TECHNICAL PROGRESS REPORTING/FINAL REPORTING TEMPLATE
(Reports must be Cumulative)

SCIRF #: 2015 I-01

PI: Swapan K. Ray, PhD

Grant Title: Novel combination therapy for neuroprotection in SCI

24-month Technical Progress Report
&
30-month Technical Progress Report
(This part is shown in bold fonts)

Aim 1: *Status (Planned, In Progress).*

Cumulative detailed description of the progress made towards the aim/sub-aim including the methods utilized:

We made further progress on the Aim 1 of this SCIRF grant award. Our cell culture models of spinal cord injury (SCI) are used to explore mechanistic inside. We carried out further experiments in our cell culture models of SCI using our novel combination therapy, as we proposed in Specific Aim 1. There is still no effective therapy for curing or successfully treating SCI in humans. Although methylprednisolone (MP) is the only recommended therapy for acute SCI, MP is found to provide partial therapeutic efficacy and its use is associated with undesirable side effects. MP is controversial therapy for acute SCI in humans. However, MP still remains as a standard therapy for acute SCI. For cell culture model of SCI, we used ventral spinal cord 4.1 (VSC4.1) motoneurons with exposure to 200 nM A23187, a calcium ionophore (CI), for 24 h. Because microRNAs (miRs) act as post-transcriptional negative regulator of many genes, expression of specific miRs can be explored for therapeutic benefits to block progression of secondary injury in SCI. Studies in our cell culture model of SCI demonstrated that miR-96 could be a neuroprotective miR, as its expression was most significantly down regulated. So, we decided overexpression of miR-96 for neuroprotection in our cell culture model of SCI. We also synthesized the novel bororetinoid BIT-5 for further neuroprotection in SCI. Our therapeutic strategy is to use combination of miR-96 overexpression and BIT-5 treatment for better neuroprotection than MP in our cell culture models of SCI. To confirm the involvement of the retinoic acid receptors (RARs), RARα and RARβ, in mediation of neuroprotection by our novel synthetic bororetinoid BIT-5 in VSC4.1 motoneurons against CI toxicity, we measured the expression of both RARα and RARβ at the mRNA level by semi-quantative reverse transcription - polymerase chain reaction (RT-PCR). After the CI insult, treatment with BIT-5 more significantly increased expression of RARα than RARβ at mRNA level in VSC4.1 cells, indicating that BIT-5 mediated neuroprotection occurred with significant

upregulation and involvement of RARα.

Describe any data analysis that was performed and include charts/tables when available:

Alteration in mRNA expression of RARα after treatment with CI and the RAR agonist BIT-5 alone and in combination in VSC4.1 cells

