Original Research

Prospects of siRNA cocktails as tools for modifying multiple gene targets in the injured spinal cord

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Impact statement

Gene therapy has reached the level of clinical trials. However, safety and efficacy are yet to be confirmed. The present study tested the prospects of gene silencing using siRNAs in a rat model of spinal cord injury. Some noteworthy observations include the effective and long-lasting silencing effects of siRNAs, inhibition of one gene's expression resulting in silencing of multiple genes in associated pathways, possibility of targeting more than one gene through siRNA cocktails, and differential gene silencing effects based on temporal changes in their expression patterns. It is argued that differential uptake of siRNAs by cells as observed and limitations in the analysis methods available can skew interpretations. Thus, this study may serve as a cautionary tale indicating that gene silencing using siRNAs for spinal cord injury can be a potential therapy, but practical issues are to be addressed in order to ensure consistency and safety.

Abstract

Gene silencing through RNA interference (RNAi) has been touted as a boon for identifying potential therapies for difficult-to-treat pathologies. In this regard, siRNA-mediated gene silencing for tackling the multifaceted pathophysiology of spinal cord injury seemed promising. The genes caspase 3 and sarm1 were targeted in the present study, using siRNAs in a rodent model of spinal cord injury, as the feasibility of concomitant silencing of more than one gene had not been previously attempted. The results indicated meager benefits in terms of functional recovery and tissue preservation. Interestingly, differential transfection efficiencies due to the heterogeneous nature of cells in the spinal cord along with variability in efficacy based on time of intervention affected the reproducibility of this approach. Complex gene interactions and inadequacies in molecular evaluation strategies further complicated the interpretation of the outcome. If these glitches are resolved through further research, gene therapy in general and RNAi, in particular, may become a mainstay approach for treating contusion spinal cord injury.

Keywords: Gene silencing, RNAi, siRNA, spinal cord injury, apoptosis, Wallerian degeneration

***Experimental Biology and Medicine* 2019; 244: 1096–1110. DOI: 10.1177/1535370219871868**

Introduction

Spinal cord injury (SCI) is a predominant cause for paralysis, next only to cerebral stroke. Contusion injuries are the most prevalent type of traumatic SCI in humans.[1] Currently, there are no validated therapies available for SCI and the widely practiced method of administering high doses of methylprednisolone within 24–48 h of injury is wrought with controversies.[2,3] While experimental studies usher several new approaches, none qualify for mainstream treatment through clinical trials. Thus, the need to explore novel therapies continues for SCI, a daunting neurological condition, for both clinicians and basic science researchers.

SCI triggers a complex pathology through cascades of molecular changes.[4] It is practically difficult to combine strategies to address these complex events. Therefore, a strategy that targets multiple factors simultaneously is in demand. Under these circumstances, RNAi through siRNA

may be a viable option, as it might be possible to target multiple pathologies (via genes) with suitable cocktails.

Manipulation of gene expression for SCI repair has been envisaged earlier. Transplantation of cells transduced to overexpress neurotrophic factors like neurotrophin-3, brain-derived neurotrophic factor, glial cell-derived neurotrophic factor, nerve growth factor, and chondroitin sulfate proteoglycan inhibitors like chondroitinase ABC to promote axonal sprouting[5,6] have been reported to be useful.

Gene manipulation of the host cells directly by overexpressing survival genes like neuroglobin[7] has also been reported. In addition to these studies which attempt to overexpress genes, shRNA-mediated silencing of axonal growth inhibitors (Nogo, myelin associated glycoprotein, nerve growth factor receptor) and gliosis inducing factors (glial fibrillary acidic protein (GFAP), Vimentin) have been reported to offer beneficial effects too.[5,8,9] Intranasal delivery of mesenchymal stem cells-derived exosomes containing phosphatase and tensin homolog (PTEN) siRNA preferentially migrated to the lesion site in the spinal cord and reduced PTEN expression leading to axonal regeneration and angiogenesis.[10] Similarly, siRNA tagged with chitosan and targeting iNOS showed significant reduction in cell death in mice compression SCI model.[11]

Interactions among genes are intertwined and complex. Therefore, permanent overexpression or suppression might lead to unforeseen complications in the long run. The potential risk of undesirable immune response and tumorigenesis associated with the use of viral vectors necessitates further validation. In this context, siRNA is a prospective candidate that can be chemically synthesized and may prove to be effective in eliciting post-transcriptional silencing, void of the risks associated with viral vector usage. In addition, their transient nature is highly desirable as the duration of gene knockdown can be regulated as required. siRNA against c-Jun was used to identify its role in axotomy, similarly the role of mitogen- and stress-activated kinase (MSK) 1 in inflammation and apoptosis after spinal cord injury was identified using siRNA coding lentiviral particles. Thus, siRNA have been used as tools to identify the role of target genes in specific conditions.[12,13]

However, siRNA-mediated transient silencing has not been extensively explored for SCI repair research. Earlier reports[14–17] while confirming the potential benefits of transient RNAi through siRNAs did not discuss critical aspects such as possible advantages of direct intraparenchymal delivery, appropriate time of intervention, cellular uptake, the possibility of silencing more than one gene, etc. In the present study, to draw proof for the concept of simultaneously silencing more than one gene, *Sarm1*, a gene known to be active in injured neurons involved in neuronal apoptosis and Wallerian degeneration[18,19] and *Casp3*, a gene known to be ubiquitously active in all the injured cells undergoing apoptosis[20,21] were selected for RNAi in a rodent model of contusion SCI.

