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High resolution nuclear magnetic resonance investigation of metabolic disturbances induced by focal traumatic brain injury in a rat model: a pilot study

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ABSTRACT

Experimental models of traumatic brain injury (TBI) provide a useful tool for understanding the cerebral metabolic changes induced by this pathological condition. Here, we report on the time course of changes in cerebral metabolites after TBI using high-resolution proton magnetic resonance spectroscopy (NMR). Extracts from adult male Sprague-Dawley rats were subjected to fluid lateral percussion and were then examined by NMR at 3, 24 and 48 h after the injury. A metabolomic approach was carried out to identify the cerebral metabolites impacted by the TBI and their quantitative temporal changes. Lactate, valine and ascorbate were the three first metabolites to be significantly modified after TBI. The quantitative elevation for these compounds last for the entire experimental time explored. Within 24 hours post-TBI, a significant elevation in choline-derivates, alanine and glucose were also measured. On the other hand, N-acetyl aspartate, a neuronal marker, and *myo*-inositol, an important organic osmolyte in the mammalian brain, were not significantly impacted in the chronic phase of TBI.

Keywords: TBI; 1H-NMR Spectroscopy; Metabolomic; Traumatic Brain

1. INTRODUCTION

Traumatic brain injury (TBI) is a worldwide problem that results in death and disability for millions of people every year. Currently, in industrialized countries it is estimated that TBI is responsible for 0.15-0.2% of deaths and that 0.2-0.3% of the population lives with

permanent disabilities [1-3]. As a result, there is real need for improved diagnosis [4], treatment and strategies for rehabilitation post TBI.

Clinical and preclinical studies have now established that brain trauma is a dynamic process characterized by two waves of lesions. The first wave corresponds to the immediate mechanical damage to the central nervous system (CNS) that occurs at the moment of impact, and the second wave, initiated at the moment of the traumatic insult, will progress over a period of time ranging from hours to days after injury [5-7]. The most common and serious consequence of TBI is then, the development of a brain edema, usually associated with a poor neurological outcome and the activation of multiple molecular pathways to counter-balance the insult [8-13]. MRS was proven useful to follow those changes; however, the limited spectral resolution associated to the low concentration of the brain metabolites limit the studies to few molecules [14]. High resolution NMR allows overcoming those drawbacks and may be used to address those metabolic modifications, often complex and implying numerous metabolic pathways [15-18]. Metabolomics has recently emerged as a powerful approach for the characterisation of the metabolic responses to stress or diseases from MS or LC data [19-21] as well as from high resolution NMR data [16,22-26]. In the present work, we took advantage of 1H-NMR potentials in term of easiness of sample preparation, sensitivity, reproducibility, high-throughput analysis without metabolites separation as well as the quantitative information that one can easily access to, especially using the ERETIC method [27].

Experimental TBI can be performed by numerous

methods and lead to either focal or diffuse brain lesions [28] with their own metabolic impairments [15,29,30]. In the presented study, we have chosen the fluid lateral percussion model that appears to be [31] the most used model and for which morphological, histo-patho-physiological, behavioural, cognitive and even biochemical data mainly on excitotoxic molecules are available [32]. Recently, two complementary works [16,31] dealing with the fluid lateral percussion model in mature rats were presented. In one hand, a metabolomic approach in the acute phase of the trauma, *i.e.* 1 hour post insult was reported, whereas on the other hand a longitudinal follow-up of the brain trauma showed a significant evolution in term of blood brain barrier permeability, water diffusion properties and blood perfusion of the brain. Taken all together, we have then investigated the metabolic changes associated with brain trauma evolution using a metabolomic approach.

2. EXPERIMENTAL

2.1. Animals and Lateral Fluid Percussion

Animal care was carried out in compliance with the relevant European Community regulations (Official Journal of European Community L358 12/18/1986).

230-270 g female Sprague-Dawley rats were supplied by Angers University Hospital animal facility; they were anaesthetized with isoflurane via a stereotactic compatible nose cone (Minerve, Esternay, France). Once induced, the animal was placed in a stereotactic frame and brain trauma was induced as previously described [31]. Briefly, a scalp incision was made, the scalp and temporal muscles were reflected, and a 2.5 mm craniotomy was carried out above the left auditory cortex, 2 mm posterior to the lateral suture. A fitting tube, connected to the fluid lateral percussion device was cemented into the open craniotomy site. A 20 ms pulse at a pressure of 2.0 ± 0.1 atm induced fluid lateral percussion (FLP) brain injury. Immediately after fluid lateral percussion, the scalp incision was sutured and the rats were allowed to recover from anaesthesia. Normothermia was maintained through the use of a heating pad placed under the animal during all surgical procedures and in the acute post-injury period. The temperature was maintained between 36.5°C and 37.5°C.

