PERSPECTIVE

Glutathione precursors shield the brain from trauma

In the United States, approximately one-third of all injury-related deaths are due to traumatic brain injury (TBI). Anyone is at risk for TBI; however, the risk is higher for athletes in contact sports, military personnel, children, and the elderly. TBI is characterized by a mild, moderate, or severe mechanical force to the head which can be further classified as blast, blunt, or ballistic. The sheer mechanical force of the impact to the head results in the primary injury including diffuse axonal injury, internal bleeding, swelling, and neuronal cell death. Secondary injury occurs over time, often weeks to months post-TBI, and is characterized by neuroinflammation, blood-brain-barrier disruption, oxidative stress, mitochondrial dysfunction, neuronal apoptosis, and other deleterious effects in the brain (Khatri et al., 2018). Recent research indicates that secondary injury from TBI may be considered a risk factor for neurodegenerative diseases occurring later in life, such as Alzheimer's disease and chronic traumatic encephalopathy. A key molecular mechanism that contributes to secondary injury after TBI is free radical damage which is induced by the aberrant production of reactive oxygen species (ROS) and reactive nitrogen species (RNS).

Free radical damage following TBI: ROS and RNS are mostly generated from dysfunctional mitochondria and neuroinflammatory cells. The brain is highly susceptible to damage induced by these free radicals and intracellular targets include protein sulfhydryls, lipid membranes, and DNA. Following TBI, cell metabolism becomes dysregulated, leading to the overproduction of ROS from damaged mitochondria. TBI disturbs the redox homeostasis resulting in a loss of essential endogenous antioxidants and a consequent further build-up of free radicals. Eventually, neurons and glia are unable to scavenge the excess ROS and RNS and the brain is exposed to oxidative and nitrosative stress, respectively. Inflammation occurring after TBI prompts the activation of microglia and astrocytes which further produce ROS and RNS, adding to the secondary injury (Khatri et al., 2018).

Glutathione (GSH) depletion following TBI contributes to secondary injury: Antioxidants are compounds that can prevent the formation of free radicals or sequester and reduce ROS and RNS through redox reactions, making them a viable therapeutic option to prevent oxidative and nitrosative stress after TBI (Khatri et al., 2018). GSH is a tripeptide that functions as an essential antioxidant and protects neurons against free radical damage in the brain (Ross et al., 2012). GSH peroxidases (GPx) use GSH to detoxify ROS, generating an oxidized form of GSH (GSSG) while reducing hydrogen peroxide to water. Glutathione reductase (GR) then utilizes NADPH to reduce the oxidized GSSG to replenish the GSH pool. The GSH to GSSG ratio is an important indicator of how the cell is managing oxidative stress and maintaining its redox balance (Ross et al., 2012).

Previous work has shown a reduction in brain GSH following TBI which may enhance the susceptibility of neurons to damage by free radicals. Ansari et al. (2008) measured the GSH/GSSG ratio and the activities of GPx and GR in a unilateral moderate cortical contusion model of TBI in the hippocampi of young adult rats. They found that the ratio of GSH/GSSG exhibited a significant time-dependent decrease post-TBI in the hippocampus ipsilateral to the injury, when compared to sham controls. The decrease was observed initially at 3 hours post-TBI and dropped to the lowest values at 24–48 hours post-TBI. The ratio of GSH/GSSG was still significantly lower than sham controls at 96 hours post-TBI. Furthermore, GPx and GR activities in the hippocampus also displayed time-dependent declines similar to that observed for the GSH/GSSG ratio (Ansari et al., 2008). In a clinical study, Bayir et al. (2002) measured GSH levels in cerebrospinal fluid of 11 infants and children who had suffered a severe TBI. They found that GSH levels were significantly decreased from day 1 post-TBI until day 7 post-TBI, when compared to healthy controls.

Dash et al. (2016) has shown that GSH precursors are also decreased after TBI. Methionine, when metabolized, generates S-adenosylmethionine (SAM). Homocysteine, an amino acid homologue of cysteine, is used to synthesize GSH under oxidative stress and is derived from SAM. The authors examined plasma levels of methionine and its metabolites in human patients 24 hours following mild and severe TBI. They found that methionine and SAM levels were significantly decreased in severe TBI patients when compared to healthy controls. Furthermore, they also observed a significant decrease in GSH precursors, cysteine and glycine, in the severe TBI group when compared to controls. Mild TBI patients also showed a decrease in methionine and glycine levels, however, this decrease was less than that measured in the severe TBI patients (Dash et al., 2016).