We demonstrate the feasibility of targeting multiple genes simultaneously albeit with their transfection efficiencies swayed by the heterogeneous cell population in the injured cord. Our observation also sheds light on some of the practical challenges that need to be resolved in order to realize the application of siRNA-based transient RNAi as a therapy for contusion SCI.

Materials and methods

Animals used, study design, and animal grouping

Adult female Sprague Dawley rats, 75–80 days of age were used for the study. The study protocol was approved by the Institutional Animal Ethics Committee (IAEC). Animals had access to food and water *ad libitum* and were maintained as per the international and national guidelines.

In the present study, siRNA for *Casp3* or *Sarm1* were injected either immediately or two weeks after SCI. Accordingly, animals used for the study were randomly assigned to various groups, details of which were given in Table 1.

For polymerase chain reaction (PCR)-based mRNA level estimations, animals were euthanized on the 3rd day and on the 18th day after injury for the SCI group and for the groups in which siRNA injections were made immediately after SCI. For animals, in which siRNA injections were made on the 15th day, mRNA levels were quantified on the 18th day alone. Protein levels were compared between the groups using Western blot, three days from the time point used for mRNA analyses. Therefore, for Western blotting, spinal cords were obtained by euthanizing animals on the 6th day and the 21st day as appropriate. For histological studies, animals were euthanized after 60 days of initial injury. For each time point and parameter mentioned above, a minimum of six animals ($n = 6$) were used under each group. The entire study design along with the (endpoint) analyses methodology adopted is represented graphically in Figure 1.

Surgical procedures—SCI creation and/or intraparenchymal injections

Under ketamine—80 mg/kg and xylazine—10 mg/kg anesthesia, spinal cord at T10 level was exposed with intact dura by standard laminectomy procedure. After immobilizing the vertebral column using spinal clamps, a contusion SCI was created by dropping a 10 g rod from 12.5 mm height using the MASCIS III Impactor (Spinal clamps and impactor were obtained from W. M. Keck Center for Collaborative Neurosciences, Rutgers University, NJ, USA). The surgical wound was closed in layers and the rats were allowed to recover from anesthesia.

Stereotaxically, injections were made into the spinal cord parenchyma at a depth of 1 mm from the dorsal surface of the dura using a flame pulled glass capillary (with a tip diameter of ~20 to 30 microns) attached to a microliter syringe (Hamilton, OH, USA) which was fitted onto a stereotaxic apparatus (Stoelting Co., USA). The dorsal vein was avoided from being pierced.

Acute and chronic postoperative animal care

Urine retention in SCI animals was relieved by manual expression of bladder twice a day until automatic bladder

Table 1. Animal grouping used in the study.

S. No.	Group name*	Description
1.	Control	Sham-operated animals
2.	SCI	Subjected to contusive Spinal Cord Injury (SCI)
3.	iCasp3	siRNA against *Casp3* injected immediately after SCI
4.	dCasp3	siRNA against *Casp3* injected two weeks after SCI
5.	iSarm1	siRNA against *Sarm1* injected immediately after SCI
6.	dSarm1	siRNA against *Sarm1* injected two weeks after SCI
7.	Combo	siRNA against *Sarm1* and *Casp3* injected immediately after SCI
8.	iScram	Scrambled siRNA injected immediately after SCI
9.	dScram	Scrambled siRNA injected two weeks after SCI
10.	Ctrl-Scram	Scrambled siRNA injected without SCI

The group names given here are used in the text to refer the concerned group animals. Thus, the prefix "i" stands for "immediate" and "d" stands for "delayed" injection of siRNAs after SCI.
*Terms used to refer the groups in the text.