Thereafter, rats were housed in temperature- and light-controlled conditions with food and water *ad libitum*. Sham-operated rats ($n = 3$) underwent the same surgery except for percussion. Three hours ($n = 3$), 24 hours ($n = 3$) and 48 hours ($n = 3$) post TBI, rats were re-anaesthetised with isoflurane. Rapid brain removal was then performed and 2-3 mm slice were dissected directly over craniectomy, the ipsi and the contralateral brain were separated. Samples were frozen in liquid nitrogen, weighted

and lyophilised. Dried brain was stored at -80°C until processing.

2.2. Tissue and Biofluids Preparation for NMR Spectroscopy

Dried brain tissues (~100 mg) were grounded in liquid nitrogen prior extraction in 5 mL of acetonitrile/water 50/50 in an ice/water bath. The homogenates were centrifuged at 400 g for 10 min at 4°C. Pellets were washed once with 3 mL of acetonitrile/water 50/50 and both supernatants were pooled and lyophilised before being reconstituted in 0.8 mL D₂O containing 0.05 wt% 3-(trimethylsilyl) propionic $-2,2,3,3\text{-d}_4$ acid (TSP). ¹H NMR spectra of tissue extract were measured at 500.13 MHz using an Avance 500 SB spectrometer with 5 mm broadband inverse probe (BBI) (Bruker Biospin, Wiessembourg, France) with non spinning samples and maintained at 298 K. One dimensional (1D) spectra were collected into 32 K data points with a spectral width of 5000 Hz and a total acquisition time of 2 min for 16 averages. Spectra were obtained using 1D version of the NOESY pulse sequence with an acquisition time of 3.28 s, a 1.5 s relaxation delay and a 40 dB field strength irradiation of the water signal during a 2.5 s presaturation delay and a 100 ms mixing delay.

All datasets were zero-filled to 64 K points and exponential line broadenings of 0.5 Hz applied before Fourier transformation. The resulting spectra were manually phased. The baseline was corrected using a quadratic function and chemical shift were calibrated using the TSP signal. Peaks were assigned using the Human Metabolome Database [33], the Magnetic resonance Metabolomics Database [34], and performing TOCSY 2D spectrum when necessary. The quantitative process was performed using MestReC 4.9.9.6 Software as previously described [35].

2.3. Post-Processing of NMR Spectra

2.3.1. Multivariate Spectral Analysis

NMR data were reduced into equidistant integral region (bucket) of the spectra (0.8 to 9.0 ppm) with a bucket width of 0.03 ppm. The spectral region from 4.50-5.50 ppm was excluded to remove variability due to suppression of the water resonance signal. Each region was integrated with AMIX software (version 3.7.10, Bruker, Karlsruhe, Germany). Each bucket was represented as the ratio of the total integral of all individual regions (X variables) to normalize for the dilution between individual samples. Partial Least Square Discriminant Analysis (PLS-DA) was performed with SIMCA P 11.0 software (Umetrics, Umea, Sweden). PLS-DA was employed as a supervised method, requiring a training set, useful for small data sets with many more variables than samples. Data were pre-processed using Pareto scaling to separate

samples according to the maximum variance detected in correlated metabolites favoring large changes. Spectral variation was reduced to a series of variables (t) explaining the largest variation in the X space, each representing correlated spectral change, and summarized in a score plot. Validation of PLS-DA was controlled with the cumulative fraction of X variation (R2X) and Y variations (R2Y), corresponding to the different classes of samples, in the three firsts components, and with the cumulative overall cross validation (Q2) modeled in the three firsts components. When the scores plot showed separated groups, a contribution plot was performed to evidence the variables that deviated from the average and contributed to the separation of the groups. Spectral regions were investigated to identify the metabolites responsible for the classification. Identification of the metabolites was performed using the Human Metabolome Database [33] and the Magnetic Resonance Metabolomics Database [34].

