

N-acetyl-L-leucine: a promising treatment option for traumatic brain injury

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Traumatic brain injury (TBI) is a mechanical injury to the brain, which can be sustained due to falls, accidents, contact sports or in combat situation. It is a serious health problem-affecting people of all ages worldwide. As per the recent epidemiological study, more than 55 million people suffer from TBI annually (GBD 2016 Traumatic Brain Injury and Spinal Cord Injury Collaborators, 2019), and its prevalence has increased by almost 8.4% between 1990 and 2016 (GBD 2016 Traumatic Brain Injury and Spinal Cord Injury Collaborators, 2019). Depending on severity, TBI can lead to premature death and disability. In long-term survivors, it is also a major risk factor for development of neurodegenerative diseases like Alzheimer's disease or Parkinson's disease (Smith et al., 2013). All together TBI causes immense emotional distress and brings huge financial burden not only to the patients and family members but also to the society (GBD 2016 Traumatic Brain Injury and Spinal Cord Injury Collaborators, 2019). Unfortunately, there is no effective pharmacological treatment available for TBI. Current therapeutic approaches are primarily focused on minimizing or alleviating injury-inflicted symptoms but do not restrict injury-induced brain damage. Thus, there is an urgent need to identify and develop pharmacological agents that can improve TBI outcomes and prevent neurodegeneration.

Pathophysiology of TBI is very complex. Primary mechanical injury to the brain triggers a cascade of biochemical events (secondary injury) that include excitotoxicity, organellar dysfunction, oxidative and endoplasmic reticulum stress and ionic imbalance (Xiong et al., 2015). These all lead to severe neuronal cell death at the early stage of injury, followed by chronic neuroinflammation and associated progressive neurodegeneration (Xiong et al., 2015). Thus, an effective treatment approach for TBI should minimize early loss of neurons and attenuate neuroinflammation. Over the past 40 years, many pharmacological agents targeting secondary injury mechanisms have been tested to treat TBI in preclinical animal models (Xiong et al., 2015). These include calcium channel blockers, antioxidants, excitatory amino acid inhibitors, N-methyl D-aspartate receptor antagonist and cell cycle inhibitors (Xiong et al., 2015). Many of these treatments demonstrated promising results in animal experiments, however, failed to show beneficial effects and/or caused adverse side effects in clinical trial in

TBI patients (Xiong et al., 2015). Therefore, there remains an urgent need to develop more effective and safer TBI treatments. Repurposing existing drugs for the treatment of TBI could be a useful way to rapidly develop effective therapy for TBI with fewer side effects.

Recently, we reported that treatment with N-acetyl-L-leucine (NALL), an acetylated derivative of amino acid leucine attenuates neuronal death and neuroinflammation in the cortical tissue of mice following controlled cortical impact induced TBI (Hegdekar et al., 2021). N-acetyl-leucine (NAL) is orally bioavailable and has been in use in France for the treatment of vertigo and vertiginous symptoms for more than 50 years (Kaya et al., 2021). It is non-toxic and no serious side effects have been reported, making it a very promising potential candidate for rapid repurposing. In an open-label clinical study, treatment with NAL was reported to have beneficial effect in lysosomal storage disease patients, specially Niemann-Pick disease type C (Kaya et al., 2021). While the racemic mixture of NAL is being used for the treatment of vertigo and in clinical trials, the levorotatory isomer (L-enantiomer) of NAL (NALL) has been identified as the active form. NALL but not its D-enantiomer demonstrated neuroprotective effects in mouse models on Niemann-Pick disease type C (Kaya et al., 2021). Accordingly, we used NALL to treat the mice orally following TBI. NALL treatment markedly improved functional deficits in mice following experimental TBI. We also detected marked attenuation of lesion volume in mice treated with NALL, demonstrating prolonged neuroprotective function of NALL after TBI (Hegdekar et al., 2021).

The neuroprotective mechanism of NALL remains unclear. Based on our study, it may be mediated through the activation of autophagy (Hegdekar et al., 2021). Autophagy is a cellular degradative process in which damaged, aged or superfluous cellular components, including proteins, protein aggregates and organelles, are enclosed within double membrane-bound organelles called autophagosomes and then delivered to lysosomes for degradation (Scervo et al., 2018). This process is extremely important to remove harmful or unnecessary cellular components and keep the intracellular environment clean in non-dividing cells like neurons. Any perturbation of autophagic process causes intracellular accumulation

of toxic components that is detrimental for neuronal survival. We have previously demonstrated that autophagy is disrupted in the mouse brain following controlled cortical impact induced TBI (Sarkar et al., 2014). Autophagosomes, their cargos, and cargo adaptor proteins such as sequestosome (SQSTM1) accumulate within the cortex and hippocampus following TBI. Initial inhibition of autophagy occurs primarily within neurons, peaks at 1 day after TBI and is associated with the neuronal death (Sarkar et al., 2014). Our data demonstrate that NALL treatment can partially restore autophagy flux and attenuate cortical cell death in the injured mice (Hegdekar et al., 2021). Consistent with improved autophagy flux, we detected reduced accumulation of both autophagosomes and SQSTM1 in the cortices of NALL-treated TBI mice. NALL treatment also markedly lowered cortical cell death in mice at 1 day after TBI (Hegdekar et al., 2021). Since autophagy is generally cytoprotective, these data suggest that neuroprotective function of NALL in TBI might be mediated through autophagic activation.