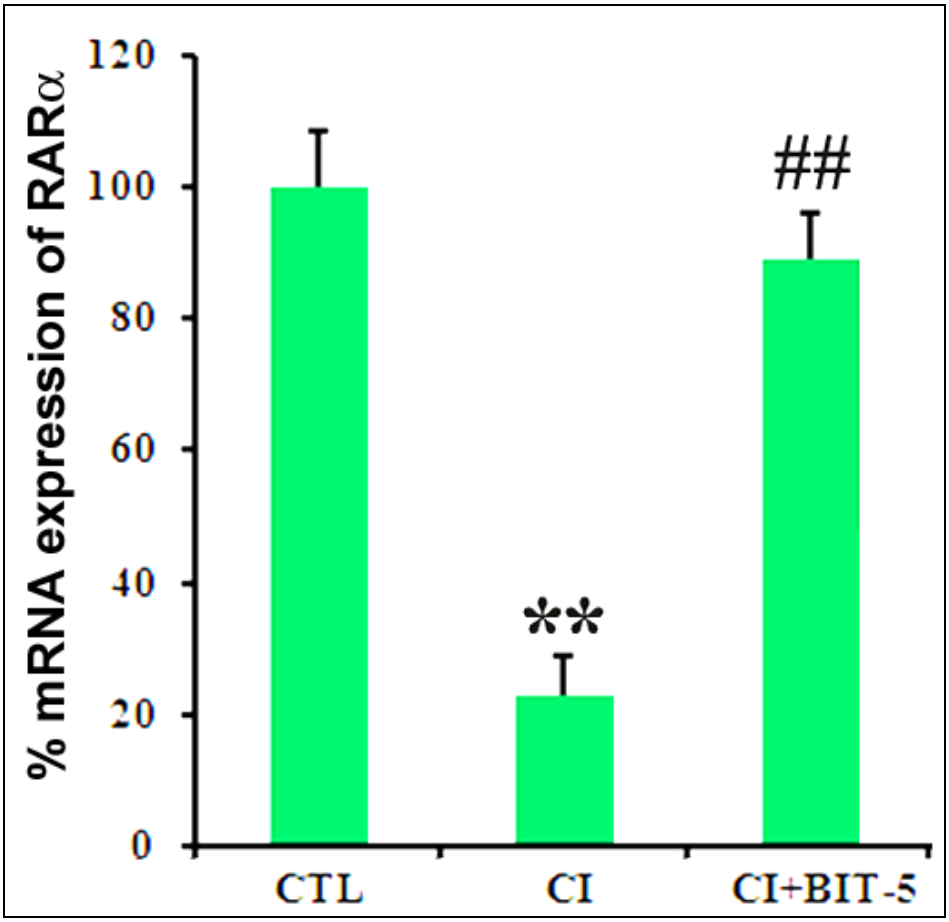

Figure 1. Percent changes in mRNA expression of RARα after treatment with CI and the RAR agonist BIT-5 in VSC4.1 motoneuron cells. First, VSC4.1 cells were exposed to 200 nM CI for 24 h and then treated with BIT-5 (10 µM) for another 24 h. Cells were harvested and semi quantitative RT-PCR was carried out using gene specific primers for RARα and GAPDH. Band intensity was quantified by ImageJ software. Significant difference between control (CTL) and CI exposure was indicated by **$p < 0.01$ and CI exposure and CI+BIT-5 treatment was indicated by ## $p < 0.01$.

Alteration in mRNA expression of RARβ after treatment with CI and the RAR agonist BIT-5 alone and in combination in VSC4.1 cells