From the above studies, it is evident that brain GSH is depleted following TBI. Additional evidence suggests that endogenous GSH plays a role in protecting neurons from the devastating effects of TBI. For instance, transgenic mice deficient in GPx displayed increased oxidative stress and mitochondrial dysfunction following a TBI induced by controlled cortical impact, when compared to non-transgenic controls (Xiong et al., 2004). Al Nimer et al. (2013) also explored a genetic component of the GSH pathway in two strains of mice, dark agouti and piebald virol glaxo (PVG^{av1}), in a weight drop model of TBI. These strains exhibit a difference in the regulation of the GSH pathway specifically at the level of the transcript for glutathione S-transferase-4. The PVG^{av1} mice display increased glutathione S-transferase-4 expression compared to dark agouti mice and demonstrated increased survival of neurons and a decrease in by-products of lipid peroxidation following TBI (Al Nimer et al., 2013). These studies further support the hypothesis that GSH plays a significant role in protecting neurons against oxidative and nitrosative stress caused by TBI.

Collectively, the above studies suggest that increasing or sustaining the available GSH pool in the brain may be a reasonable approach to limit secondary injury post-TBI. GSH is synthesized by the enzymes γ-glutamylcysteine ligase and glutathione synthetase. The limiting precursor for GSH synthesis is the amino acid cysteine. Thus, agents which augment the available cysteine pool act as GSH precursors and could be used to enhance GSH synthesis making the brain more resilient to TBI (preventative) or improving recovery post-TBI (restorative).

GSH precursors as treatment options for TBI: A few studies have shown the neuroprotective effect of treatment with GSH precursors post-TBI. N-acetylcysteine (NAC) is an antioxidant precursor to GSH and cysteine analog which can induce GSH synthesis or scavenge ROS itself. Hicdonmez et al. (2006) explored the beneficial effect of NAC in a closed head free fall cortical impact TBI model in rats. NAC was given to rats 15 minutes post-TBI and rats were sacrificed at 2 and 12 hours post-TBI. Administration of a single dose of NAC post-TBI significantly decreased malondialdehyde levels (an index of lipid peroxidation), increased superoxide dismutase and GPx levels, and protected neurons when compared to untreated rats that received TBI (Hicdonmez et al., 2006). These findings indicate that NAC can protect neurons from oxidative stress through the increased activity of antioxidants such as superoxide dismutase and GPx. Another study tested the importance of the excitatory amino acid carrier type 1 (EAAC1), a membrane transporter that participates in the neuronal uptake of cysteine and maintains the levels of GSH in the brain. In this study, EAAC1 receptor knock-out mice were subjected to a controlled cortical impact TBI. When mouse hippocampi were analyzed 3 and 24 hours post-TBI, superoxide production was significantly increased, and neuronal death was doubled in the $EAAC1^{-/-}$ mice, when compared to wild-type mice subjected to TBI. Inflammation was also significantly increased in the $EAAC1^{-/-}$ mice when compared to wild-type mice evidenced by an increase in microglial activation at 1-week post-TBI. Interestingly, pretreatment with NAC significantly reduced neuronal death and superoxide production in the $EAAC1^{-/-}$ mice. These observations designate a crucial role for the EAAC1 transporter in GSH production and neuronal protection against the secondary injury induced by TBI (Choi et al., 2016). S-nitrosoglutathione (GSNO) is a metabolite of GSH which can combat oxidative stress. GSNO has the ability to scavenge RNS and is seven-fold more capable than GSH to combat against oxidative stress caused by peroxynitrite (Khan et al., 2009). When GSNO was administered to rats 2 hours after a controlled cortical impact TBI, the integrity of the blood brain barrier was preserved, apoptosis was reduced, and macrophages showed reduced inducible nitric oxide synthase expression when compared to vehicle-treated injured animals. TBI animals treated with GSNO also significantly recovered neurobehavioral functions, such as motor deficits and cognitive impairment, as evaluated by the rotarod task and sensorimotor measurements when compared to untreated TBI animals (Khan et al., 2009). Studies have also explored the neuroprotection exhibited by γ-glutamylcysteine ethyl ester (GCEE), a cell-permeable form of γ-glutamylcysteine that up-regulates GSH production in the brain. Reed et al. (2009) administered GCEE to adult rats approximately 10 min following a weight drop model of TBI, followed by euthanasia at 24 hours post-TBI. The results showed that GCEE signifi-

cantly reduced levels of 3-nitrotyrosine, an indicator of nitrosative stress, and protein carbonyls, a marker of protein oxidation, to values like those of sham controls.

