FINAL REPORT

PROJECT TITLE:	An investigation using microdialysis into traumatic brain injury (diffuse axonal injury) with and without hypoxia
ORGANISATION:	National Trauma Research Institute & Alfred Health
CHIEF INVESTIGATORS	Prof Jeffrey Rosenfeld & A/Prof Cristina Morganti-Kossmann
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1. Project findings (Scientific)

Background:
Traumatic brain injury (TBI) is the leading cause of mortality and morbidity in those under the age of 45 years, worldwide, ahead of HIV and malaria. Apart from the severity of the traumatic incident itself, there are numerous factors that may worsen the neurological outcome of the patients. Post-traumatic hypoxia is a well established insult that is known to extensively aggravate neurological outcome and increase mortality rates. Hypoxia, defined as a failure of ventilation, is a common sequel following trauma, whether it be due to lung contusions, flail chest injury or central apnoea resulting from the brain injury itself. The shortest episode of hypoxia after TBI has been reported to exacerbate adverse outcome. Studies investigating the effects of post-traumatic hypoxia have focused on histopathological, physiological and behavioural aspects and demonstrated worsened parenchymal damage, with prolonged cerebral oedema.

Neuroinflammation is common to both TBI and hypoxia, with activation of cytokine expression and cellular infiltration in the central nervous system (CNS) documented in both pathologies. Little is known about the synergistic effect of both TBI and hypoxia on the inflammatory reaction in the CNS, even though they commonly occur together in the clinical setting. Thus the purpose of this study was to characterise the neuroinflammatory response in post-traumatic hypoxia, using both an experimental rat model of traumatic axonal injury (TAI) and patients with severe TBI. To this end, diagnostic and prognostic markers were also investigated. The state of metabolic disarray in the brain provides an opportunity to measure metabolites as indicators of impending damage. The release of glial and neuronal proteins into the cerebrospinal fluid and serum allows for the assessment of injury severity and the possible prediction of outcome.

Currently, mostly radiological measures exist for the investigation of TBI, and the validation of adjunctive biochemical tests would provide valuable information to clinicians, patients and their families.

Animal Studies:

Methods:
Experimental traumatic axonal injury (TAI) was performed using the Marmarou model (1994). Post traumatic hypoxia was induced by ventilation with 10%/90% O_2/N_2 for 30 minutes to produce mild-severe hypoxia (SaO_2~50-60%). Four groups of animals are included in this study: (a) TAI only, (b) TAI followed by a 30 min hypoxia, (c) hypoxia insult only and (d) sham control (no TAI or hypoxia). Brain tissue was collected at 2, 24, 48, 72 and 96 h post trauma (n=6 rats per time point per group) for analysis of cytokine (IL-1β, IL-6) concentration in brain homogenates.

Microdialysis was performed in a small subset of animals, with microdialysis samples (microdialysate) collected every 3 hours for 5 days. Sensorimotor tests were also performed at 24 hours after trauma to monitor neurological impairment and improvement after TBI using previously validated methods. Brains and serum were collected at sacrifice at various timepoints. Brain oedema was also determined and brain tissue, serum and microdialysate were analysed by enzyme-linked immuno-sorbent assay (ELISA) for a subset of cytokines (IL-1α, IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, TNF-α, IFN-γ and GM-CSF), and specific CNS proteins (glial fibrillary acidic protein (GFAP), myelin basic protein (MBP), S100B and neuron specific enolase (NSE)). Brain metabolites in rat microdialysate (glucose, lactate, pyruvate, lactate/pyruvate ratio, glycerol and glutamate) were also analysed using CMA ISCUS microdialysis analyser.

Results: Neurological Outcomes
The sensorimotor tests used to assess neurological deficit and recovery, consist of Rotarod, walking on a 2cm beam, and the latency of adhesive tape removal from frontal paws (n=10 rats per group). The animals were trained for these tests twice on the week immediately before injury. Subsequently the rats were traumatised and tested every day during the first week post-injury, and twice a week during the week 2.

The maximum speed TAI and TAI+hypoxia animals able to walk were significantly reduced between day 1 (8.2±2.1 rpm and 3.4±1.6, respectively) and 6 (18.8±2.5 and 12.8±2.8) ($P<0.05$). Although, the TAI+hypoxia animals walked slower than the TAI animals, no significant difference was observed between groups. All animals were unable to balance or walk on the 2cm beam at day 1 post injury, although the function progressively improved over time, both treatment groups were unable to walk normally on the beam at day 14. TAI and TAI+hypoxia rats showed a significant increase in latency of tape removal at day 1 after injury (1.7±0.2 min and 1.8±0.2, respectively) compared with sham (0.18±0.04 min) ($P<0.05$). They showed progressive decrease in latency of tape removal over time and both TAI and TAI+hypoxia rats were not different to sham at 2 weeks.

