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The prognostic value of brain extracellular fluid nitric oxide metabolite after traumatic brain injury

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Keywords: Traumatic Brain Injury, Microdialysis, Nitric Oxide, Critical Care, Patient Monitoring

Abstract

Introduction: Nitric oxide (NO) is a compound with both protective and damaging effects on neurons. Quantification of NO metabolites in humans is limited by sample contamination with blood. *In vivo* cerebral microdialysis may offer an alternative approach as sampling of extracellular fluid (ECF) adjacent to neurons becomes possible. We investigate the prognostic value of brain ECF NO metabolites in patients with traumatic brain injury (TBI).

Methods: A prospective case cohort of 195 ECF samples collected from 11 cases over four days following TBI were collected. Nitrate and nitrite concentration ([NOx]) were quantified using a vanadium based colorimetric assay.

Results: Early ECF NOx levels (<48 hours post TBI) were significantly higher in non-survivors (median 59.2 µmol/L, n=7) compared to survivors (23.3 µmol/L, n=4)(p=0.04). Late (48-96 hours) ECF NOx levels remained higher in non-survivors 47.9 µmol/L compared to survivors 23.0 µmol/L but this was not of significant (p=0.29). Receiver operator characteristic analysis show an optimized cutoff level for ECF NOx of 26.5 µmol/L measured <48 hours post TBI for predicting non-survival (sensitivity 100%, specificity 75%).

Conclusion: Early ECF NOx levels are of prognostic value after TBI. ECF NOx may be a useful biomarker for treatment trials targeted at nitric oxide metabolism.

INTRODUCTION

Nitric oxide (NO) is a free radical involved in diverse physiological and pathophysiological processes in the central nervous system. After traumatic brain injury (TBI), NO may have both protective and damaging effects(1) and this relates to NO's role in cerebral blood flow regulation(2), its reaction with superoxide to form peroxynitrite, and its inhibition of cytochrome c oxidase(3). TBI remains the major cause of death in young people and survival of TBI is associated with significant morbidity. Improved prognostication after TBI might facilitate patient selection for aggressive management strategies, which may improve mortality but carry the risk of increased morbidity if patient selection is suboptimal.

Due to its short half life, it is difficult to measure nitric oxide concentration directly, but measurement of the concentration of downstream NO metabolites, nitrate and nitrite (NOx), is an accepted method of estimating endogenous NO production(4). NOx concentration ([NOx]) in the cerebrospinal fluid (CSF) of patients after TBI has been reported (5,6) but the presence of subarachnoid hemorrhage, as commonly occurs after TBI, can influence CSF [NOx] measurement(4) and might reduce the usefulness of CSF [NOx] as a marker of secondary injury.

Cerebral microdialysis is an established technique that allows focal measurement of brain extracellular fluid (ECF) biochemistry(7,8). As the microdialysis catheter is placed in brain tissue, it can be used to assess [NOx]

closer to NO's site of action than that measured in CSF and localise the measurement to a particular area of interest. Microdialysate [NOx] in the brains of rats post cortical impact injury correlates well with direct NO electrode readings(1).

In this study we measure the concentration of nitrite and nitrate in the brain extracellular fluid of patients with traumatic brain injury and compare it with patient outcome.

2. Materials and Methods

2.1 Patients and sample preparation

This study was approved by the Joint Research Ethics Committee of the National Hospital for Neurology and Neurosurgery and the Institute of Neurology and, as all patients were unconscious at the time of the study, written assent was obtained from their personal representatives.

We studied eleven adults with TBI undergoing protocolised management to control cerebral perfusion pressure (CPP) and intracranial pressure (ICP) on the neurocritical care unit. The patients were all male with median age 26.5 years (range 17-68). As part of routine clinical monitoring of brain ECF biochemistry each patient had a commercially available microdialysis catheter (CMA 70, CMA/Microdialysis, Solna, Sweden) with a 10 mm dialysis membrane length and 20 kDa molecular weight cut-off inserted into their brain tissue through a skull bolt (Technicam Ltd, Newton Abbott, UK). Probe positioning followed the recommendations of the consensus meeting on microdialysis in neurointensive

care(9). The probe was perfused with artificial CSF (Perfusion Fluid CNS, CMA), using a CMA 106 pump, at a rate of 0.3 µL/min.