We recently demonstrated that impairment of neuronal autophagy after TBI is caused by cytosolic phospholipase A2-mediated lysosomal damage (Sarkar et al., 2020). We observed that early inhibition of cytosolic phospholipase A2 by arachidonyl trifluoromethyl ketone attenuated lysosomal damage, enhanced autophagy flux and improved functional outcomes after TBI (Sarkar et al., 2020). Thus, restoration of autophagy after TBI requires both increase in initiation of autophagy and attenuation of lysosomal damage. Mammalian target of rapamycin (mTOR) plays an important role in regulating autophagy. mTOR directly inhibits autophagy as well as prevents nuclear translocation of transcription factor EB, a master regulator of autophagy and lysosomal genes (Scervo et al., 2018). Inhibition of mTOR by rapamycin or torin1 stimulates autophagy and induces lysosomal biogenesis, and inhibition of mTOR by rapamycin can attenuate neuronal death in mice after TBI (Nikolaeva et al., 2016). It has been shown that amide derivative of NAL inhibits mTOR function (Hidayat et al., 2003). This suggests that NALL-induced upregulation of autophagy might be mediated through inhibition of mTOR, thus enhancing both initiation of autophagy and lysosomal biogenesis.

In our study, NALL treatment improved autophagy flux and restricted neuronal death after moderate TBI. Since, TBI generates large amount of damaged tissue and cellular components, acute activation of autophagy after TBI may be extremely important for the removal of these neurotoxic components. This may be highly beneficial not only for the moderate injury used in our experiments but also for other injury severities. This includes

mild injury, which unlike moderate or severe TBI does not cause any significant neuronal death but induces axonal injury (Bolton-Hall et al., 2019). Autophagy plays crucial role in maintaining axonal homeostasis. Thus, activation of autophagy by NALL after injury could be extremely beneficial in preventing axonal stress and injury and subsequent behavioral deficits following mild TBI. However, further studies will be required to confirm the beneficial effect of NALL following mild TBI.

TBI is associated with acute and chronic neuroinflammation, which involves activation of quiescent glial cells, recruitment of peripheral macrophages to the injury site and release of both pro and anti-inflammatory cytokines (Barrett et al., 2017). These are involved in regulating both reparative and damaging events in the injured brain and contribute to progressive neurodegeneration. Release of pro-inflammatory cytokines by the activated microglia and macrophages further activates resting glial populations, which can secrete additional neurotoxic modulators (Barrett et al., 2017). Our study demonstrated marked reductions in the expression of proinflammatory cytokine IL-1 β in the cortices of TBI mice treated with NALL (Hegdekar et al., 2021). We also detected marked decrease in *Nox2* gene expression in the injured mouse cortices following NALL treatment. IL-1 β is involved in inflammasome mediated inflammatory responses and can trigger activation of astrocytes, while NOX2 enhances production of reactive oxygen species (Barrett et al., 2017). NALL-mediated attenuation of inflammatory gene-expression suggests that in addition to its neuroprotective function, NALL is also effective in reducing neuroinflammation in TBI. While the mechanisms of anti-inflammatory activity of NALL remain to be clarified, they might be extremely beneficial over a wide spectrum of injury severity.

Our study demonstrated functional improvements in both memory and motor skills and reduction in lesion volume in TBI mice treated with NALL (Figure 1). We expect this is primarily due to the observed attenuation of neurodegeneration and neuroinflammation following NALL treatment. These data support development of NALL as a potential future TBI treatment. However, additional preclinical assessments will be necessary, as our study has been carried out using only young male mice. Beneficial effects of NALL will need to be assessed in females and in aged mice of both sexes. The latter will be particularly important, as incidence of TBI is higher and outcomes worse among the elderly population.

In summary, our study demonstrates the importance of early inhibition of neuronal

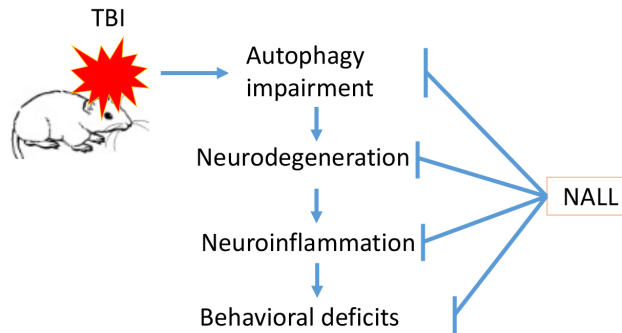


Figure 1 | Neuroprotective function of N-acetyl-L-leucine (NALL) in traumatic brain injury (TBI).

NALL treatment partially restores autophagy flux, attenuates neurodegeneration and neuroinflammation and improves functional recovery in mice after TBI.

death and neuroinflammation in limiting life-long functional deficits after TBI. While NALL has been in use for vertigo and is under clinical trials for Niemann-Pick disease, it has not been tested clinically in TBI patients. Our study provides the scientific basis for NALL as a potential therapy for TBI and hence provides scientific foundation for its future clinical trial in TBI.

This work was supported by IntraBio and NIH funding (R01NS091218 and R01NS091218) to MML.

We thank Ms. Kelly Sikorski, Senior Program Manager of the Department of Anesthesiology, University of Maryland School of Medicine, Baltimore, for proof reading the manuscript.

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Date of submission: July 9, 2021

Date of decision: August 10, 2021

Date of acceptance: September 14, 2021

Date of web publication: February 8, 2022

<https://doi.org/10.4103/1673-5374.335146>

How to cite this article: Sarkar C, Lipinski MM (2022) N-acetyl-L-leucine: a promising treatment option for traumatic brain injury. *Neural Regen Res* 17(9):1957-1958.

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P-Reviewer: Xiong Y; C-Editors: Zhao M, Liu WJ, Qiu Y; T-Editor: Jia Y