Results: Oedema
TBI is known to induce cerebral oedema both in animals and humans. In rat experiments (n=6 rats per group per time point), we have shown that brain water content, indicative of brain oedema, was significantly increased at 24h in TAI+hypoxia animals (79.29±0.22%) when compared with the sham control brain (78.81±0.14%) ($P<0.05$). Trend increases were also observed at 24h TAI (79.27±0.14%), 48h TAI (79.14±0.26) and TAI+hypoxia (79.31±0.18)

animals, but there was no significant difference between the groups. The water content returned to normal level at 96h after both TAI and TAI+hypoxia.

Results: Cytokine Measurements

In rat brain, IL-6 concentration showed early increase after TAI+hypoxia with significant elevation at both 24h and 48h (12.7±2.0 pg/mg protein and 11.3±1.8, respectively) when compared with sham levels (6.7±0.7 pg/mg) ($P<0.05$). This increase in IL-6 concentration was maintained until 96h post TAI+hypoxia (12.6±3.2), although it did not reach significance at this time when compared with sham. In comparison, TAI alone had lower IL-6 concentration in brain homogenates at early post-trauma period, and a significant increase only occurred at 96h post TAI (10.6±1.2 pg/mg, $P<0.05$)). A significant increase in IL-6 was observed at 24h in TAI-hypoxia rats over TAI only (12.7±2.0 vs. 8.3±0.6, $P<0.05$). Brain IL-1β concentrations were significantly elevated at 2h in both TAI and TAI+hypoxia groups (2.4±1.6 and 3.1±0.6) compared with sham (1.8±0.2). IL-1β returned to the basal level at 24h after TAI alone, while in TAI+hypoxia group it remained significantly increased at 24h (2.4±0.2) over both sham (1.7±0.2) and TAI only (1.8±0.1) animals ($P<0.05$).

Results: Changes in Brain Metabolism

Markers of aerobic and anaerobic metabolism (glucose, lactate and lactate/pyruvate (L/P) ratio) showed significant overall increases in the trauma/hypoxia group, indicating an overall shift to anaerobic metabolism. Cytokine concentrations in rat brain microdialysate showed late changes, with non-significant elevation of selected pro-inflammatory cytokines (IL-2 and TNF-α) and decreases in the anti-inflammatory cytokines (IL-4, IL-6 & IL-10).

Results: Histological changes

Accumulation of amyloid precursor protein (APP) and neurofilament (n=6 rats per group per time point) was observed in the corpus callosum and pyramidal tracts of the brainstem. The TAI+hypoxia group showed swollen axons and retraction bulbs at both 1 and 7 days. In comparison, the TAI alone group showed only the retraction bulbs at 1 and 7 days with no axonal swelling. Infiltrated macrophages and activated microglia were localised to the corpus callosum (39±13 cells/section) and optic tracts (217±30 cells/section) at 7 days following injury in the TAI+hypoxia group, and to a lesser extent the TAI group (corpus callosum 24±6 cells; optic tracts 142±49 cells/section). No infiltrated macrophages or activated microglia were observed in sham animals. Reactive astrocytes staining with GFAP is currently ongoing and are being analysed.

Human Studies:

Methods:

Cerebrospinal fluid (CSF) and serum samples were collected daily from 39 TBI patients with a pre-intubation post-resuscitation Glasgow Coma Scale (GCS) <9 and an external ventricular drain (EVD) in situ, admitted to the Alfred Hospital. Hypoxia was determined by a $SaO_2<90\%$ or clinical evidence of cyanosis or apnoea in the field. The presence of focal or diffuse brain injury was classified according to the Marshall Criteria and neurological outcome was assessed at 6 months using the Extended Glasgow Outcome Score (GOSE). Subsets of patient samples were analysed as described for the rodents, for cytokines, CNS proteins and metabolites.

A total of 20 patients have been recruited consisting of 9 TBI+hypoxia patients and 11 TBI-normoxia patients. For each patient, ventricular CSF drained with the purpose to decrease the intra-cranial pressure was collected as accumulative samples over 24 h for 5 days from the time of hospital admission after injury.

Results: Epidemiological
In severe TBI patients, the hypoxic and normoxic groups were not significantly different from each other in terms of epidemiological characteristics, apart from primary type of brain injury (focal or diffuse).

Results: Changes in Brain Metabolism
The L/P ratio showed a significant overall increase in the hypoxic patients, thus indicating hypoxic tissue damage.