Systemic data including CCP and ICP were collected electronically. After routine measurement of standard microdialysis variables, including lactate:pyruvate ratio (LPR), using the CMA 600 analyser (CMA/Microdialysis, Solna, Sweden), the microdialysate samples were stored at -80 ^{o}C prior to NOx assay.

Microdialysate sampling was continued until invasive intracranial monitoring was no longer clinically indicated and at that stage the microdialysis catheter was removed. In any event only samples from the first 4 days post injury were included in this analysis. Peak CSF [NOx] occurs within the first 48 hours post TBI(5,6) and so samples for analysis were divided into those taken up to 48 hours, and those taken 48-96 hours, post TBI.

2.2 NOx Assay

*W*e used a vanadium-based assay for nitrite/nitrate measurement in biological specimens as described by Miranda *et al*.(10).

Reagents: Sulfanilamide (SULF), *N*-(1-Napthyl) ethyl-enediamine dihydrochloride (NEDD), vanadium (III) chloride (VCl_3), sodium nitrite, sodium nitrate, hydrochloric acid were purchased from Sigma-Aldrich (Poole, Dorset, UK). Water was purified with Milli-RO and Millli-Q systems (Millipore).

The assay was performed in a standard flat-bottomed 96-well polystyrene microtitre plate, containing 50 µl/well of standard or sample in duplicate.

Immediately before commencing the assay, Griess reagents: SULF(50µl) and

NEDD(50µl) were premixed and added to each well. Nitrite mixed with Griess reagents forms a chromophore from the diazotization of sulfanilamide by acidic nitrite followed by coupling with bicyclic amines such as *N*-1-(Napthyl) ethylenediamine. Subsequently, the vanadium solution was added in a volume of 100 µl/well, the contents mixed vigorously and the plate left to incubate for 60 minutes at 37 ^{o}C. The absorbance at 540 nm was measured to assess the total level of nitrite and nitrate (NOx) in the samples.

2.3 Statistical Analysis

Data were analysed using SAS software (version 9.1, SAS Institute, Cary, NC, USA). Wilcoxon rank sum test was used to compare variables in survivors and non-survivors. Because of a non-Gaussian distribution the median (interquartile range) are presented. Receiver operator characteristic (ROC) analyses are used to determine the sensitivity and specificity of threshold variable values for non-survival. Two tailed exact p values are presented throughout and values of less than 0.05 are considered significant.

3. Results

195 microdialysate samples were analysed from 11 patients. Microdialysate [NOx] <48 hours post TBI was significantly higher in non-survivors (n=7, 59.2 (31.8-150.6) µmol/l compared to survivors 23.3 (15.2-32.9) µmol/l (n=4, p=0.04). Microdialysate [NOx] 48 to 96 hours post TBI was 23.0 (21.8-35.2) in survivors (n=4) and 47.9 (37.6-60.6) in non-survivors (n=5) (p=0.29) (figure 1). There was no significant difference in microdialysate [NOx]

over time for either subgroup although there was a trend toward microdialysate [NOx] increasing over time in survivors and decreasing over time in non-survivors. For the period <48 hours post TBI, receiver operator characteristic (ROC) analysis revealed microdialysate [NOx]>26.5 μmol/l predicted non-survival with sensitivity 100% and specificity 75% and the area under the curve was 0.89. For the period 48-96 hours post TBI microdialysate [NOx]>24.1 μmol/l predicted non-survival with sensitivity 100% and specificity 75% and the area under the curve was 0.75 (figure 2).

There were no significant differences in ICP, CPP or microdialysate lactate pyruvate ratio between survivors and non-survivors for either time period.