Although the above studies highlight the effects of different GSH precursors against secondary injury in TBI, many of them lack an assessment of motor and cognitive function. Observing the effect of GSH precursors on motor function and cognition would allow for an evaluation of recovery following TBI and would be more relevant to future clinical applications in humans. Furthermore, many of the GSH precursors discussed above have primarily been tested as restorative treatments for TBI. A preventative treatment would be highly beneficial in protecting high-risk populations from TBI. We have recently studied the effects of a non-denatured whey protein supplement, Immunocal®, in a mouse model of moderate TBI induced by controlled cortical impact (Ignowski et al., 2018). Unlike the above studies, Immunocal® was administered as a preventative treatment and the ability of Immunocal® to restore motor and cognitive deficits post-TBI was also measured. Immunocal® is replete with cystine and γ-glutamylcysteine and has been shown to act as a cysteine delivery system that boosts GSH levels *in vivo* (Ross et al., 2012). CD1-Elite male mice were pretreated orally with Immunocal® for a period of 28 days prior to TBI. Following TBI, the mice were assessed for motor deficits using the beam walk and rotarod. Mice were also assessed for spatial learning and memory using Barnes maze testing. TBI mice pretreated with Immunocal® performed significantly better than untreated TBI mice in all these tests. Immunocal® pretreated mice also displayed a significantly higher preserved brain GSH/GSSG ratio measured at 72 hours post-TBI than measured in untreated TBI mice. These data indicate that Immunocal® provides ample cysteine which is converted into GSH in the brain. With supplementation of Immunocal®, cells can overcome excess ROS and RNS and maintain a balance of the GSH redox state. Furthermore, TBI mice pretreated with Immunocal® also displayed a two-fold reduction in brain malondialdehyde and a preservation of brain-derived neurotrophic factor, when compared to untreated TBI mice at 72 hours post-TBI. Lastly, we found that Immunocal® pretreated mice subjected to TBI displayed significantly less demyelination of the corpus callosum and decreased numbers of degenerating neurons when compared to untreated TBI mice.

Concluding remarks: The above findings indicate that various GSH precursors may be viable neuroprotective agents against the secondary injury caused by TBI (**Figure 1**). Immunocal® would be particularly beneficial for high-risk populations due to its preventative effects against secondary injury mechanisms induced by TBI and due to its ability to significantly improve deficits in motor and cognitive function. Amelioration of secondary injury could be crucial in the long term. Pre-treatment with GSH precursors could lessen the severity of motor and cognitive impairments and ultimately improve an individual's quality of life after TBI. Furthermore, TBI increases the risk of developing serious neurodegenerative diseases, such as Alzheimer's disease. Pre-treatment with GSH precursors could reduce this risk in the long term. Further research should be done with Immunocal® and other compounds which increase or enhance GSH production, as these could be considered safe and effective treatments for TBI.

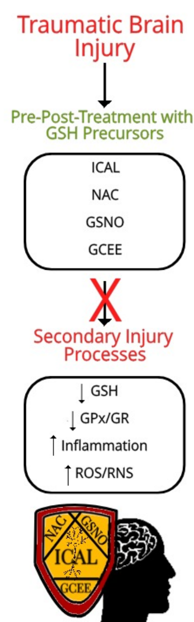

Figure 1 GSH precursors protect the brain from multiple secondary injury processes induced by traumatic brain injury.
Brain trauma results in decreased GSH and GSH-dependent enzyme activities. Trauma also increases inflammation and production of free radicals. GSH precursors act to restore or preserve the GSH pool, scavenge free radicals, and attenuate inflammation. GSH: Glutathione; ICAL: Immunocal®; NAC: N-acetylcysteine; GSNO: s-nitrosoglutathione; GCEE: γ-glutamylcysteine ethyl ester; GPx: GSH peroxidase; GR: GSH reductase; ROS: reactive oxygen species; RNS: reactive nitrogen species.