2.3.2. Multivariate Spectral Analysis

Quantification of metabolite peaks was performed with the ERETIC peak as a quantitative reference [27]. Calibration of this peak, which had the same intensity in all spectra of brain extracts, was made with reference to a 2 mM creatine solution. Metabolite concentration was calculated according to the following equation:

$$C_X = \frac{A_X}{A_E \cdot N_X} \cdot \frac{A_{ER} \cdot N_R}{A_R} \cdot C_R \cdot \frac{V_{D2O}}{W_{DT}}$$

where C_X is the concentration of the metabolite, N_X is the number of protons for the frequency of the peak quantified, and A_X and A_E are the areas of the metabolite and

ERETIC peaks, respectively, in the spectrum. A_R and A_{ER} are the measured areas of the creatine and ERETIC peaks, respectively, for the creatine reference, N_R is the number of protons resonating for creatine, C_R is the concentration of creatine (2 mM). The concentrations of each metabolites was then calculated in μ moles by gram of dried tissue taking account the weight of dried tissue (W_{DT}) and the volume of D_2O (V_{D2O}) for the reconstitution of the lyophilized sample.

3. RESULTS

PLS-DA score plots show evidence for a separation between tissue extracted at the site of TBI with respect to time (**Figure 1(a)**), and tissue extracted at the opposite with respect to time (**Figure 1(b)**). For the two 3D score plot the cross validation score (Q2) is at least equal to 50% of the fraction of Y variation (R2Y) confirming the robustness of the model used. Difference between ipsi and contralateral brain content, for each time point, was evaluated using PLS-DA score plots and is presented in **Figure 2**. At all experimental times, contra and ipsilateral brain separation were confirmed by high Q2 values. Indeed, at 3, 24 and 48 hours the cross-validation score were respectively equal to $Q2 = 80.2\%$; $Q2 = 98.8\%$ and $Q2 = 92.5\%$. Using loading plots corresponding to these score plots, four metabolites influencing the separation were identified and selected for a quantitative analysis. Those metabolites were identified as valine H3 (-CH₃, 1.07 ppm), lactate H3 (-CH₃, 1.33 ppm), alanine H3 (-CH₃, 1.44 ppm) and choline-derivates (-N-(CH₃)₃,