4. Discussion

This prospective study shows that it is feasible to reliably quantify NOx from minute amounts of human ECF using a rapid and high throughput technique. The main finding of this study is that ECF NOx samples in the initial 48 hours after TBI were of prognostic value. This data is in line with results from CSF [NOx] in humans after TBI (5). The main advantage ECF NOx levels have over CSF NOx levels is that sample contamination with systemic NOx levels coming from the blood is less likely.

The potential of this measurement is suggested by the fact that we saw significant differences in microdialysate [NOx] between the outcome groups in the period <48 hours post TBI whilst finding no differences in other measured variables. ROC analysis indicates effective discrimination between survivors and

non-survivors for microdialysate [NOx] <48 hours post TBI as indicated by the large area under the ROC plot. Interestingly this overlaps with the time interval of accelerated neuronal loss following TBI(8).

Our results suggest that microdialysate [NOx] might be a useful prognostic marker after TBI and that its prognostic value may be greatest early after injury, during the first 48 hrs in this study. This is important because if prognostication of patient outcome is to guide clinical decision making it must be possible to do so as soon as possible after TBI.

This study presents data from a small number of patients and the threshold value we suggest must therefore be viewed as a preliminary result. We present these results to demonstrate the prognostic potential of microdialysate [NOx] and for evaluation in future studies. These microdialysate concentrations are dependent on the relative recovery of NOx and hence the microdialysis catheter type and perfusate flow rate and in this study we use standard methods(7). Automatisation of the colorimetric method(10) used should be possible and thus allow for high throughput bedside measurements of ECF NOx levels from much larger patient cohorts.

The incidence of mortality after TBI in our cohort is high and we believe that this is related to our selection of patients for cerebral microdialysis monitoring. In our centre, patients in whom sedation is stopped shortly after admission following either conservative or surgical management are not monitored using cerebral microdialysis and this study cohort therefore represents the more severely injured end of the spectrum of our TBI admissions.

Microdialysate [NOx] has been previously correlated with regional cerebral blood flow in patients with TBI(2). In the above study no significant correlation was found between microdialysate [NOx] and neurological outcome, which is in apparent contrast with earlier results reported by the same group where the highest microdialysate [NOx] in the first 5 days after TBI were found in patients surviving with moderate or severe disability as opposed to non-survivors or those making a good recovery, with non-survivors having lower microdialysate [NOx] than survivors(11). This seems to be at variance with our findings, perhaps reflecting the complexity of NO's neuroprotective and neurotoxic roles, although our results are more consistent with previous CSF studies (5).

Animal and human studies suggest that the peak in NO occurring > 6 hours post TBI is primarily related to expression of inducible nitric oxide synthase (iNOS)(1) and, as none of our patients were studied <6 hours post TBI, the elevated microdialysate [NOx] we observed in non-survivors may also to be due to iNOS. Most studies suggest that NO produced by iNOS has detrimental effects and that inhibition of iNOS activity after TBI might have benefit(1), although this is not universally accepted. The present findings are in line with the basic science literature.

We would like to stress that, as NO is required for a number of vital physiological functions, any future treatment aiming to completely block NO production or receptor binding is likely to have deleterious effects. However, combining ECF NOx levels with a validated biomarker for neurodegeneration (eNfH)(8) might allow determination of the *in vivo* concentration at which

neurotoxicity occurs and thus help establish a target treatment threshold for NOx lowering strategies.

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Figure Legends

Figure 1: Microdialysate nitrite/nitrate concentration ([NOx]) for survivors and non-survivors of traumatic brain injury for the periods less than 48 hours, and 48-96 hours, post traumatic brain injury (ns=not significant).

Figure 2: Receiver operator characteristic analysis for microdialysate nitrite/nitrate concentration ([NOx]) as predictor of non-survival during the first 48 hours (A) and 48-96 hours (B) post traumatic brain injury.

