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**Received,** October 3, 2006.
**Accepted,** March 16, 2007.

# Temporal Window of Metabolic Brain Vulnerability to Concussions: Mitochondrial-related Impairment—Part I

**OBJECTIVE:** In the present study, we investigate the existence of a temporal window of brain vulnerability in rats undergoing repeat mild traumatic brain injury (mTBI) delivered at increasing time intervals.

**METHODS:** Rats were subjected to two diffuse mTBIs (450 g/1 m height) with the second mTBI delivered after 1 (n = 6), 2 (n = 6), 3 (n = 6), 4 (n = 6), and 5 days (n = 6) and sacrificed 48 hours after the last impact. Sham-operated animals were used as controls (n = 6). Two further groups of six rats each received a second mTBI after 3 days and were sacrificed at 120 and 168 hours postinjury. Concentrations of adenine nucleotides, N-acetylated amino acids, oxypurines, nucleosides, free coenzyme A, acetyl CoA, and oxidized and reduced nicotinamide adenine dinucleotides, oxidized nicotinamide adenine dinucleotide phosphate, and reduced nicotinamide adenine dinucleotide, reduced nicotinamide adenine dinucleotide phosphate nicotinic coenzymes were measured in deproteinized cerebral tissue extracts (three right and three left hemispheres), whereas the gene expression of N-acetylaspartate acylase, the enzyme responsible for N-acetylaspartate (NAA) degradation, was evaluated in extracts of three left and three right hemispheres.

**RESULTS:** A decrease of adenosine triphosphate, adenosine triphosphate /adenosine diphosphate ratio, NAA, N-acetylaspartylglutamate, oxidized and reduced nicotinamide adenine dinucleotide, reduced nicotinamide adenine dinucleotide, and acetyl CoA and increase of N-acetylaspartate acylase expression were related to the interval between impacts with maximal changes recorded when mTBIs were spaced by 3 days. In these animals, protracting the time of sacrifice after the second mTBI up to 1 week failed to show cerebral metabolic recovery, indicating that this type of damage is difficult to reverse. A metabolic pattern similar to controls was observed only in animals receiving mTBIs 5 days apart.

**CONCLUSION:** This study shows the existence of a temporal window of brain vulnerability after mTBI. A second concussive event falling within this time range had profound consequences on mitochondrial-related metabolism. Furthermore, because NAA recovery coincided with normalization of all other metabolites, it is conceivable to hypothesize that NAA measurement by $^{1}$H-NMR spectroscopy might be a valid tool in assessing full cerebral metabolic recovery in the clinical setting and with particular reference to sports medicine in establishing when to return mTBI-affected athletes to play. This study also shows, for the first time, the influence of TBI on acetyl-CoA, N-acetylaspartate acylase gene expression, and N-acetylaspartylglutamate, thus providing novel data on cerebral biochemical changes occurring in head injury.

**KEY WORDS:** Brain vulnerability, Concussion, Energy metabolism, N-acetylaspartate metabolism, Repeat or multiple concussions, Second impact syndrome

*Neurosurgery 61:379–389, 2007* DOI: 10.1227/01.NEU.0000280002.41696.D8 www.neurosurgery-online.com

The evaluation and management of mild traumatic brain injury (mTBI), defined as a traumatically induced alteration in mental status, not necessarily with loss of consciousness, is affected by significant variability either in the clinical setting or in sports medicine.

Patients experiencing mTBI and attending emergency rooms often do not see a specialist (neurosurgeon or neurologist) for consultation and, in most cases, receive no specific advice and/or care. The lack of guidance is even more noticeable in sports medicine because of the higher incidence of mTBI, particularly in contact sports played at either the professional or amateur level. In fact, the fundamental issue of when to return to play after an episode of mTBI is still a matter of debate (22, 23, 40, 51). In strict correlation with this problem is another open question on what diagnostic tests are best applied to these patients, particularly those defined as asymptomatic (12, 16). At present, in addition to history-taking, symptoms indicated by the patient, physical examination, and cognitive neuropsychological tests are widely used to assess the mildly injured athlete and often to establish the time scale of return-to-play (32, 33, 45).

In the past decades, the general knowledge on traumatic brain injury (TBI) has greatly improved (46), demonstrated in the release of excitatory amino acids (1, 43), onset of oxidative stress (44, 56), overproduction of nitric oxide (11, 18), impairment of mitochondrial functions (27, 48, 49), failure of energy metabolism (53, 54), and induction of apoptosis (24, 29), some of the most important molecular events in the evolution of damage in the injured brain. Experimental studies on animals subjected to different models of repeated mTBI have indicated cognitive deficits and histological brain damage (25, 28). Recently, by using the impact acceleration model of diffuse TBI, we were able to confirm the hypothesis of a metabolically "vulnerable brain," originally proposed by Giza and Hovda (15) and Hovda et al. (20, 21), demonstrating that a second mTBI may result in catastrophic damage to mitochondrial energy metabolism depending on the lag time between the two traumatic events (55). However, a more definite picture of the effect of the time interval between two mTBIs on brain metabolism, as well as more robust evidence of whether the mitochondrial dysfunction observed is partly or totally reversible, is still lacking.

Previous data from our laboratory highlighted in N-acetylaspartate (NAA) a valuable biochemical marker in monitoring deterioration or recovery after mTBI, confirming the diagnostic value of measuring this metabolite in the assessment of the cerebral metabolic state after head injury. In particular, we demonstrated a close relationship among trauma severity, depression of energy metabolism, and NAA in rats (48, 49, 53), as well as a correlation between trauma severity and extracellular NAA release in patients sustaining TBI (4). Moreover, data from numerous preclinical and clinical studies have demonstrated the relevance of NAA measurement in many cerebral pathologies, particularly in view of its noninvasive in vivo quantification by $^{1}$H-NMR spectroscopy (9, 19, 31).

In accordance with what was reported previously, particularly the role and measurement of NAA, we now describe the results of an extensive screen of markers of mitochondrial-related functions obtained in rats subjected to two mTBIs spaced by 1, 2, 3, 4, and 5 days. Besides measuring adenine nucleotides, oxypurines, nucleosides, CoA-SH, acetyl-CoA, and NAA values, we determined the concentrations of the NAA-related compounds N-acetylglutamate (NAG) and N-acetylaspartatylglutamate (NAAG) as well as the expression of N-acetylaspartoacylase (ASPA), the enzyme responsible for NAA hydrolysis into aspartate and acetate.