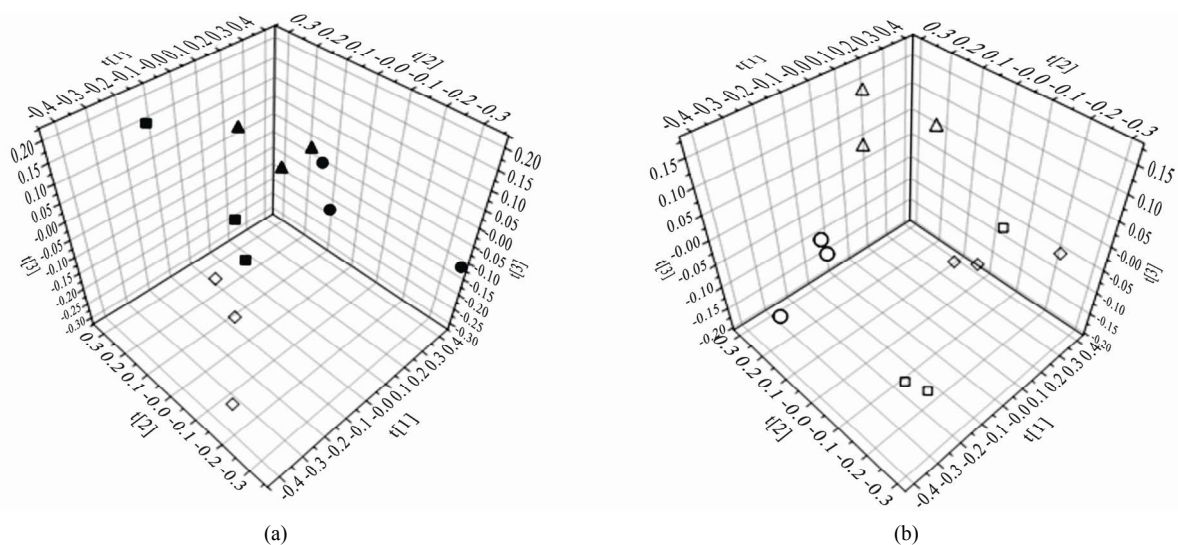


Figure 1. Score plots of the NMR spectra of tissue extracts. Each point corresponds to a spectrum after a bucketing of 0.03 ppm. (a) score plot showing the differences between the four classes (Y variable) sham (\diamond), ipsilateral at 3 hours (\blacksquare) 24 hours (\bullet) and 48 hours (\blacktriangle); $R2X = 72.3\%$, $R2Y = 98.3\%$ and $Q2 = 92.5\%$. (b) score plot showing the differences between the four classes sham (\diamond), contralateral at 3 hours (\square), 24 hours (\circ) and 48 hours (\triangle); $R2X = 71.6\%$, $R2Y = 82.2\%$ and $Q2 = 44.1\%$.

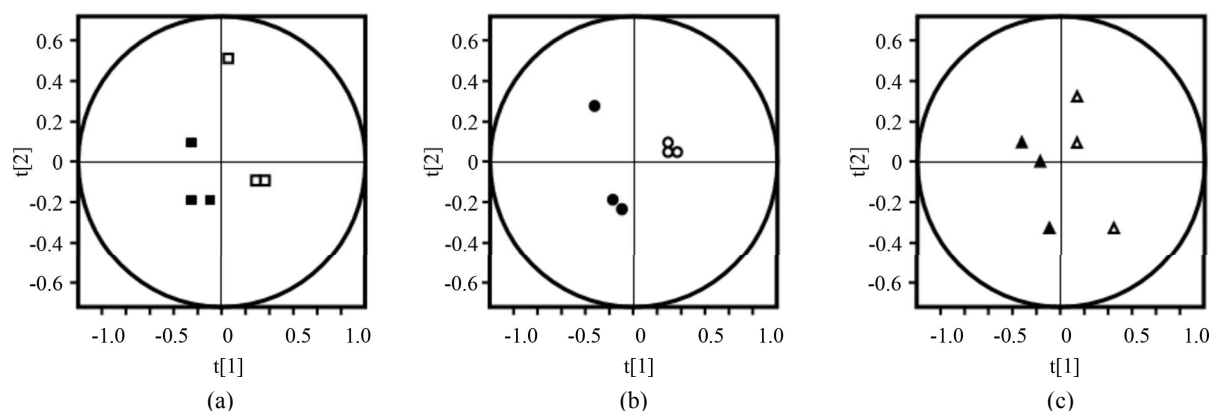


Figure 2. Score plots of the NMR spectra of tissue extracts evidencing the difference between ipsi and contralateral for each experiment times; (a) ipsi (■) and contralateral (□), for 3 hours, R2X = 79.3%, R2Y = 95.7%, Q2 = 80.2%; (b) ipsi (●) and contralateral (○) for 24 hours, R2X = 70.7%, R2Y = 99.9%, Q2 = 98.8%; (c) ipsi (▲) and contralateral (△) for 48 hours, R2X = 72.3%, R2Y = 98.3%, Q2 = 92.5%.

3.19-3.23 ppm). However as the number of data set included for the metabolomic analysis is limited, a quantitative analysis of eleven metabolites that can be identified from the high resolution 1H-NMR spectrum of a rat brains (**Figure 3**), was also performed. Those metabolites were N-acetyl aspartate H6 (-CH₃, 2.00 ppm), GABA H4 (-CH₂, 2.28 ppm), glutamate H3 and H4 (-CH₂, 2.35 ppm), succinate H2 (-CH₂, 2.42 ppm), citrate H3 (CH₂, 2.82 ppm), alpha-ketoglutarate H4 (-CH₂, 3.01 ppm), creatine/Phosphocreatine (N-CH₃, 3.04 ppm), taurine H3 (CH₂-N, 3.42 ppm), myo-inositol H2(CHOH, 4.05 ppm), ascorbate (H5 -CH 4.52 ppm), glucose-1-β