Figure 1

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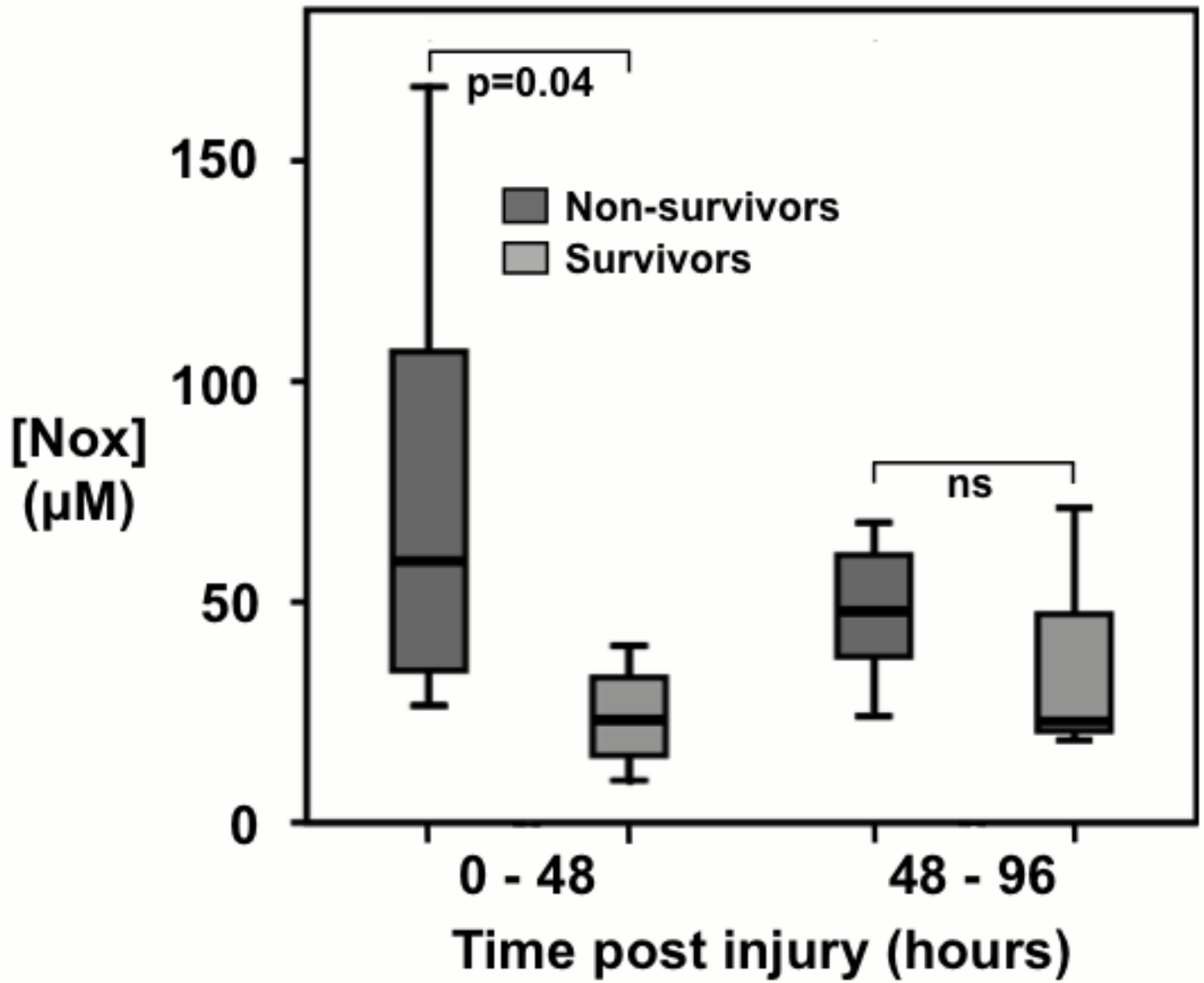

Figure 2

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