Results: Cytokine Analysis
CSF cytokine levels were measured using Bio-Rad Multi-Plex ELISA assay. A stronger increase of IL-4 (day1 TBI+hypoxia: 0.77±0.13 pg/ml; TBI+normoxia: 0.31±0.02 pg/ml), GM-CSF (day1 TBI+hypoxia: 28.89±13.13 pg/ml; TBI+normoxia: 5.59±2.37 pg/ml), IFN-γ (day 1 TBI+hypoxia: 52.66±29.50 pg/ml; TBI+normoxia: 14.97±5.48 pg/ml) and TNF-α (day1 TBI+hypoxia: 10.44±3.09 pg/ml; TBI+normoxia: 3.65±1.11 pg/ml) were observed in the CSF of TBI+hypoxia patients as compared with TBI+normoxia patients during the first 3 days after trauma. This data clearly show that inflammation is exacerbated in the CSF of patients suffering from the combination of TBI and a post-traumatic hypoxic insult.

With regard to inflammatory cytokines, there was a significant elevation of IL-2 in the serum of the normoxic patient cohort above the hypoxic group. No significant alterations in cytokine concentrations were seen as a result of post-traumatic hypoxia, however interestingly, there were non-significant decreases of the anti-inflammatory cytokines, IL-4 and IL-10, in the hypoxic group.

Results: Protein markers
Patients' CSF analysis also demonstrated higher concentration of NSE, S100β and MBP at day 1 post-injury in TBI+hypoxia patients as compared to patients with trauma alone which gradually decreased over the following 5 days. Control CSF was collected from elective neurosurgical patients without TBI and other inflammatory neuropathologies and showed that these protein markers were under detection limit of the assay.

Of the protein markers measured in serum and CSF, serum GFAP levels were significantly elevated in the hypoxic patient cohort, but no differences were found in NSE, MBP or S100β. When correlated with outcome, CSF concentrations of cytokines (IL-2, IL-6, IL-8, IL-10, TNF-α, IFN-γ and GM-CSF) and all CNS proteins showed negative correlations with GOSE. Day 5 levels of NSE showed significant predictive potential. Certain cytokines (IL-2, IL-6, IL-8, IL-10), NSE and most metabolites (pyruvate, glycerol, glutamate and the L/P ratio) also demonstrated a significant capacity to distinguish between focal and diffuse brain injury.

2. Publications and Presentations

Abstracts

Nguyen P., Bye N., Rancan M., Henning R., Rosenfeld J., Kossmann T., Morganti-Kossmann M.C. (2004) Evaluation of markers for differentiation of traumatic axonal injury with or without hypoxia. Australian Neuroscience Society, Melbourne, Australia.

Nguyen P., Agyapomaa D., Kossmann T., Morganti-Kossmann M.C. (2005). Post-traumatic hypoxia following experimental TBI exacerbates cerebral inflammation and neurological impairment. Trauma Research Symposium, Melbourne Australia.

Nguyen P., Agyapomaa D., Kossmann T., Morganti-Kossmann M.C. (2005). Post-traumatic hypoxia following experimental TBI exacerbates cerebral inflammation and neurological impairment. Alfred Research Week, Melbourne Australia.

Nguyen, P., Agyapommaa, D., Kossmann, T., Morganti-Kossmann, M.C. Post-traumatic hypoxia following experimental TBI exacerbates cerebral inflammation, brain metabolism and neurological impairment. (Poster) 8th International Neurotrauma Symposium, Rotterdam, May 2006. Journal of Neurotrauma 23:770, 2006

Satgunaseelan, L., Yan, E.B., Nguyen, P., Agyapomaa, D., Kossmann, T., Morganti-Kossmann, MC. (2007) The effect of post-traumatic hypoxia on neuroinflammation, protein marker release & brain metabolism after TBI. 7th *IBRO World Congress*, Melbourne

Satgunaseelan, L., Bye,N., Agyapomaa, D., Nguyen, P., Kossmann, T., Morganti-Kossmann, MC. (2007) The diagnostic role of protein markers and cytokines in focal and diffuse traumatic brain injury. 7th *IBRO World Congress*, Melbourne

Hellewell S, Yan EB., Agyapommaa D., Morganti-Kossmann MC. (2008) Post-traumatic hypoxia worsens neuropathological damage in an animal of diffuse axonal injury. Alfred Research Week, 20-24 October, Melbourne, Australia.

Hellewell S, Yan EB., Agyapommaa D., Morganti-Kossmann MC. (2009) Post-traumatic hypoxia exacerbates brain damage in an animal of diffuse axonal injury. ANS Meeting Canberra 27-30 January, Australia

Manuscripts

No publications have been published to date.

Degrees

Dr Laveniya Satgunaseelan, Bachelor of Medical Science, Monash University 2006
Ms Sarah Hellewell, Bachelor of Science, Monash University 2008

3. Use of funds

$15,000.00 was received with $15,000.00 used for direct research costs.

4. Other research funding

This research project has contributed to obtaining further funding that has included:

National Health and Medical Research Council Grant #384240 (2007-2009)
Victorian Neurotrauma Initiative Project Grant DP009 (2007-2010)
Victorian Neurotrauma Initiative Early Career Fellowship – Edwin Yan (2009-2011)