The aim of this study was to evaluate the effect of the time interval between impacts on the reversibility of mitochondrial dysfunction and metabolism of NAA and NAA-related compounds to define the concept of a temporal window of brain metabolic vulnerability after repeat concussions.

## MATERIALS AND METHODS

### Experimental Protocol of Repeat mTBI

All surgical procedures were approved by the Ethical Committees of Tor Vergata University and Catholic University according to international standards and guidelines.

Male Wistar rats weighing 300 to 350 g were used in this study. They were fed with a standard laboratory diet and water ad libitum in a controlled environment and were randomly selected to receive two mTBIs spaced by the following time intervals: 1 day (Group 1, n = 6); 2 days (Group 2, n = 6); 3 days (Group 3, n = 6); 4 days (Group 4, n = 6); and 5 days (Group 5, n = 6). Rats that experienced skull fracture, seizures, or nasal bleeding, and those that did not survive the impacts were excluded from the study. Sham-injured animals (n = 6) acted as the control group. All rats were initially anesthetized with halothane anesthesia (4%) followed by an intraperitoneal injection of propofol (23 mg/kg). Monitoring of respiratory parameters, body temperature, and arterial blood gases as well as animal preparation for trauma and conditions to induce diffuse mTBI are as described in detail elsewhere (14, 30).

Before receiving the second mTBI, the rats were again anesthetized. Sham-injured animals were subjected to the same protocol of anesthesia administration (interval of 1 day between two anesthesias), but no mass was ever dropped. The animals were sacrificed 48 hours after the second impact; sham-operated animals were sacrificed 48 hours after the last anesthesia. To evaluate the eventual cerebral metabolic recovery, two additional groups of six rats each were subjected to two mTBIs delivered 3 days apart and were sacrificed at 120 and 168 hours postinjury.

### Cerebral Tissue Processing and High-performance Liquid Chromatography Analyses of Metabolites

An in vivo craniectomy was performed in all animals during anesthesia with the aid of an operative microscope. After carefully removing the rat's skull, the brain was exposed and sharply cut along the sagittal fissure; the two hemispheres were removed with a surgical spatula and quickly dropped in liquid nitrogen. This sample processing procedure, separation of the two hemispheres, was rendered possible by the model adopted to induce the diffuse mTBI, which has been demonstrated to cause symmetrical damage in the two hemispheres (14, 30). After the wet weight (w/w) determination, one hemisphere was deproteinized as previously reported (26, 55). Aliquots of each deproteinized hemisphere were filtered through a 0.45-μm HV Millipore filter (Millipore Corp., Billerica, MA) and loaded (20 μL) onto a Hypersil C-18, 250 × 4.6 mm, 5-μm particle size column, provided with its own guard column (Thermo Fisher Scientific, Milan, Italy) and connected to a high-performance liquid chromatography (HPLC) apparatus consisting of a SpectraSystem P2000 pump system (Thermo Fisher Scientific) and a highly-sensitive UV6000LP diode array detector (Thermo Fisher Scientific) equipped with a 5-cm light path flow cell and setup between 200 and 300 nm wavelength. Data acquisition and

analysis were performed using a personal computer and the ChromQuest software package provided by the HPLC manufacturer (Thermo Fisher Scientific).

NAA, NAG, NAAG, and metabolites related to tissue energy state and mitochondrial function (ATP, ADP, AMP, oxypurines, nucleosides, $NAD^+$, NADH, $NADP^+$, NADPH, CoA-SH, and acetyl-CoA) were separated, in a single chromatographic run, according to a gradient modification of an existing ion-pairing HPLC method formerly set up in our laboratory (52). Assignment and calculation of the compounds of interest in chromatographic runs of tissue extracts were carried out at either 206 (NAA, NAG, NAAG) or 260 nm wavelength by comparing retention times, absorption spectra, and areas of peaks with those of peaks of chromatographic runs of freshly prepared ultrapure standard mixtures with known concentrations. For each group of animals (including controls), three right and three left hemispheres were used to perform the HPLC biochemical evaluation of the repeat mTBI-injured brain.

### Tissue Preparation and Reverse Transcriptase-polymerase Chain Reaction Analysis of N-acetylaspartate Acylase Expression

Total ribonucleic acid (RNA) was extracted by homogenizing the right or left hemisphere tissue in a monophasic solution of phenol and guanidine isothiocyanate (Trizol; Invitrogen Life Technologies, Carlsbad, CA) using the Ultra-Turrax homogenizer (Janke & Kunkel, Staufen, Germany) at 24,000 rpm/minute to produce a final 10% homogenate (weight:volume). The addition of 0.2 mL chloroform/1 mL of Trizol and vigorous agitation was followed by centrifugation at 12,000 $\times$ *g* for 15 minutes at 4°C to separate the aqueous and organic phase. The upper RNA-containing aqueous phase was carefully collected and RNA precipitated by mixing with excess isopropyl alcohol at room temperature for 10 minutes followed by centrifugation at 12,000 $\times$ *g* for 10 minutes at 4°C. Supernatant was discarded and the precipitated RNA was washed with 75% ethanol and centrifuged again at 7500 $\times$ *g* for 5 minutes at 4°C. At the end of the procedure, RNA was dissolved in RNase-free, doubly distilled water.

Reverse transcriptase-polymerase chain reaction was performed using the SuperScript III One-step System with Platinum *Taq* Deoxyribonucleic Acid (DNA) Polymerase (Invitrogen Life Technologies) performing both complementary DNA synthesis and polymerase chain reaction amplification in a single reaction using gene-specific primers and target RNAs from total RNA.