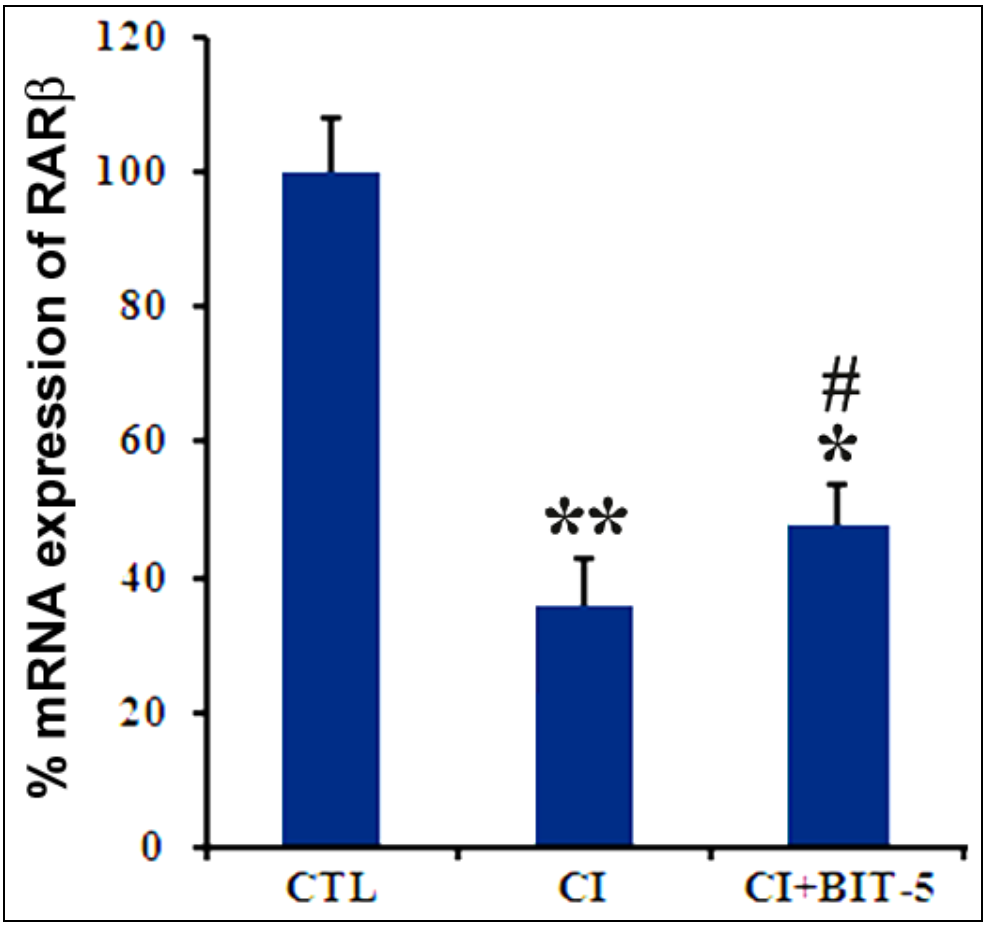

Figure 2. Percent changes in mRNA expression of RARβ after treatment with CI and the RAR agonist BIT-5 in VSC4.1 motoneuron cells. First, VSC4.1 cells were exposed to 200 nM CI for 24 h and then treated with BIT-5 (10 μM) for another 24 h. Cells were harvested and semi quantitative RT-PCR was carried out using gene specific primers for RARα and GAPDH. Band intensity was quantified by ImageJ software. Significant difference between control (CTL) and any treatment was indicated by *$p < 0.05$ or **$p < 0.01$, and CI exposure and CI+BIT-5 treatment was indicated by # $p < 0.05$.

Aim 1: ***Status (Planned, In Progress).***

Cumulative detailed description of the progress made towards the aim/sub-aim including the methods utilized:

During the last 30-month period, we conducted additional experiments to make further progress on Aim 1 of this grant award from the SCIRF. We continued using our well-established cell culture model of spinal cord injury (SCI) to achieve the objectives as we proposed in the Specific Aim 1 of this grant. So far no pharmacological agents have been effective for treating successfully of

curing the devastative consequences of SCI. However, methylprednisolone (MP) as a recommended therapy is still being used for treating acute SCI. It should be noted that MP is controversial therapy for acute SCI as it causes severe side effects. The pathogenesis in SCI is associated with multiple damaging pathways and factors. So, a monotherapy is unlikely to produce optimal therapeutic effects. Currently, our laboratory and also other laboratories are exploring the therapeutic effects of novel combination therapy in SCI. For our investigation, we are exploring molecular mechanisms of efficacy of a novel combination of two agents for targeting secondary injury pathways for enhancement of neuroprotection in SCI. In our cell culture model of SCI, we observed that exposure of ventral spinal cord 4.1 (VSC4.1) motoneurons to 200 nM A23187, a calcium ionophore (CI), for 24 h induced significant neuronal death. It is well established that miRNAs (miRs) can post-transcriptionally regulate the entire set of genes. Thus, miRs are attractive candidates as upstream regulators of the secondary SCI progression. In our studies in cell culture model of SCI, we found that miR-96 (a neuroprotective miR) was most significantly down regulated. We planned overexpression of miR-96 for neuroprotection in our cell culture model of SCI. We synthesized the novel bororetinoid BIT-5 to evaluate its neuroprotective effects as well in SCI. So, we used combination of miR-96 overexpression and BIT-5 treatment for enhancement of neuroprotective effects in our cell culture model of SCI. Briefly, VSC4.1 cells were seeded at a concentration of $5x10^5$ cells per well in 6-well plates. On the next day, cells were transfected separately with pre-miRs of miR-96 oligomeric RNA at 50 nM final concentration using 20 µl Lipofectamine 2000 reagent and Opti-MEM medium following the manufacturer's protocol (Invitrogen, Carlsbad, CA, USA). After 12 h, transfection medium was replaced by fresh growth medium containing 2% fetal bovine serum (FBS) and nothing (control) or 200 nM CI. After 24 h of incubation, cells were treated with 10 µM BIT-5 and incubated for another 24 h. Enhancement of autophagy following combination therapy could be a cell survival strategy for increasing neuroprotection in CI insulted cells. Upregulation of Belin-1, an early molecular marker of autophagy, could indicate neuroprotection following a therapeutic treatment. On the other hand, down regulation of mechanistic target of rapamycin (mTOR), a well-known inhibitor of autophagy, could clearly indicate progression of autophagy for providing neuroprotection following therapeutic treatment. We are performing more experiments (e.g., fura-2 assay, specific mRNA analysis, Western blotting) in this mono-culture and also co-culture models of SCI to generate a complete set of results for delineating mechanism of neuroprotection.

