**10-month Technical Progress Report**

Enolase is a multifunctional glycolytic enzyme involved in growth control, hypoxia, immune activation, inflammation, allergic responses, and cancer. Spinal cord injury (SCI) is a devastating debilitating condition whose progressive pathological changes include complex and evolving molecular cascades, and insights into the role of enolase in multiple inflammatory events have not yet been fully elucidated. Neuronal damage following SCI can be characterized by an elevation of neuron-specific enolase (NSE), which may play an important role in the secondary injury pathology in SCI. To date, only few studies have focused on the role of NSE in the alterations of inflammatory processes after SCI, and insights into the role of NSE in SCI-mediated dysfunctions remain largely unknown. **We hypothesized that increased NSE expression after SCI promotes inflammatory events in spinal cord, activates plasmin-mediated degradative pathways leading to activation of inflammatory cytokines/chemokines, which aggravate secondary damages of SCI.** We also hypothesized that inhibition of NSE by a novel small molecule inhibitor ENOblock (C31H43FN8O3) may attenuate secondary damage following rat SCI, protecting neurons and improving functions. A long-term goal of this proposal is to develop NSE-targeted therapeutic intervention for treatment of individuals with SCI. To test these hypotheses, we proposed two specific aims:

**Specific Aim 1:** Examine and compare NSE expression/activity in neurons and glial cells after acute SCI, and investigate whether NSE levels correlate with the inflammatory cell infiltration and production of inflammatory cytokines/chemokines in rats following SCI.

**Specific Aim 2:** Investigate whether treatment of animals with a novel enolase inhibitor ENOblock attenuates inflammatory events and neuron death after acute and chronic SCI, and improve locomotor function. Studies are planned to determine NSE levels in serum and spinal cord samples, assess cytokine/chemokine levels in sera and tissues, analyze infiltrating cells, inflammatory factors (NO, Cox-2, etc.), gliosis, myelin loss, axonal degeneration and neuronal cell death, and evaluate locomotor function (BBB scale) after SCI. The mechanistic insights gained from this proposal may lead to the novel discovery of enolase as a critical target in SCI, and ENOblock as a potential therapy to promote functional recovery in human SCI.

ENOblock is a novel small molecule which is the first, non-substrate analogue that directly binds to enolase and inhibits its expression and activity. It is the first reported enolase inhibitor that is suitable for biological assays, and ENOblock treatment has been shown to target cell-surface-bound
enolase to modulate cell growth through downregulation of AKT and BCL-xL. ENOblock also reduces intracellular enolase expression and activity, and decreases the ability of cells to adapt to the hypoxic inflammatory condition.

A significant progress has been made towards the Specific Aim 1. Our recent studies suggest that NSE is markedly elevated after acute SCI, and ENOblock treatment attenuated NSE levels in serum and spinal cord tissues, decreased inflammatory cytokines/chemokines in serum, and spinal cord tissues (Manuscript Submitted). Thus, reduction of enolase expression and activity may have therapeutic potential in attenuating inflammatory mediators in acute SCI. Although NSE functions extend beyond glycolytic activity, it lacks traditional sorting signals. Thus, the question remains as to how NSE becomes surface exposed and play multiple roles in hypoxia, ischemia, and possibly neuronal death after SCI. Cell surface enolase activates plasminogen, which is a serine protease present in serum as an inactive proenzyme, and is converted by tissue-type plasminogen activator (tPA) or urokinase plasminogen activator (uPA) to active plasmin, and to degrade ECM proteins. The diagrammatic representation in Figure 1 implicates some of the adverse effects of cell surface NSE after SCI (Haque et al, Metab Brain Dis, in press).

Our SCIRF Pilot Project as well as Investigator Initiated Research Project examined serum NSE levels and cytokines/chemokines in SCI/vehicle and SCI/ENOblock treated rats, and submitted a manuscript for publication to the journal Neurochemical Research. To achieve other goals stated in Aim 1, we evaluated serum metabolic enzymes in both injured and treated animals using Discovery Metabolic Arrays. Briefly, rats were anesthetized with ketamine (80 mg/kg)/xylazine (10 mg/kg), and laminectomy was performed at T10. The spine was immobilized with a spinal stereotactic device, and SCI was induced using a modified method by Perot of dropping a constant weight (5 gm) from a height of 8 cm onto an impounder (0.3 cm in diameter) gently placed on the spinal cord. ENOblock was purchased from Bioscience (Catalog No. A11840), dissolved in vehicle (0.01% DMSO in saline), and was administered (100 µg/kg, 100 µl in volume) at 15 min and 24 h post-injury via intravenous injection. Vehicle-treated animals received the same volume of vehicle (100 µl). Sham animals received T10 laminectomy. Blood and tissue samples were collected 48 h post-injury. The results indicated alterations of several metabolic factors in ENOblock treated rats (Figure 2). We examined the connecting peptide called C-peptide, which can exert insulin-independent biological effects on cells, and is known to act as a bioactive peptide with anti-inflammatory properties. Data from metabolic arrays showed that serum C-peptide levels were significantly elevated in SCI-ENO rats as compared to SCI-vehicle rats (Fig. 2A). We also examined leptin levels in SCI, which are implicated in the pathogenesis of chronic inflammation. The elevated circulating leptin levels increase the risk of developing cardiovascular diseases, type II diabetes, or degenerative disease including inflammatory autoimmune disease (e.g., multiple sclerosis) and