Primers were designed with the 0.2 version of the Primer3 Input software developed by the Whitehead Institute for Biomedical Research (Cambridge, MA) and using the sequences of *Rattus norvegicus* ASPA (Gene Bank Accession Number NM_024399) published by the National Center for Biotechnology Information as a template. Primer sequences of ASPA, amplifying a 500-bp fragment, were:

- ASPA forward 5′-GACCTGCATGGCTCCATTAC-3′
- ASPA reverse 5′-GGATGCTTTTTGCGTTGAGT-3′

For the semiquantitative analysis, the reference gene of hypoxantine phosporibosyltransferase (HPRT) from *Rattus norvegicus* (GeneBank Accession Number NM_012583) was selected. Primer sequences for HPRT, amplifying a 282-bp fragment, were:

- HPRT forward 5′-CCTGCTGGATTACATTAAAGCGCTG-3′
- HPRT reverse 5′-CTTCGAGAGGTCCTTTTCACCAGC-3′

Reverse transcriptase–polymerase chain reaction was performed using 10 ng RNA for each reaction in a T Gradient Thermocycler (Biometra, Goettingen, Germany) with the following cycling parameters:

50°C, 30 minutes; 94°C, 4 minutes (94°C, 60 s; 58°C, 120 s; 72°C, 120 s) 28×; 72°C, 10 minutes for ASPA messenger RNA (mRNA).

50°C, 30 minutes; 94°C, 4 minutes (94°C, 60 s, 60°C, 120 s, 72°C, 120 s) 30×; 72°C, 10 minutes for HPRT mRNA.

Both cycling parameters of reverse transcriptase-polymerase chain reaction and RNA amount were selected on purpose to satisfy criteria of linearity to be used for semiquantitative ASPA expression. Reverse transcriptase-polymerase chain reaction products of each brain sample were then loaded on a 1.5% agarose gel and electrophoresed at 10 V/cm for 60 minutes. Amplified cDNAs of either ASPA or HPRT of a given sample were allowed to run in the same lane to minimize the subsequent densitometric band evaluation performed using the ImageMaster VDS TotalLab software (Amersham Pharmacia Biotech, Little Chalfont, United Kingdom).

For each group of animals (including controls), three left and three right hemispheres were used to perform the molecular biological evaluation of the repeat mTBI-injured brain.

### Statistics

Differences in the various groups were tested by one-way analysis of variance and Fisher's probable least-squares difference post hoc test. Differences were corrected for multiple comparisons and were considered statistically significant at a *P* value of less than 0.05.

## RESULTS

### Effect of Trauma on Physiological Parameters

A complete analysis of the variation of physiological parameters in this experimental model is thoroughly described elsewhere (14, 30, 55). Briefly, the mortality rate in doubly impacted animals was significant only with a 3-day interval and was less than 10%. No animals died when subjected to single mTBI or double concussion with 1-, 2-, 4-, and 5-day intervals. Animals subjected to the second mTBI experienced a short period of apnea for approximately 5 seconds immediately after impact with no alterations of heart rate and an initial decrement of systolic arterial blood pressure by 15 $\pm$ 4 mmHg (pressure changes were transient and short-lasting with return to preimpact values in approximately 120 s). It is worth stressing that no change in any of the aforementioned parameters was observed during the remaining recording times. Blood gases and pH determined throughout the first 30 minutes after brain impact showed no significant variations with respect to values recorded in control sham-injured rats.

### Posttraumatic Alteration of Energetic Metabolism

*Table 1* summarizes data referring to variations in brain hemisphere extracts (three right and three left hemispheres) of metabolites related to mitochondrial functions (adenine nucleotides, CoA-SH, acetyl CoA) after repeat mTBIs at different time intervals. An upside down, bell-shaped trend for ATP changes was observed by increasing the interval between mTBIs with residual ATP being 68.3, 59.3, 47.2, 58.7, and 86.1% of the control value when the second mTBI was delivered 1, 2, 3, 4, and 5 days after the first impact, respectively. Although ADP significantly increased only in rats of Group 3 (mTBI-3 d-mTBI), AMP elevation was recorded in all doubly concussed rats except Group 5 (mTBI-5 d-mTBI) with varying proportions depending on the

**TABLE 1. Concentrations of adenine nucleotides, CoA-SH, and acetyl-CoA determined by high-performance liquid chromatography in brain hemisphere extracts (three right and three left hemispheres) of rats subjected to two mild traumatic brain injuries delivered at different intervals (controls are represented by sham-operated animals)[a]**

| | ATP | ADP | AMP | $\Sigma_{NT}$ | CoA-SH | Acetyl-CoA |
|---|---|---|---|---|---|---|
| Controls | 2318.03 | 206.38 | 32.44 | 2556.85 | 27.72 | 38.13 |
| | (209.12) | (19.12) | (5.12) | (198.13) | (1.76) | (4.65) |
| mTBI-1 days-mTBI | 1583.42[b] | 236.68 | 47.67[b] | 1867.77[b] | 22.81[b] | 23.97[b] |
| | (136.43) | (35.07) | (5.29) | (224.36) | (3.22) | (1.11) |
| mTBI-2 days-mTBI | 1374.16[b] | 245.38 | 62.90[b] | 1682.44[b] | 20.78[b] | 21.55[b] |
| | (165.56) | (46.24) | (9.69) | (205.03) | (3.49) | (3.14) |
| mTBI-3 days-mTBI | 1094.61[b] | 337.86[b] | 70.74[b] | 1503.21[b] | 21.07[b] | 9.41[b] |
| | (139.14) | (46.38) | (15.51) | (179.68) | (3.59) | (0.53) |
| mTBI-4 days-mTBI | 1360.09[b] | 265.12 | 50.23[b] | 1675.44[b] | 20.54[b] | 26.01[b] |
| | (116.17) | (37.06) | (7.49) | (189.51) | (2.16) | (1.57.) |
| mTBI-5 days-mTBI | 1994.88[b] | 226.73 | 37.28 | 2258.89 | 24.35 | 33.92 |
| | (156.33) | (31.43) | (6.72) | (242.77) | (3.68) | (3.89) |

[a] ATP, adenosine triphosphate; ADP, adenosine diphosphate; AMP, adenosine monophosphate; $\Sigma_{NT}$, ATP + ADP + AMP; CoA-SH, uncombined coenzyme A; mTBI, mild traumatic brain injury. Each value is the mean (standard deviation) of six different animals and is expressed as nmol/g wet weight.
[b] Significantly different from corresponding values recorded in controls, $P < 0.05$.

interval between concussions. A decrease in the sum of adenine nucleotides ($\Sigma_{NT}$ = ATP + ADP + AMP) occurred in all repeat injuries, exactly mirroring ATP depletion. However, in Group 5, the $\Sigma_{NT}$ was not significantly different from that recorded in control animals. The same pattern was observed when measuring the concentrations of CoA-SH, revealing a significant, albeit slight, decrease in all doubly injured rats, with the exception of the 5-day interval group. Unlike CoA-SH, acetyl-CoA underwent a progressive fall with increase in time to second mTBIs up to 3 days when the lowest mean value of 9.41 nmol/g w/w (−75.32%, $P < 0.001$ with respect to controls) was recorded. Again, rats subjected to insults spaced by 5 days showed acetyl-CoA values not significantly different from controls.