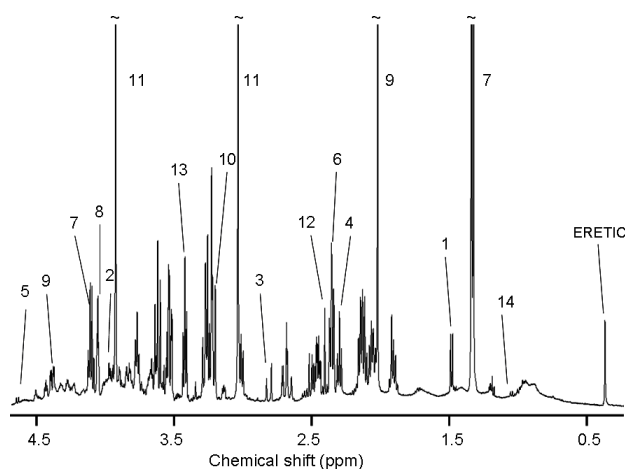


Figure 3. Section of a representative 1H NMR spectrum of the polar metabolites extracted from rat brain 48 hours post-TBI. The metabolites quantified in **Table 1** are assigned as (1) alanine, (2) ascorbate, (3) citrate, (4) γ -aminobutyric acid, (5) glucose, (6) glutamate, (7) lactate, (8) myo-inositol, (9) N-acetylaspartate, (10) phosphocholine and glycerophosphocholine, (11) phosphocreatine and creatine, (12) succinate (13) taurine and (14) valine.

(5.2 ppm). **Table 1** summarizes the quantitative analysis of 1H NMR observable metabolites content in the brain extracts obtained from animals at increasing times after TBI. Absolute concentrations are presented as mean \pm sd and expressed in μ mol per g of dry tissue as we have previously shown that in this TBI model, the brain water content evolves with time [31]. Valine, lactate, alanine, choline derivatives, ascorbate and glucose appeared significantly modified with TBI.

4. DISCUSSION

The primary injury induced by TBI consists of a rapid deformation of the brain, leading to rupture of cell membranes, escape of intracellular contents, and disruption of blood flow, resulting in necrotic cell death. Secondly a complex series of biochemical, structural and molecular changes lead to cellular damage and loss. The main biochemical perturbations associated with TBI involve the release of excitotoxic glutamate, the production of reactive oxygen species, the disruption of membranes, the impairment of mitochondrial functions and the neuronal death.

4.1. Excitotoxic Injury

Pathologic release of excitatory amino acid neurotransmitters such as glutamate or aspartate have been reported both in animal models [9,36] and humans [37]. The subsequent activation of glutamate receptors, results in the influx of Na⁺, efflux of K⁺, and subsequent Ca²⁺ influx into the cell causing cellular swelling (cytotoxic edema) and the excitotoxic destruction of cells through direct or indirect pathways [32]. MR studies have although always failed to demonstrate a significant initial increase in glutamate level after focal or diffuse TBI, and when modifications of glutamate levels were seen, a significant

Table 1. Metabolite NMR assignments and their concentrations (expressed in $\mu\text{mol/g}$ dried tissue) in rat brain as a function of time post-TBI. Data are presented as mean \pm SD. Significant differences (ANOVA) are indicated in boldface if values are different from t_0 and/or italic if different from the contralateral at the same time point.

