury epidemiology in Europe. **Acta Neurochir (Wien) 148:**255–268, 2006
75. Tallan HH: Studies on the distribution of N-acetyl-L-aspartic acid in brain. **J Biol Chem 224:**41–45, 1957
76. Tavazzi B, Di Pierro D, Amorini AM, Fazzina G, Galvano M, Lupi A, et al: Direct NAD(P)H hydrolysis into ADP-ribose(P) and nicotinamide induced by reactive oxygen species: a new mechanism of oxygen radical toxicity. **Free Radic Res 33:** 1–12, 2000
77. Tavazzi B, Di Pierro D, Bartolini M, Marino M, Distefano S, Galvano M, et al: Lipid peroxidation, tissue necrosis, and metabolic and mechanical recovery of isolated reperfused rat heart as a function of increasing ischemia. **Free Radic Res 28:**25–37, 1998
78. Tavazzi B, Signoretti S, Lazzarino G, Amorini AM, Delfini R, Cimatti M, et al: Cerebral oxidative stress and depression of energy metabolism correlate with severity of diffuse brain injury in rats. **Neurosurgery 56:**582–589, 2005
79. Tavazzi B, Vagnozzi R, Di Pierro D, Amorini AM, Fazzina G, Signoretti S, et al: Ion-pairing high-performance liquid chromatographic method for the detection of N-acetylaspartate and N-acetylglutamate in cerebral tissue extracts. **Anal Biochem 277:**104–108, 2000
80. Tavazzi B, Vagnozzi R, Signoretti S, Amorini AM, Belli A, Cimatti M, et al: Temporal window of metabolic brain vulnerability to concussions: oxidative and nitrosative stresses—part II. **Neurosurgery 61:**390–396, 2007
81. Thies RL, Autor AP: Reactive oxygen injury to cultured pulmonary artery endothelial cells: mediation by poly(ADP-ribose) polymerase activation causing NAD depletion and altered energy balance. **Arch Biochem Biophys 286:**353–363, 1991
82. Truckenmiller ME, Namboodiri MA, Brownstein MJ, Neale JH: N-Acetylation of L-aspartate in the nervous system: differential distribution of a specific enzyme. **J Neurochem 45:** 1658–1662, 1985
83. Vagnozzi R, Marmarou A, Tavazzi B, Signoretti S, Di Pierro D, del Bolgia F, et al: Changes of cerebral energy metabolism and lipid peroxidation in rats leading to mitochondrial dysfunction after diffuse brain injury. **J Neurotrauma 16:**903–913, 1999
84. Vagnozzi R, Signoretti S, Cristofori L, Alessandrini F, Floris R, Isgrò E, et al: Assessment of metabolic brain damage and recovery following mild traumatic brain injury: a multicentre, proton magnetic resonance spectroscopic study in concussed patients. **Brain** [epub ahead of print], 2010
85. Vagnozzi R, Signoretti S, Tavazzi B, Cimatti M, Amorini AM, Donzelli S, et al: Hypothesis of the postconcussive vulnerable brain: experimental evidence of its metabolic occurrence. **Neurosurgery 57:**164–171, 2005
86. Vagnozzi R, Signoretti S, Tavazzi B, Floris R, Ludovici A, Marziali S, et al: Temporal window of metabolic brain vulnerability to concussion: a pilot 1H-magnetic resonance spectroscopic study in concussed athletes—part III. **Neurosurgery 62:**1286–1296, 2008
87. Vagnozzi R, Tavazzi B, Di Pierro D, Giardina B, Fraioli B, Signoretti S, et al: Effects of increasing times of incomplete cerebral ischemia upon the energy state and lipid peroxidation in the rat. **Exp Brain Res 117:**411–418, 1997
88. Vagnozzi R, Tavazzi B, Signoretti S, Amorini AM, Belli A, Cimatti M, et al: Temporal window of metabolic brain vulnerability to concussions: mitochondrial-related impairment—part I. **Neurosurgery 61:**379–389, 2007
89. van der Naalt J: Prediction of outcome in mild to moderate head injury: a review. **J Clin Exp Neuropsychol 23:**837–851, 2001
90. Vos PE, Battistin L, Birbamer G, Gerstenbrand F, Potapov A, Prevec T, et al: EFNS guideline on mild traumatic brain injury: report of an EFNS task force. **Eur J Neurol 9:**207–219, 2002
91. Wilson JX, Gelb AW: Free radicals, antioxidants, and neurologic injury: possible relationship to cerebral protection by anesthetics. **J Neurosurg Anesthesiol 14:**66–79, 2002
92. Wroblewska B: NAAG as a neurotransmitter. **Adv Exp Med Biol 576:**317–325, 2006
93. Wu A, Ying Z, Gomez-Pinilla F: Vitamin E protects against oxidative damage and learning disability after mild traumatic brain injury in rats. **Neurorehabil Neural Repair 24:**290–298, 2010
94. Yoshino A, Hovda DA, Kawamata T, Katayama Y, Becker DP: Dynamic changes in local cerebral glucose utilization following cerebral conclusion in rats: evidence of a hyper- and subsequent hypometabolic state. **Brain Res 561:**106–119, 1991
95. Yu SW, Wang H, Poitras MF, Coombs C, Bowers WJ, Federoff HJ, et al: Mediation of poly(ADP-ribose) polymerase-1-dependent cell death by apoptosis-inducing factor. **Science 297:**259–263, 2002
96. Zhong C, Zhao X, Sarva J, Kozikowski A, Neale JH, Lyeth BG: NAAG peptidase inhibitor reduces acute neuronal degeneration and astrocyte damage following lateral fluid percussion TBI in rats. **J Neurotrauma 22:**266–276, 2005
97. Zoratti M, Szabò I: The mitochondrial permeability transition. **Biochim Biophys Acta 1241:**139–176, 1995

Manuscript submitted July 14, 2010.
Accepted September 13, 2010.
Address correspondence to: Stefano Signoretti, M.D., Division of Neurosurgery, Department of Neurosciences Head and Neck Surgery, S. Camillo Hospital, Circonvallazione Gianicolense 183, 00100 Rome, Italy. email: stefano.signoretti@tiscali.it.