As a result of energy metabolism impairment, noteworthy alterations of the products of ATP catabolism (sum of oxypurines: hypoxanthine + xanthine + uric acid, and sum of nucleosides: inosine + adenosine) were observed in rats of Groups 1 (mTBI-1 d-mTBI), 2 (mTBI-2 d-mTBI), 3 (mTBI-3 d-mTBI), and 4 (mTBI-4 d-mTBI) as illustrated in *Fig. 1*. Also, rats of Group 5 (mTBI-5 d-mTBI) showed values of ATP catabolites similar to those recorded in sham-injured rats.

## Variation of N-acetylated Amino Acids and N-acetylaspartate Acylase Gene Expression

In *Figure 2*, data of NAA, NAG, and NAAG variations in brain hemisphere extracts (three right and three left hemispheres) are illustrated. Although NAG did not significantly change in any animal group, NAA and NAAG showed a similar trend with increasing depletion by increasing the time between the two concussions up to 3 days, when minimal mean values were recorded (3808.8 and 303.94 nmol/g w/w, respectively; $P < 0.001$). In rats receiving the second mTBI 5 days after the first impact, NAA was 7555.3 nmol/g w/w and NAAG was 613.75 nmol/g w/w, values not significantly different from sham-injured rats. To appreciate better the similarity between changes in NAA concentration and the mitochondrial oxidative phosphorylating capacity, *Figure 3* compares NAA and ATP/ADP ratio, as calculated from data reported in *Table 1*, in the various groups of animals. Minimal values of NAA (−58.5% with respect to controls; $P < 0.001$) and of the ATP/ADP ratio (−71.1% with respect to controls; $P < 0.001$)

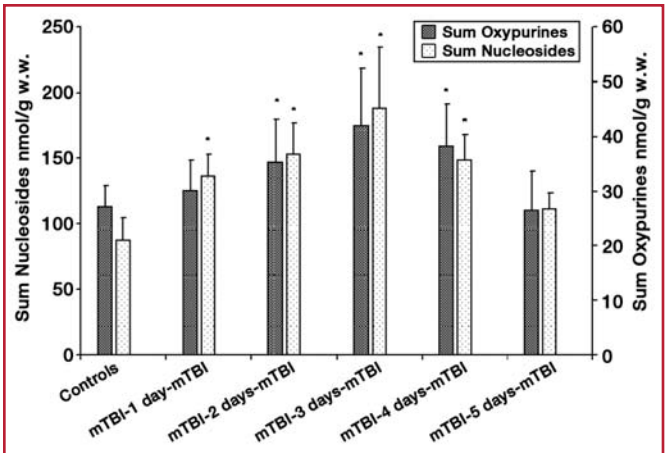


**FIGURE 1.** *Bar graph showing changes of sum of oxypurines (hypoxanthine + xanthine + uric acid) and sum of nucleosides (inosine + adenosine) recorded in deproteinized hemisphere extracts (three right and three left hemispheres) of control rats and rats subjected to repeat mTBIs at different time intervals. Each histogram is the mean of six animals. Standard deviations are represented by* vertical bars. Asterisk, *$P < 0.05$ with respect to controls.*

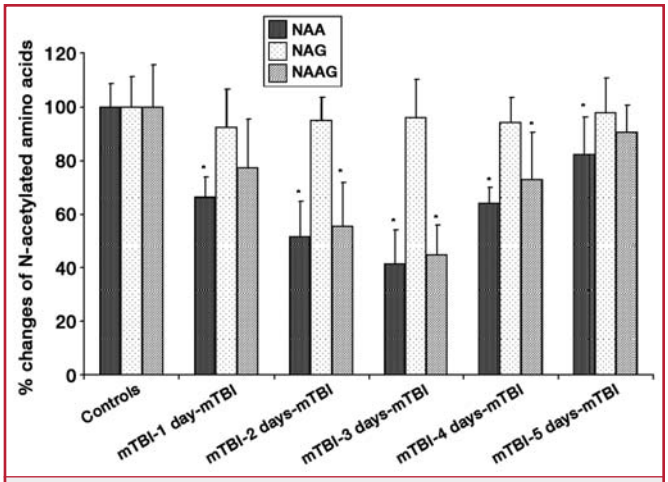


**FIGURE 2.** *Bar graph showing the percent N-acetylaspartate, N-acetylglutamate, and N-acetylaspartylglutamate variations recorded in deproteinized hemisphere extracts (three right and three left hemispheres) of control rats and rats subjected to repeat mTBIs at different time intervals. Each histogram is the mean of six animals. Standard deviations are represented by* vertical bars. Asterisk, *$P < 0.05$ with respect to controls.*

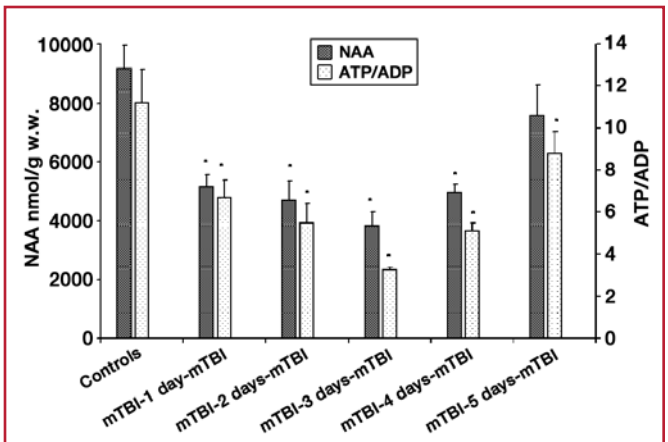


**FIGURE 3.** *Bar graph showing the variations* of *N-acetylaspartate concentrations and ATP/ADP ratio (calculated from ATP and ADP values reported in* Table 1*) recorded in deproteinized hemisphere extracts (three right and three left hemispheres) of control rats and rats subjected to repeat mTBIs at different time intervals. Each histogram is the mean of six animals. Standard deviations are represented by* vertical bars. Asterisk, *$P < 0.05$ with respect to controls.*