Lilia Koza, Daniel A. Linseman*
Department of Biological Sciences, University of Denver, Denver, CO, USA (Koza L, Linseman DA)
Knoebel Institute for Healthy Aging, University of Denver, Denver, CO, USA (Linseman DA)
***Correspondence to:** *Daniel A. Linseman, PhD, Daniel.Linseman@du.edu.*
***orcid:** 0000-0001-8774-1976 (Daniel A. Linseman)*
***Received:** January 12, 2019*
***Accepted:** March 5, 2019*

***doi:** 10.4103/1673-5374.257520*
***Copyright license agreement:** The Copyright License Agreement has been signed by both authors before publication.*
***Plagiarism check:** Checked twice by iThenticate.*
***Peer review:** Externally peer reviewed.*
***Open access statement:** This is an open access journal, and articles are distributed under the terms of the Creative Commons Attribution-NonCommercial-ShareAlike 4.0 License, which allows others to remix, tweak, and build upon the work non-commercially, as long as appropriate credit is given and the new creations are licensed under the identical terms.*
***Open peer reviewers:** Edwin Estefan Reza-Zaldívar, Biotecnología Médica y Famacéutica, Mexico; Creed Stary, Stanford University, USA.*
***Additional file:** Open peer review reports 1 and 2.*

References

Al Nimer F, Ström M, Lindblom R, Aeinehband S, Bellander BM, Nyengaard JR, Lidman O, Piehl F (2013) Naturally occurring variation in the glutathione-S-transferase 4 gene determines neurodegeneration after traumatic brain injury. Antioxid Redox Signal 18:784-794.
Ansari MA, Roberts KN, Scheff SW (2008) Oxidative stress and modification of synaptic proteins in hippocampus after traumatic brain injury. Free Radic Biol Med 45:443-452.
Bayir H, Kagan VE, Tyurina YY, Tyurin V, Ruppel RA, Adelson PD, Graham SH, Janesko K, Clark RS, Kochanek PM (2002) Assessment of antioxidant reserves and oxidative stress in cerebrospinal fluid after severe traumatic brain injury in infants and children. Pediatr Res 51:571-578..
Choi BY, Kim IY, Kim JH, Lee BE, Lee SH, Kho AR, Jung HJ, Sohn M, Song HK, Suh SW (2016) Decreased cysteine uptake by EAAC1 gene deletion exacerbates neuronal oxidative stress and neuronal death after traumatic brain injury. Amino Acids 48:1619-1629.
Dash PK, Hergenroeder GW, Jeter CB, Choi HA, Kobori N, Moore AN (2016) Traumatic brain injury alters methionine metabolism: implications for pathophysiology. Front Syst Neurosci 10:36.
Hicdonmez T, Kanter M, Tiryaki M, Parsak T, Cobanoglu S (2006) Neuroprotective effects of N-acetylcysteine on experimental closed head trauma in rats. Neurochem Res 31:473-481.
Ignowski E, Winter AN, Duval N, Fleming H, Wallace T, Manning E, Koza L, Huber K, Servoka NJ, Linseman DA (2018) The cysteine-rich whey protein supplement, Immunocal®, preserves brain glutathione and improves cognitive, motor, and histopathological indices of traumatic brain injury in a mouse model of controlled cortical impact. Free Radic Biol Med 124:328-341.
Khan M, Im YB, Shunmugavel A, Gilg AG, Dhindsa RK, Singh AK, Singh I (2009) Administration of S-nitrosoglutathione after traumatic brain injury protects the neurovascular unit and reduces secondary injury in a rat model of controlled cortical impact. J Neuroinflammation 6:32.
Khatri N, Thakur M, Pareek V, Kumar S, Sharma S, Datusalia AK (2018) Oxidative stress: major threat in traumatic brain injury. CNS Neurol Disord Drug Targets 17:689-695.
Reed TT, Owen J, Pierce WM, Sebastian A, Sullivan PG, Butterfield DA (2009) Proteomic identification of nitrated brain proteins in traumatic brain-injured rats treated postinjury with gamma-glutamylcysteine ethyl ester: Insights into the role of elevation of glutathione as a potential therapeutic strategy for traumatic brain injury. J Neurosci Res 87:408-417.
Ross EK, Gray JJ, Winter AN, Linseman DA (2012) Immunocal® and preservation of glutathione as a novel neuroprotective strategy for degenerative disorders of the nervous system. Recent Pat CNS Drug Discov 7:230-235.
Xiong Y, Shie FS, Zhang J, Lee CP, Ho YS (2004) The protective role of cellular glutathione peroxidase against trauma-induced mitochondrial dysfunction in the mouse brain. J Stroke Cerebrovasc Dis 13:129-137.

P-Reviewers: Reza-Zaldívar EE, Stary C; C-Editors: Zhao M, Li JY; T-Editor: Liu XL