![Figure 2](Image). ENOblock treatment differentially influences metabolic enzymes after SCI in rats. SCI rats were treated with vehicle alone or ENOblock (100µg/kg, twice, intravenously), and then blood samples were obtained 48h post-injury. Sham operated rats (T10 laminectomy) were used as controls. Serum c-peptide, leptin, and amylin levels were analyzed by using Discovery rat metabolic arrays. Results obtained from serum samples (duplicate analyses) of three rats suggested that ENOblock treatment significantly downregulated leptin and amylin levels while elevated c-peptide as compared with vehicle treated SCI rats. Statistical analyses were performed using Student’s t-test.
cancer. Interestingly, serum leptin levels were significantly inhibited by ENOblock treatment (Fig. 2B). We also examined the peptide hormone, amylin, which is strongly associated with inflammatory markers in a variety of injury conditions. Data suggested that ENOblock treatment significantly reduced amylin levels in SCI-ENO rats as compared to SCI-vehicle rats (Fig. 2C), suggesting that enolase may be an important target to design novel therapeutics for SCI.

Since early administration (15 min) of ENOblock had beneficial effects, we wanted to investigate if injection of ENOblock at later time points (1 h, 2 h and 4 h post-SCI) may yield differential outcomes in SCI rats (Scheme I). Rats were treated with ENOblock (100µg/kg) at 15 min, 1 h, 2 h and 4 h after SCI, followed by analysis of serum samples for c-peptide and leptin levels at 48 h. Data showed that ENOblock treatment of rats at different time points (15 min-4 h) had a similar trend in modulating metabolic enzymes especially c-peptide and leptin after SCI (Figure 3). Thus, administration of ENOblock at a reasonable window of time (15 min-4 h) after SCI contributed to induction of anti-inflammatory responses in the host.

Scheme I: Time-dependent effects of ENOblock on metabolic enzymes in SCI.

A

![Graph A](image)

B

![Graph B](image)

**Figure 3. Effects of time-dependent administration of ENOblock on metabolic enzymes after SCI in rats.** SCI rats were treated with vehicle alone or ENOblock at different time points as indicated, and blood samples were obtained 48h post-injury. Sham operated rats (T10 laminectomy) were used as controls. Serum c-peptide (A) and leptin (B) levels were analyzed by using Discovery Rat Metabolic Arrays. Data suggest that administration of ENOblock downregulates leptin levels while increases c-peptide levels as compared with vehicle treated SCI rats. Statistical analyses were performed using Student's t-test. *p<0.05, ns=not significant.

Primary injury to spinal cord would lead to secondary injury causing gliosis, inflammation, and
neurodegeneration but treatment of animals with ENOblock may inhibit these inflammatory events in SCI, inducing neuroprotection.

The status of inflammatory molecule (MMP-9), microglial activation (Iba1), and astrogliosis (GFAP) was also monitored by immunohistochemistry (Figure 4). Analysis of SCI tissue samples by immunohistochemistry confirmed that ENOblock inhibited MMP-9 protein expression, and decreased gliosis (Iba1 & GFAP), which may have occurred through reduction of elevated NSE in rats. These data are consistent with our earlier findings that serum cytokines/chemokines were also diminished after ENOblock treatment. Overall, elevation of NSE is deleterious as it promotes extracellular degradation and production of inflammatory cytokines/chemokines and metabolic factors which activates glia and damages neurons. Thus, reduction of NSE by ENOblock may have potential therapeutic implications in acute SCI.

In addition, during the last 10 months, the PI presented data in the ASN meeting (March 18-22, 2017) in Little Rock, Arkansas. The PI submitted two abstracts for presentation in the ASN-2017 meeting. The PI also submitted a related manuscript for publication in Neurochemical Research. The PI also published a research paper in Molecular Neurobiology, and a review article in the Proceedings of Neurosciences. We thank SCIRF for supporting this Investigator Initiated Project.

**Publications:**

Manuscripts accepted or submitted (related):


Manuscripts published (unrelated):

**Abstracts Published:**