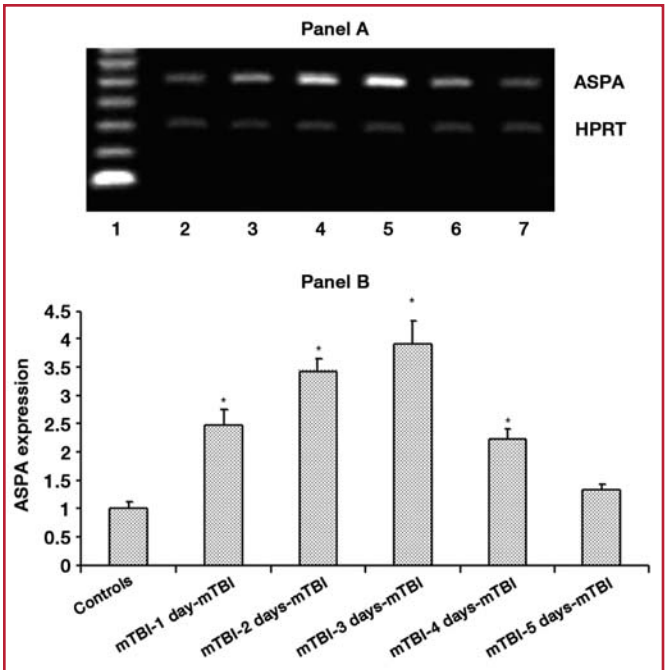


**FIGURE 4.** *Variations in the cerebral gene expression of N-acetylaspartate acylase recorded in deproteinized hemisphere extracts (three left and three right hemispheres) of control rats and rats subjected to repeat mTBIs at different time intervals.* **A**, *representative gel electrophoresis of amplified mRNA of ASPA and hypoxanthine phosporibosyltransferase (the housekeeping gene selected as reference) of control rats and rats subjected to repeat mTBIs with increasing time interval.* Line 1, *DNA ladder (size markers);* line 2, *controls;* line 3, *mTBI at a 1 day interval;* line 4, *mTBI at a 2-day interval;* line 5, *mTBI at a 3-day interval;* line 6, *mTBI at a 4-day interval;* line 7, *mTBI at a 5-day interval.* **B**, *bar graph showing the semiquantitative mRNA values of N-acetylaspartate acylase expression as calculated by using mRNA of hypoxanthine phosporibosyltransferase expression as reference in control rats and rats subjected to repeat mTBIs with increasing time interval. Controls were represented by sham-injured animals. Each histogram is the mean of six animals. Standard deviations are represented by* vertical bars. Asterisk, *$P < 0.05$ with respect to controls.*

were observed in rats receiving mTBIs 3 days apart. Conversely, the 5-day interval produced a 17.6% depletion of NAA (not significant) and a 21.6% of the ATP/ADP ratio ($P < 0.05$) with respect to control rat values.

Results of the analysis of mRNA transcript of the ASPA gene determined in hemispheres (three left and three right hemispheres) of control rats and rats subjected to repeat mTBIs are shown in *Figure 4*, *A* and *B*. *Figure 4A* illustrates a representative gel electrophoresis of amplified mRNA of HPRT (the housekeeping gene selected as reference) and ASPA clearly indicating no change in HPRT expression; instead, there was an effect of the time interval between concussions on the ASPA gene expression. *Figure 4B* illustrates the semiquantitative mean values of ASPA expression, which shows a progressive increase of the mRNA transcript of the ASPA gene as a result of increasing the time interval between concussions up to 3 days. In fact, in rats in Group 3 (mTBI-3 d-mTBI), a maximal fourfold increase of ASPA expression was recorded. Animals in Group 5 (mTBI-5 d-mTBI) had values of mRNA for ASPA comparable to those recorded in controls.

## Posttraumatic Changes of Nicotinic Coenzymes

*Table 2* summarizes changes of oxidized and reduced nicotinic coenzymes in deproteinized hemisphere extracts (three right + three left hemispheres) of control rats and all double injury groups. Remarkable $NAD^+$ and $NADP^+$ variations occurred by

**TABLE 2. Concentrations of oxidized and reduced nicotinic coenzymes determined by high-performance liquid chromatography in brain hemisphere extracts (three right and three left hemispheres) of rats subjected to two mild traumatic brain injuries delivered at different intervals (controls are represented by sham-operated animals)[a]**

| | $NAD^+$ | NADH | $NAD^+$/NADH | $NADP^+$ | NADPH |
|---|---|---|---|---|---|
| Controls | 492.55 | 13.68 | 36.01 | 23.55 | (0.84) |
| | (59.15) | (1.12) | (2.47) | (2.86) | 6.78 |
| mTBI-1 days-mTBI | 354.35[b] | 11.78 | 30.35 | 17.69[b] | 7.07 |
| | (34.08) | (2.07) | (2.11) | (2.51) | (0.53) |
| mTBI-2 days-mTBI | 286.48[b] | 9.88[b] | 29.00 | 12.90[b] | 8.44 |
| | (12.35) | (1.42) | (3.26) | (2.67) | (1.01) |
| mTBI-3 days-mTBI | 184.84[b] | 7.33[b] | 25.22[b] | 10.92[b] | 7.31 |
| | (49.80) | (0.51) | (2.64) | (1.59) | (0.92) |
| mTBI-4 days-mTBI | 310.13[b] | 9.30[b] | 33.35 | 16.10[b] | 8.34 |
| | (41.28) | (1.02) | (4.75) | (3.07) | (1.14) |
| mTBI-5 days-mTBI | 402.74 | 10.37[b] | 38.83 | 21.40 | 5.89 |
| | (27.85) | (0.97) | (3.70) | (2.15) | (0.44) |

[a] $NAD^+$, oxidized nicotinamide adenine dinucleotide; NADH, reduced nicotinamide adenine dinucleotide; $NADP^+$, oxidized nicotinamide adenine dinucleotide phosphate; NADPH, reduced nicotinamide adenine dinucleotide phosphate; mTBI, mild traumatic brain injury. Each value is the mean (standard deviation) of six different animals and is expressed as nmol/g wet weight.
[b] Significantly different from corresponding values recorded in controls, $P < 0.05$.