Metabolite	Group and chemical shift	Sham	3 hours		24 hours		48 hours	
			Traumatized Brain	Contralateral Brain	Traumatized Brain	Contralateral Brain	Traumatized Brain	Contralateral Brain
Valine	1.07; dd; -CH3	0.06 \pm 0.01	<i>0.11 \pm 0.03</i>	0.06 \pm 0.01	<i>0.09 \pm 0.01</i>	0.06 \pm 0.01	<i>0.09 \pm 0.01</i>	0.08 \pm 0.02
Lactate	1.33; d; -CH3	9.05 \pm 0.77	10.84 \pm 1.64	9.73 \pm 1.64	14.01 \pm 1.28	12.54 \pm 1.56	14.70 \pm 0.77	12.02 \pm 1.09
Alanine	1.44; d; -CH3	0.63 \pm 0.15	0.81 \pm 0.37	0.65 \pm 0.04	0.93 \pm 0.03	0.65 \pm 0.04	0.84 \pm 0.12	0.66 \pm 0.06
NAA	2.00; s; -CH3	3.67 \pm 0.76	3.84 \pm 0.65	3.58 \pm 1.27	2.77 \pm 0.83	4.43 \pm 0.13	3.08 \pm 0.47	3.42 \pm 0.93
GABA	2.28; t; -CH2	2.11 \pm 0.69	2.02 \pm 0.82	2.22 \pm 1.02	2.04 \pm 0.51	2.27 \pm 0.23	2.01 \pm 0.33	2.28 \pm 0.49
Glutamate	2.35; t; -CH2	5.97 \pm 0.74	6.15 \pm 1.00	5.88 \pm 0.73	5.78 \pm 0.90	6.55 \pm 0.59	6.39 \pm 0.13	6.85 \pm 0.75
Succinate	2.42; s; -CH2	0.75 \pm 0.30	0.83 \pm 0.27	0.80 \pm 0.31	0.93 \pm 0.15	0.96 \pm 0.07	0.87 \pm 0.15	0.84 \pm 0.15
Citrate	2.82; dd; -CH2	1.00 \pm 0.27	0.92 \pm 0.10	1.22 \pm 0.31	0.83 \pm 0.02	0.87 \pm 0.06	1.04 \pm 0.28	1.51 \pm 0.53
PCr and Cr	3.04; s; N-CH3	4.92 \pm 0.95	5.29 \pm 1.04	5.28 \pm 1.29	4.79 \pm 0.54	5.72 \pm 0.46	5.23 \pm 0.44	5.69 \pm 0.30
PC and GPC	3.20; s; N-(CH3) ₃	0.43 \pm 0.12	0.48 \pm 0.11	0.41 \pm 0.13	0.47 \pm 0.04	0.46 \pm 0.04	0.63 \pm 0.07	0.52 \pm 0.16
Taurine	3.42; t; N-CH2	2.64 \pm 0.66	3.00 \pm 0.74	2.99 \pm 0.58	3.00 \pm 0.29	3.35 \pm 0.14	3.32 \pm 0.09	3.44 \pm 0.35
Myoinositol	4.05; t; -CHOH	5.01 \pm 3.04	5.07 \pm 1.40	6.22 \pm 2.05	4.66 \pm 0.85	4.86 \pm 0.59	4.77 \pm 0.50	5.86 \pm 1.69
Ascorbate	4.52; d; -CH	0.03 \pm 0.05	0.19 \pm 0.14	0.08 \pm 0.13	0.13 \pm 0.01	0.22 \pm 0.05	0.17 \pm 0.02	0.19 \pm 0.06
Glucose	5.20; d; -CH	0	0	0	0.25 \pm 0.09	0	0.17 \pm 0.14	0

decrease [16,38], ranging from -40% to -15% was observed. In the present study, the glutamate appeared not significantly modified regardless of the experimental time. The discrepancy between glutamate levels determined using NMR and other quantitative techniques such as microdialysis may be discussed according to the pool of glutamate assessed. First of all, microdialysis measured glutamate level corresponding to the extracellular pool of this compound whereas the NMR glutamate level measured on tissue extract corresponds to the total glutamate, *i.e.*, the extracellular pool and the intra-neuronal pool, which taken as an all cannot be significantly changed, at least in the early phase of the injury. Later on, the extracellular glutamate which has moved from the neurons to the extracellular space, may be up-taken by astrocytes and converted to glutamine [39] leading to an overall decrease in glutamate level as revealed using NMR.

4.2. Oxidative Stress

Despite a short half-life in biological tissue but according to their high reactivity, oxygen species such as superoxide anion, hydroxylradical, peroxynitrite and nitric oxide produce in TBI induce brain tissue oxidative damage [40,41]. It is therefore important to evaluate the levels of the two main water-soluble antioxidants, *i.e.* glutathione and ascorbate. However and with respect to the low concentration of glutathione [42], this compound is not detectable in this study as in a previous one using NMR [16]. Concerning ascorbate, a significant increase is detectable at all time points in the traumatized brain but also in the contralateral hemisphere at day 1 and day 2 compared to the ascorbate level measured in shams. The increase of ascorbate has never been observed and is striking as it suggests a massive *de novo* synthesis of

ascorbate [41,42]. It has previously been shown that an increase in extracellular ascorbate is observed after TBI as the result of the release of an intracellular pool [43] and in order to limit the oxidative damages linked to the potential pro-oxidant interactions with metal ions that are released as tissue damage occur [40,41,44]. However, when working with tissues extracts, the measured ascorbate corresponds to the total pool, *i.e.* intra and extracellular, and therefore, the increase must correspond to an influx/*de novo* synthesis of ascorbate to the brain. Nevertheless, one may evoked the circadian evolution of ascorbate as a potential artefact even though the drop from maximal value during the night to minimal value during the day occurs within 3 hours [45] and that all rats were operated at least 2 hours after the day-cycle was powered on.