Describe any data analysis that was performed and include charts/tables when available:

Upregulation of Belin-1 following treatment with our novel combination therapy (miR-96 and BIT-5) in the CI exposed VSC4.1 cells showed occurrence of autophagy for providing neuroprotection to the motoneurons (Figure 1).

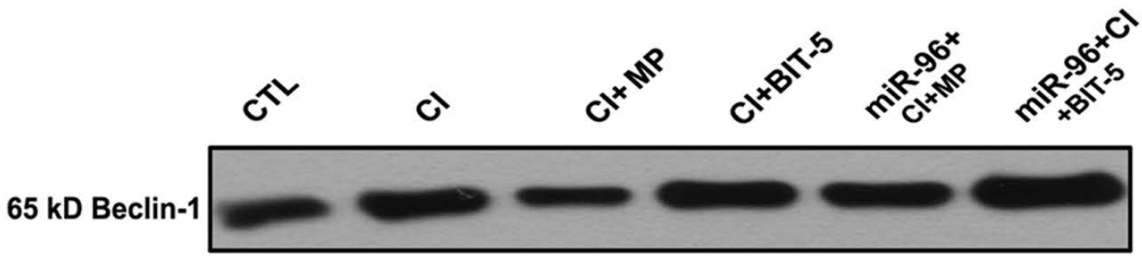

Figure 1. Western blotting for determination of induction of autophagy following treatments with single or combination therapy in the CI exposed VSC4.1 cells. Cells were first transfected with 50 nM miR-96 mimic and incubated for 12 h. Then, cells were exposed to 200 nM CI for 24 h and treated with 1 μM MP or 10 μM BIT-5 and further incubated for another 24 h. Control cells without transfection was also investigated. At the end of incubation, cells were harvested and protein samples were subjected to Western blotting for examining the increased levels of expression of Beclin-1, a marker of induction of autophagy.

Down regulation of mTOR (an inhibitor of autophagy) following treatment with our novel combination therapy (miR-96 and BIT-5) in the CI exposed VSC4.1 cells showed progression of autophagy for providing neuroprotection to the motoneurons (Figure 2).

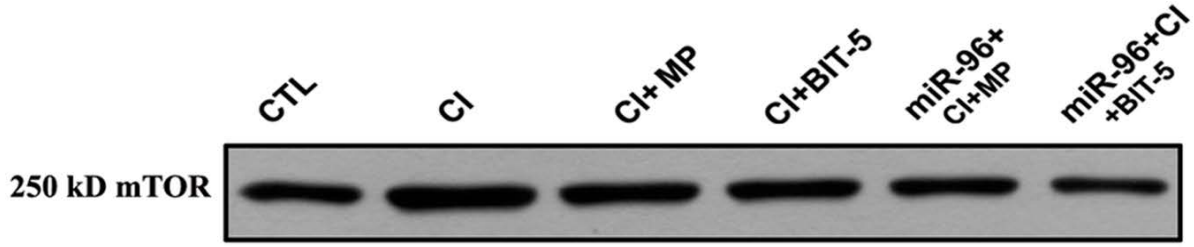

Figure 2. Western blotting for determination of progressio of autophagy following treatments with single or combination therapy in the CI exposed VSC4.1 cells. Cells were first transfected with 50 nM miR-96 mimic and incubated for 12 h. Then, cells were exposed to 200 nM CI for 24 h and treated with 1 μM MP or 10 μM BIT-5 and further incubated for another 24 h. Control cells without transfection was also investigated. At the end of incubation, cells were harvested and protein samples were subjected to Western blotting for examining the decreased levels of expression of mTOR, an inhibitor of autophagy.

Aim 2: *Status (Planned, In Progress).*

Cumulative detailed description of the progress made towards the aim/sub-aim including the methods utilized:

We made further progress on our proposed Specific Aim 2. Our cell culture studies established the mechanisms for therapeutic efficacy of the combination of miR-96 overexpression and BIT-5 treatment in SCI. So, we used this combination therapy in acute SCI in male Sprague-Dawley rats. In this acute SCI animal model, we kept using MP as a standard therapy. Conventionally, 48 h post SCI is regarded as an acute SCI model in rats. So, we continued using this acute SCI rat model for our further in vivo therapeutic studies. Briefly, adult male Sprague-Dawley rats (200-250 g, Charles River) were used to induce moderately severe SCI (40 g.cm force) at T10. As controls, sham (laminectomy only) animals and also SCI plus vehicle (dimethyl sulfoxide or DMSO) animals were used in our studies. We employed control and treatment groups for therapeutic studies. After treatments for 48 h, we surgically removed SCI lesion area segments (1-cm long) from all the animals and used these segments for histopathological and molecular studies. All these experiments were designed to validate and establish the increase in neuroprotection after treatment with novel combination therapy (miR-96 overexpression and BIT-5 treatment) in acute SCI in rats. Inducible cyclooxygenase (COX-2) plays an important role in inflammation following SCI and therefore therapeutic inhibition of COX-2 is highly desirable for prevention of pathogenesis in SCI. We currently produced results showing changes in expression of COX-2 at mRNA and protein levels in SCI tissues. We used the uniform expression of β-actin as the house-keeping control. The spinal cord tissues from acute SCI rats plus vehicle (untreated control) showed increase in expression of COX-2 compared to corresponding tissues from sham rats. Monotherapy negligibly decreased expression of COX-2 in the SCI lesion but the combination of miR-96 and BIT-5 substantially decreased expression of COX-2 in the SCI lesion segment.

Describe any data analysis that was performed and include charts/tables when available:

RT-PCR experiments showed the changes in expression of mRNA of the inflammatory molecule COX-2 in acute SCI rats

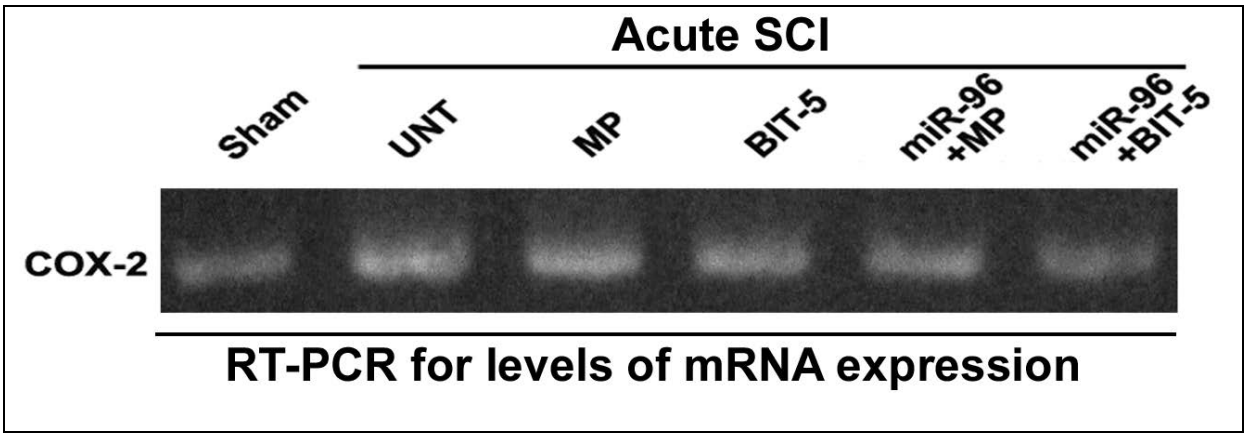

Figure 3. RT-PCR experiments to examine changes in expression of COX-2 in the SCI lesion segments in acute SCI rats. After 48 h of SCI induction (without treatment or treatments), rats were sacrificed, RNA samples were isolated and subjected to RT-PCR

using gene specific primers against COX-2 to show its down regulation leading to the highest neuroprotection due to treatment with the combination of the therapeutic agents (miR-96 + BIT-5).

Western blotting showed the highest decrease in expression of COX-2 molecule in acute SCI rats after the treatment with combination of miR-96 and BIT-5