increasing the time interval between concussions. Again, minimal amounts of both oxidized nicotinic coenzymes were recorded when the second mTBI was delivered 3 days after the first impact (−62.47 and −53.63%, respectively; $P < 0.001$ with respect to controls), whereas values near to those of controls were observed in rats in Group 5 (mTBI-5 d-mTBI). Of the two reduced nicotinic coenzymes, NADPH did not show significant changes in any groups of animals. Conversely, NADH levels were significantly diminished in rats of Groups 2 (mTBI-2 d-mTBI), 3 (mTBI-3 d-mTBI), and 4 (mTBI-4 d-mTBI). As a consequence of the concomitant changes in $NAD^+$ and NADH, significant variations of the $NAD^+$/NADH ratio were observed in rats in Groups 2 (mTBI-2 d-mTBI) and 3 (mTBI-3 d-mTBI) only.

### Differential Severity of Metabolic Alterations

The different severity of metabolic brain alterations in hemisphere extracts (three right and three left hemispheres) of rats in which the second impact was delivered after 3 days is supported by data summarized in *Table 3*. For the sake of clarity, only values of ATP, NAA, NAAG, $NAD^+$, and acetyl-CoA are included. It is evident that no amelioration was produced by prolonging the time of recovery after the second impact up to 168 hours (7 d), thus indicating that metabolic activities, particularly those related to mitochondrial functions, were still severely altered at that time point in rats in Group 3 (mTBI-3 d-mTBI).

## DISCUSSION

Increasing focus on mTBI in both the clinical setting and in sports medicine has produced a wealth of information on postimpact brain metabolic changes. However, questions on when the brain has fully recovered from a concussive injury and what parameters are suitable for assessing such recovery are still unanswered. Both problems are at the center of a continuous debate in sports medicine surrounding an issue of fundamental importance for athletes who have experienced mTBI: when to return to play. Although obtained in an animal model, the data described in the present study provide a series of results that might be helpful in elucidating the aforementioned issues. With respect to our previous study (55), validating the hypothesis of metabolic vulnerability of the mildly injured brain proposed by Giza and Hovda (15) and Hovda et al. (20, 21), the present data allow us to make two main inferences: 1) the lag time between repeat mTBIs is the crucial factor affecting the reversibility of cerebral metabolic alterations; and 2) if a second mTBI takes place within the temporal window of metabolic vulnerability, severe, difficult to reverse brain damage will occur.

Several studies have been conducted in sports medicine to evaluate the differences between groups of athletes who experienced single or repeat mTBI (10, 17, 38, 46, 57). These reports described cumulative effect of multiple concussions, highlighting the possible occurrence of the second impact syndrome (7, 8, 10). It should, however, be recalled that there is debate about the existence of this particular clinical entity (34), although cases with catastrophic consequences have been reported (7, 8). At present, no indication of what determines the potential for catastrophic effects of cumulative mTBIs is available. According to our data, the time interval between concussions is the critical variable to decide the fate of the postconcussed brain; if a second mTBI occurs outside the window of metabolic brain vulnerability, then a full metabolic recovery is possible; other-

**TABLE 3. Concentrations of brain metabolites in rats subjected to repeat mild traumatic brain injuries 3 days apart; sacrificed 48, 120, or 168 hours after the last impact[a]**

| | ATP | NAA | NAAG | $NAD^+$ | Acetyl-CoA | ASPA expression |
|---|---|---|---|---|---|---|
| Sacrificed 48 hr after last mTBI | 1094.61 | 3808.8 | 303.94 | 184.84 | 9.41 | 3.91 |
| | (139.14) | (480.80) | (50.28) | (49.80) | (0.53) | (0.42) |
| Sacrificed 120 hr after last mTBI | 1246.84 | 3543.5 | 287.59 | 230.55 | 7.69 | 3.78 |
| | (207.31) | (340.82) | (53.12) | (61.31) | (1.51) | (0.55) |
| Sacrificed 168 hr after last mTBI | 988.45 | 3866.0 | 334.76 | 200.44 | 10.33 | 4.11 |
| | (142.54) | (235.81) | (71.58) | (36.92) | (2.09) | (0.79) |

[a] ATP, adenosine triphosphate; NAA, N-acetylaspartate; NAAG, N-acetylaspartylglutamate; NAD+, oxidized nicotinamide adenine dinucleotide; ASPA, N-acetylaspartate acylase; mTBI, mild traumatic brain injury. Each value is the mean (standard deviation) of six different animals. ATP, NAA, NAAG, NAD+, and acetyl-CoA are expressed as nmol/g wet weight. ASPA expression is calculated by considering the value of the gene expression of the housekeeping gene, hypoxanthine phosporibosyltransferase.

wise, the risk of irreversible brain metabolic damage increases significantly. We believe that the existence of such a temporal window is one of the most important findings of our study. This notion may be used as the basis for establishing the safe return to play of mildly head-injured athletes. However, translating these experimental time scales into human values is a rather complex matter. In general, the time scale of metabolic events in rats is much shorter than in humans, and it would not be unreasonable to assume that these periods of postinjury pathophysiological changes are longer lasting in humans (5, 6). The main implication of our experimental data is that within a reasonable timeframe of the injury, the metabolic effects of concussion are dangerously cumulative, although information on the kinetics of recovery of brain metabolites in humans during this time period is not available.

## The Apparently Invisible Post-concussive Damage

The data reported in this study indicate that a wide interrelated series of metabolites are deeply affected by the occurrence of a second mTBI within the brain vulnerability window. The majority of them have the common feature of being related to the mitochondrial activity of energy supply. All of them converge to cause what can be defined as "apparently invisible" post-concussive brain damage. Adenine nucleotides, NAA, acetyl-CoA, and oxidized nicotinic coenzymes all showed the same pattern of variation by extending the lag time between impacts, and all showed values similar to controls when the second concussion was delivered at 5 days. Of particular note are the variations of acetyl-CoA in view of its dual role as a fundamental compound for the reducing equivalent supply through the Krebs cycle activity and as the acetyl group donor in the NAA biosynthetic reaction. To our knowledge, this is the first report showing that cerebral acetyl-CoA concentration is remarkably decreased after repeat mTBI. The diminished availability of this compound should have a negative consequence on the continuous flow of NADH necessary for the electron transport chain, thus playing an important role in the deeply decreased mitochondrial phosphorylating capacity (decrease of the ATP/ADP ratio) and leading to a profound drop in ATP concentration as observed in Group 3 rats (mTBI-3 d-mTBI). The almost 50% decrease in NADH recorded in these animals is consistent with this hypothesis and corroborates the concept of a deep tricarboxylic acid cycle involvement in the energy state impairment. To this end, it is worth underlining that in our study, a slight but significant decrease of 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 $NAD^+$ level rather than to hypoxic/ischemic-like conditions as also indicated by the normal blood gases recorded also in these animals within the first 30 minutes of injury (data not shown).