4.3. Membrane Markers

In the very early phase after TBI, clear evidence for a decrease in the levels of phosphocholine and glycerophosphocholine were previously reported [16,38], evidences that only occurred during the acute phase. Indeed, as soon as 3 hours post TBI, phosphocholine and glycerophosphocholine levels recover there pre-TBI levels prior to significantly increase with time [16,38,42]. In the present study, the same pattern is observed with no statistical changes 3 hours post trauma followed by a significant increase that reaches 50% within 2 days. In the initial acute phase, primary lesions result in membrane disruption and ions leakage that may activate for example Ca^{2+} dependant phospholipases and lead to a global decrease of the choline pool. In the chronic phase, the choline elevation was attributed to larger membrane degradation/repair, demyelination, inflammatory reaction and glial reaction [14,46,47]. Gliosis is associated

with elevation in inositol level and is therefore observed in the late phase post TBI [14,48] even though astrocytes damage are reported as early as 30 min post FLP induced TBI [49]. In our experiment, variations were limited in magnitude (ca. 10%) and never significant.

4.4. Neuronal Damage

The most prominent peak of the normal high resolution 1H-NMR spectra is that of NAA. This compound is known since long to be located within neurons and is therefore traditionally used as marker of neuronal integrity [50]. In the acute phase, NAA remained unchanged in the ipsi lateral brain but, even though not significant, decreased later on by about 20%. In previous studies, NAA was reported reduced as early as 1 hour post TBI [14,16] (range -15; -60%) and associated with neuronal injury [14]. Differences in magnitude and dynamic of NAA change may reflect the milder traumatic model used in the present study. Even though extracellular NAA is transported into astrocytes [51] to be rapidly hydrolyzed into acetate and aspartate [52], none of these catabolites were changed after TBI.

4.5. Energy Metabolism and Lactate

FLP induced TBI was demonstrated to induce a transient but significant 50% reduction in cerebral blood flow (CBF) within the ipsilateral brain, and a chronic reduction of up to 80% in contralateral hemisphere [31]. Despite the CBF reduction in the contralateral brain, and apart from lactate and ascorbate, none of the measured metabolites were significantly impaired, suggesting that the transient hypoperfusion was not deleterious for the brain. However, in the traumatized brain, the mechanical insult associated with the hypoperfusion induces perturbation in the energy metabolism. Very prominent is the marked and constantly increased characteristic doublet around 1.32 ppm, corresponding to lactate. Different sources for lactate after trauma are known. Initially, a continuous production of lactate via glycolysis might occur. Indeed, the increased energy demand to re-establish ionic homeostasis leads to increased glucose utilization, resulting in an increase in lactate [53]. Moreover, lactate may also arise from astrocytes metabolism through the astrocyte-neuron lactate shuttle or a Ca²⁺ mediated mitochondrial dysfunction with impaired ATP production of the respiratory chain was reported in TBI [14,54]. Even though possible, the contribution of infiltrated inflammatory cells or of necrotic contusion core, to lactate production may be marginal as neither are massively present in this TBI model [31]. Finally, as the time between TBI and the NMR analysis increase, free glucose is detected in the traumatized cortex as previously shown using ¹³C-labeled glucose [29].

4.6. GABA and Other Biomarkers

GABAergic neurotransmission has been extensively studied following TBI [55,56] and extracellular level of GABA following cortical TBI contusion in rats were proven either increased or stable [9,16,17,57]. For example, in a recent study where metabolites levels were followed with a high temporal resolution, a 25-fold increase in protective GABA was measured 15 min post TBI, to recover a pre-TBI within 45-60 min [9]. In the present study, GABA level was not significantly modified at the time points studied. On the other hand, and as previously reported, levels of numerous amino-acid are affected in focal or diffuse brain trauma [16,17]. In this longitudinal study, levels of valine and alanine were significantly increased with time.

4.7. Conclusions

Numerous studies have looked at metabolite profiles following TBI and complementary or opposite data are available. Two main points have to be highlighted once dealing with TBI. On one hand, the type of TBI, *i.e.* diffuse or focal and on the other hand the timing between TBI and analysis. In the first half-hour post-TBI, irrespective of the model, major changes occur such as hemodynamic drop [31,58], Blood Brain Barrier permeation [59] and metabolic perturbations [9,16]. For the latest, a substantial increase in metabolites is usually observed corresponding probably to the leakage from the intracellular space leading to the aggravation of the initial lesion.

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