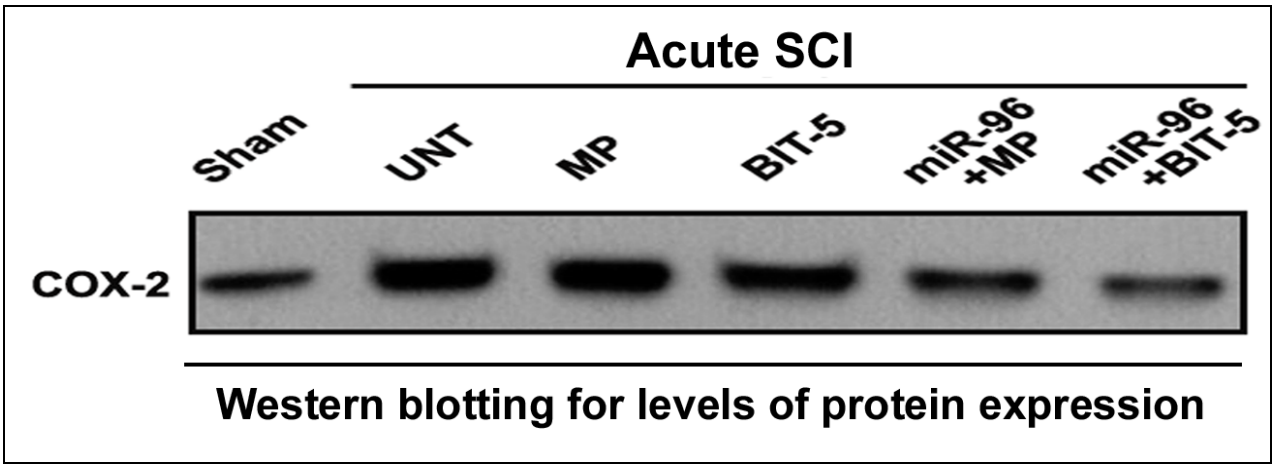

Figure 4. Western blotting for changes in expression of COX-2 at protein level in the SCI lesion segments in acute SCI rats. After 48 h of SCI induction (without treatment or treatments), rats were sacrificed, protein samples were extracted and subjected to Western blotting using specific antibody against COX-2 to show its down regulation leading to the highest neuroprotection due to treatment with the combination of therapeutic agents (miR-96 + BIT-5).

Aim 2: ***Status (Planned, In Progress).***

Cumulative detailed description of the progress made towards the aim/sub-aim including the methods utilized:

During the 30-month period, we continued using our well-established in vivo rat model of SCI to perform more experiments on Aim 2 of this grant award from the SCIRF. Using our experimental therapeutic agents miR-96 and BIT-5 alone and also in combination, we conducted our experiments in acute SCI in male Sprague-Dawley rats (Charles River). We still used MP as a standard therapy in our animal model of acute SCI. For all in vivo therapeutic studies in acute SCI, we employed adult male Sprague-Dawley rats (weighing 200-250 g) for induction of moderately severe SCI (40 g.cm force) at T10 by using our standardized weight-drop method. We used sham (laminectomy only) animals and also SCI plus vehicle (dimethyl sulfoxide or DMSO) animals as controls. For assessing prevention after treatments in animal model of acute SCI, we included six groups: sham (laminectomy without SCI), untreated (UNT) SCI, SCI plus MP, SCI plus BIT-

5, SCI plus combination of experimental therapeutic agents (miR-96 mimics + MP), and SCI plus combination of experimental therapeutic agents (miR-96 mimics + BIT-5). The UNT animals received <1% DMSO as vehicle. After treatments for 48 h, we surgically removed SCI lesion area segments (1-cm long) from all the animals and used these segments for histopathological and molecular studies. We isolated total RNA samples from the segments, prepared mRNA, and performed RT-PCR was performed using gene specific primers against IL-1β, IL-6, and β-actin (control) to show the down regulation of inflammatory cytokines for neuroprotection.

Describe any data analysis that was performed and include charts/tables when available:

Analysis of mRNA expression by RT-PCR experiments indicated the changes in levels of the inflammatory cytokine IL-1β in acute SCI rats after the therapeutic treatments (Figure 3).