Because brain metabolism is mainly based on glucose utilization, the main site of acetyl-CoA cerebral generation is at the pyruvate dehydrogenase level. According to previous results (47), pyruvate dehydrogenase is particularly sensitive to pathological conditions of increased oxidative stress, because this can markedly decrease the activity of this multienzymatic complex (47). On the other hand, in the reaction catalyzed by aspartate N-acetyltransferase, the enzyme responsible for NAA biosynthesis (35), low acetyl-CoA levels should certainly reduce the velocity of NAA production and contribute to the NAA depletion after repeat mTBI observed when the second concussion was delivered at 3 days. Therefore, acetyl-CoA availability might represent the phenomenon effectively linking the parallel NAA and ATP changes observed in many previous head injury studies (48, 49, 52, 55).

## Dynamics of Posttraumatic N-acetylaspartate Reduction

Based on the data on changes in ASPA gene expression, it appears that NAA variations are not simply attributed to a decreased rate of biosynthesis. In accordance with the hypothesis of different compartmentation for NAA biosynthesis (neuronal mitochondria) and degradation (oligodendrocytes) (2), it is conceivable that NAA decrease occurs in two distinct phases and with two different mechanisms: 1) in the first phase, independently from the severity of injury, a change in mitochondrial

permeability (13) causes an increased velocity of NAA outflow from neurons to the extracellular space. Simultaneously, mitochondrial impairment leads to diminished NAA synthesis. In the case of reversible brain damage such as single mTBI or repeat mTBIs in which the second impact occurs outside the brain vulnerability window, recovery of mitochondrial functions takes place with normalization of rate of NAA efflux and biosynthesis (NAA level close to controls and no increase in ASPA expression); 2) in single, severe TBI 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 gene 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. It is worth mentioning that this is the first report showing changes of ASPA gene expression in head injury. 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.

The reversibility of NAA depletion after mTBI and the availability of a noninvasive modality for measuring its cerebral level by $^1$H-NMR spectroscopy (9, 19, 31) seem to indicate in NAA measurement a valid diagnostic tool for answering the question of when to return to play after an mTBI. Combined with neuropsychological tests (12, 22, 23, 33, 40, 41, 45), $^1$H-NMR spectroscopy might represent a useful investigation to carry out on mTBI-affected athletes to ascertain the full recovery of cerebral metabolic functions before returning them to full activity. NAA, perfectly mirroring the metabolic changes of all the aforementioned energy-related biomarkers, ultimately reflects the overall cerebral metabolic status and we believe that the large scale application of $^1$H-NMR spectroscopic NAA evaluation would render visible the "apparently invisible" postconcussive brain damage.

## Significance of N-acetylaspartylglutamate Variations

Our data show, for the first time, that the second main cerebral N-acetylated amino acid (NAAG) was markedly affected by repeat mTBIs. Unlike NAA, several biological activities were clearly demonstrated for NAAG either under physiological (58) or pathological conditions (3, 37). With regard to head injury, the beneficial effects connected to the inhibition of glutamate carboxypeptidase II, also known as N-acetylated alpha-linked acidic dipeptidase, are of particular interest in models of neuropathies, stroke, and focal TBI (36, 59). 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 (3, 37, 50). Because glutamate release is a well-documented adverse phenomenon occurring even in mTBI (1, 43), it is plausible that the NAAG decrease (correlated with the lag time between repeat mTBIs) observed in our experiments might contribute to this mechanism, thus resulting in increased brain damage.

## The Brain Vulnerability Timing: A Stimulating Peculiarity

The results of the present study confirm previous data from our laboratory indicating that maximal metabolic alterations (highest decrease of NAA and energy-related metabolites) were recorded in animals receiving two mTBIs 3 days apart (55). However, it must be underlined that the present experiments revealed an apparent contradiction in the timing of the temporal window of brain vulnerability. In fact, under our experimental conditions, mitochondrial-related metabolic changes progressively increased with the time between concussions up to 3 days, when the maximal damaging effects of repeat mTBIs were observed. In this regard, it should be recalled that cerebral concussions produce functional but not anatomic damages. Bearing this in mind, the apparent paradox might be explained by assuming that the cell energy-dependent recovery processes are at their maximal intensity 3 days after mTBI. Therefore, a second impact at this time point has profound effects because of minimal "metabolic buffering capacity" to counteract all the known early changes of mTBI (15, 20, 21, 25, 28, 52, 54). On the other hand, this apparent paradox might well explain why athletes practicing certain types of sports, particularly boxing, in which they are exposed to repeat concussions do not experienced the so-called second impact syndrome. Too close concussions, occurring while boxing in the same match, very rarely produce (7) the same effects as two mTBIs occurring days or weeks apart, probably because in these latter cases, the second mTBI lies in the temporal window of brain vulnerability. This finding and the consequent explanation might help to overcome the skepticism about the real entity of second impact syndrome (34).