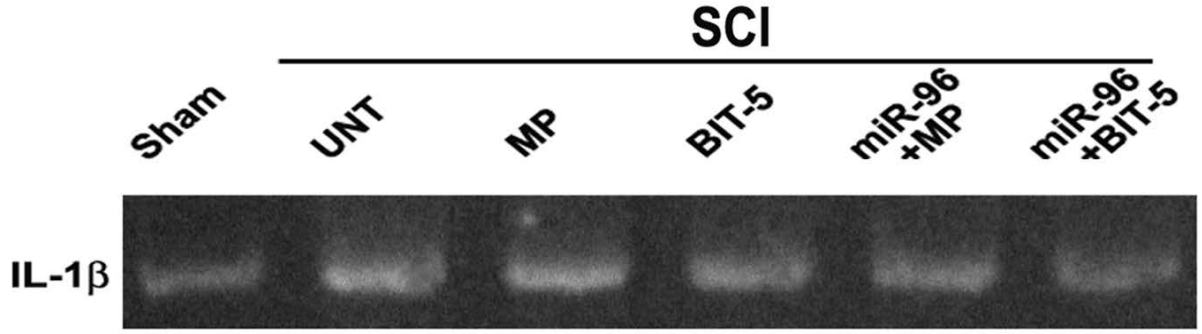

Figure 3. Assessment of changes in mRNA expression of IL-1β by RT-PCR experiments using the SCI lesion segments from acute SCI rats following treatments. After 48 h of SCI induction (without treatment or treatments), we sacrificed the rats using standard procedure, isolated RNA samples, and performed RT-PCR using the gene specific primers against IL-1β. Our data showed that combination of miR-96 and MP was not better than MiR-96 and BIT-5 in down regulating IL-1β in acute acute SCI rats. The highest down regulation of IL-1β occurred for the highest neuroprotection after the treatment with our novel combination therapy (miR-96 + BIT-5).

Determination of mRNA expression by RT-PCR experiments clearly showed the changes in levels of the inflammatory cytokine IL-6 in acute SCI rats after the therapeutic treatments (Figure 4).

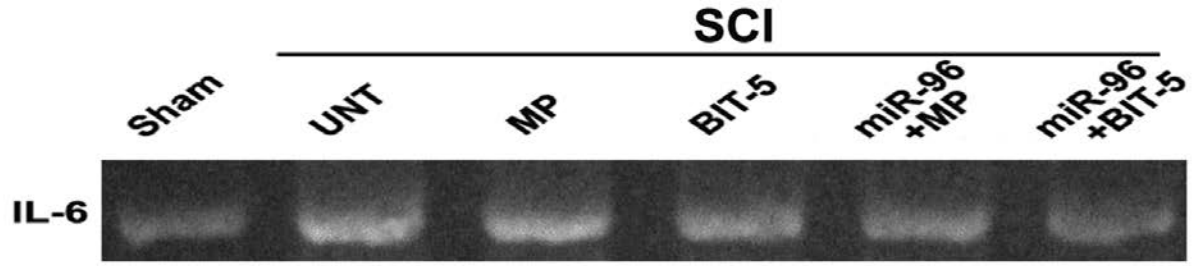

Figure 4. Determination of mRNA expression of IL-6 by RT-PCR experiments using the SCI lesion segments from acute SCI rats following treatments. After 48 h, we sacrificed the rats using standard procedure, isolated RNA samples, and performed RT-PCR using the gene specific primers against IL-6. Our data showed the highest down regulation of IL-6 for the highest neuroprotection after the treatment with our novel combination therapy (miR-96 + BIT-5).

List all articles, publications, presentations, grant applications or grant awards related to the SCIRF:

We produced peer-reviewed publication (shown below) related to SCI work in our laboratory. We also presented results from our SCI cell culture studies in a recent research meeting. Moreover, we submitted R01 grants (Ray as Co-PI) in collaboration with an investigator at Icahn School of Medicine at Mount Sinai (New York City, NY).

Publication (peer-reviewed publication on SCI):

Raghava N, Das BC, Ray SK (2017) Neuroprotective effects of estrogen in CNS injuries: insights from animal models. *Neuroscience and Neuroeconomics* 6: 15–29.

List all articles, publications, presentations, grant applications or grant awards related to the SCIRF:

Our work produced results for preparation and publication of articles in peer-reviewed journals. Our laboratory also presented SCI results in the scientific meetings. Besides, we recently submitted an R21 grant (Ray as PI) for funding our research by the National Institutes of Health.

Publications (in peer-reviewed journals):

Miller LG, Jr., Young JA, Ray SK, Wang G, Purohit S, Banik NL, Dasgupta S (2017) Sphigosine toxicity in MS and EAE; Evidence for ceramide generation via serine-palmitoyltransferase activation. Neurochem. Res. 42:2755-2768.

Dasgupta S, Ray SK (2017) Insights into abnormal sphingolipid metabolism in multiple sclerosis: targeting ceramide biosynthesis as a unique therapeutic strategy. Ther. Targets Neurol. Dis. 4:e1558.