## Key Issues for Future Investigations

From the results presented in this study, some concluding remarks may be drawn: 1) in the clinical setting, more attention should be given to mTBI in view of a possible, however unlikely, second mTBI occurrence in the same patient within the temporal window of metabolic brain vulnerability; 2) in sports medicine, the realization of such a temporal window, the existence of a biochemical marker suitable for monitoring the cerebral metabolic status (NAA), the availability of a rapid, noninvasive, ready-to-use technique for assessing such a parameter in humans ($^1$H-NMR spectroscopy), suggest the use of NAA evaluation by $^1$H-NMR spectroscopy (in combination with neuropsychological tests, standard clinical examination, and symptom checklists) as a discriminating diagnostic tool for establishing when to return concussed athletes to play. This technique might also be useful in validating neuropsychological tests in view of recent concerns raised on the reliability of these as the main guide for establishing the full recovery after concussion in athletes (42). Moreover, NAA evaluation by $^1$H-NMR spectroscopy might also be helpful in improving the management and evaluation of mTBI in the clinical setting (39); 3) most of the biochemical changes occurring in TBI have the mitochondria as the main actor: reversing mitochondrial impairment signifies a favorable outcome; 4) once more, TBI appears as a multifaceted pathological entity requiring simultaneous administration of different new drugs in a multistrat-

egy, yet unexplored, pharmacological approach (according to what we report, to include antioxidants to protect pyruvate dehydrogenase, glycolytic stimulators to increase pyruvate generation, and oxidative phosphorylation-independent ATP production, stimulators of mitochondrial metabolism, inhibitors of NADH-glycohydrolase, and poly-ADP-ribose polymerase to protect $NAD^+$ concentration, protectors of mitochondrial ionic permeability to counteract neuronal NAA efflux, inhibitors of glutamate carboxypeptidase II to reduce NAAG-deriving glutamate formation). In our opinion, efforts should be put in evaluating the effects of such a multiple, metabolism-protective, pharmacological approach in various models of TBI.

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## Acknowledgments

This work has been supported in part by research funds of University of Rome Tor Vergata, Catholic University of Rome, and University of Catania.


# COMMENTS

Delineation of the degree and extent of mitochondrial dysfunction is currently a hot topic in current neurotrauma research. These authors provide some novel findings with respect to prolonged and perhaps irreversible mitochondrial dysfunction after repeated mild traumatic brain injury (TBI).

The "window of vulnerability" defined in this study, maximum effects seen when injuries were separated by 3 days, is difficult to explain. The authors postulate that this represents a "timepoint of minimal metabolic buffering capacity," but this is highly speculative and runs counter to other studies that show maximal metabolic/neurotransmitter/cerebral blood flow derangements within 12 to 48 hours after minor TBI, with subsequent progressive normalization. Additionally, the authors' contention that their data corroborate "severe, difficult to reverse brain damage" with a second injury in their window of vulnerability requires close scrutiny, as there were no functional measures undertaken and all animals were sacrificed within 48 hours of the last impact injury.

The potential use of proton magnetic spectroscopy in patients/athletes to define complete metabolic recovery after minor TBI is certainly appealing. Given the recent controversy over the National Football League-funded concussion studies and the limitations of neuropsychological testing, having an objective basis on which to make return-to-activity/play recommendations would significantly advance our clinical care of these patients.


**Jack E. Wilberger**
*Pittsburgh, Pennsylvania*


This latest report of the authors' work in this area raises several intriguing questions. One point is the potential utility of 1H-nuclear magnetic resonance spectroscopy for measuring N-acetyl aspartate levels as an indicator of an athlete's readiness for safe return to competition after brain injury. Another issue is the suggestion that a second blow to the head immediately after the first, as in boxing, may not be as potentially dangerous as delivery of the second blow at a specific time interval after the first, when the brain is in the window of vulnerability. Such innovative concepts represent ripe areas for future inquiry.


**Alex B. Valadka**
*Houston, Texas*


The authors report neurochemical results obtained from rodents after enduring two experimental concussions separated by 1, 2, 3, 4, or 5 days. Besides confirming that the brain is vulnerable to a second insult, the results indicate that apparently the degree of vulnerability is greatest for mitochondrial function at 3 days after the first injury. Furthermore, when the second concussion occurs 3 days after the first one, the resulting neurochemical markers of mitochondrial dysfunction persisted for at least 7 days.

Within the field of neurotrauma, little basic experimental neuroscience has been directed toward the topic of mild TBI. Clinically referred to as a concussion, mild TBI has been reported to have consequences affecting cellular function even in the absence of a loss of consciousness or cell death. This fact has produced great concern in the field of sports medicine, in which physicians try to determine guidelines from which to advise athletes to return to play after mild TBI. In

addition, they are faced with advising athletes when it is time for them to retire from their respective sport given the number of mild TBIs. These two issues (return-to-play and retirement) are still debated in the clinical neurological and neurosurgical literature. Consequently, the work described in the current study by Vagnozzi et al. has important clinical relevance. The degree and extent of neurochemical change and the corresponding neuronal (and glial) consequences have yet to be evaluated. However, the demonstration that mitochondrial function appears to be compromised to a greater degree when a second insult occurs is very enlightening.

In general, most scientists would hypothesize that the degree of vulnerability would be greatest immediately after the first insult. Although Vagnozzi et al. report vulnerability 24 hours after the first injury, their results suggest that postinjury Day 3 is when the brain exhibits its greatest degree of vulnerability. It is not known whether this "delayed" response primarily reflects the neurochemistry measured or if this is a common finding across all measures of cellular vulnerability. It has been well documented that neurochemical and neurometabolic cascades are initiated after a single mild TBI. These cascades have both a temporal and regional component. It is, therefore, possible that as one or more of these cascades take their normal postinjury course, an accumulation of factors and/or ions summate to achieve a heightened level of cellular crisis which, in the case of the current work, reached its peak at 3 days postinjury.

The animal modeling of repeated mild TBI is very challenging. The first insult necessarily causes changes with the brain that could have a direct effect on the biomechanics of the secondary insult. For example, the primary insult could cause changes in cerebral compliance and/or vascular responsiveness. Both of which could make the second biomechanical load much different in terms of severity or extent of tissue involvement. This leads to the concept of primary injury severity dictating the degree and extent of secondary vulnerability, which was not addressed by Vagnozzi et al., but what appears to be an accepted principle in the clinical literature. What Vagnozzi et al. did manipulate was time between insults, which, in and of itself, is an important experimental design. The overriding effect of injury severity as it interacts with time has yet to be comprehensively addressed.

In summary, the neurochemical results reported by Vagnozzi et al. appear to be sensitive and robust. These results support the conclusion that after a second concussion, neurochemical markers of mitochondrial function reveal a disturbance. In addition, this disturbance is most pronounced when insults are spaced apart by 3 days. Clearly, these data would suggest that, at the very least, cells are compromised in their ability to function and that they may have significant mitochondrial dysfunction, which can lead to their death.

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