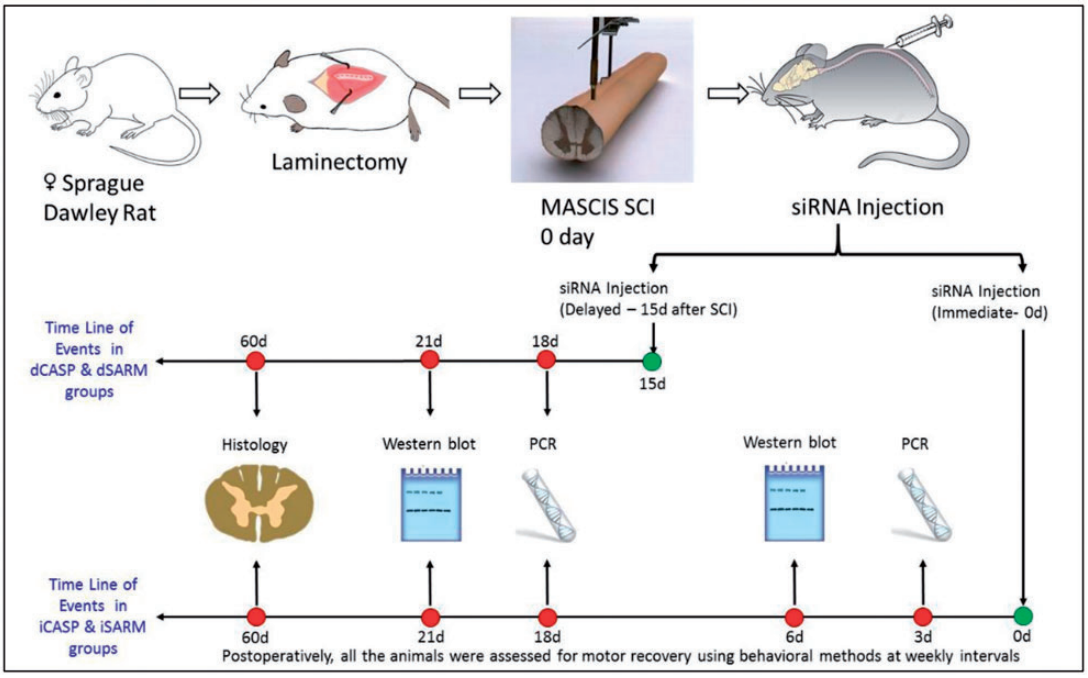

Figure 1. Graphical representation of the study design. SCI: spinal cord injury. (A color version of this figure is available in the online journal.)

was established. Prophylactic antibiotics, ampicillin—20 mg/kg/day and gentamycin—2 mg/kg/day were given *b.i.d.* through intramuscular injections for three days following surgery. Postoperative period of these animals was uneventful in general. In the event of urinary tract infections, an additional course of antibiotics effectively addressed the condition.

siRNAs used for the experiments

Individual flexitube siRNA were procured from Qiagen (Hilden, Germany). The sequence details of siRNA cocktails for Sarm1 were given in Table 2, and Table 3 lists the sequence details of siRNA cocktails against Casp3. AllStars Negative Control siRNA tagged with Alexa Fluor 555, a proprietary sequence from Qiagen (Hilden, Germany) that does not share homology with any known mammalian gene was used as negative control.

Table 2. Sarm1 siRNA sequence details.

Name	Sequence
siSARM_1	
SENSE	5′-ACGACAAUACAGCAUAUGATT-3′
ANTISENSE	5′-UCAUAUGCUGUAUUGUCGUTG-3
TARGET	5′-CAACGACAATACAGCATATGA-3′
siSARM_2	
SENSE	5′-GACCUAGGCAUGAAAUCAATT-3
ANTISENSE	5′-UUGAUUUCAUGCCUAGGUCTG-3′
TARGET	5′-CAGACCTAGGCATGAAATCAA-3′
siSARM_3	
SENSE	5′-GAUGGAAUGGUGCCCUCUATT-3′
ANTISENSE	5′-UAGAGGGCACCAUUCCAUCTG-3′
TARGET	5′-CAGATGGAATGGTGCCCTCTA-3′
siSARM_4	
SENSE	5′-GCCUAGGCGUGAUCUUGAATT-3′
ANTISENSE	5′-UUCAAGAUCACGCCUAGGCCG-3′
TARGET	5′-CGGCCTAGGCGTGATCTTGAA-3′

The cocktail of siRNAs used against Sarm1 includes four individual siRNAs targeting four different sites in the Sarm1 mRNA as given in this table.

Table 3. Casp3 siRNA sequence details.

Name	Sequence
siCASP3_1	
SENSE	5′-GAAGGAACUCACAUUAUAATT-3′
ANTISENSE	5′-UUAUAAUGUGAGUUCCUUCCT-3′
TARGET	5′-AGGAAGGAACTCACATTATAA-3′
siCASP3_2	
SENSE	5′-GGAACAGACCAUAAUACCATT-3′
ANTISENSE	5′-UGGUAUUAUGGUCUGUUCCTG-3′
TARGET	5′-CAGGAACAGACCATAATACCA-3′
siCASP3_3	
SENSE	5′-AGGUCGUCAUUGAACAAUATT-3′
ANTISENSE	5′-UAUUGUUCAAUGACGACCUGG-3′
TARGET	5′-CCAGGTCGTCATTGAACAATA-3′
siCASP3_4	
SENSE	5′-CAAAUUGUUGACUAUUAGATT-3′
ANTISENSE	5′-UCUAAUAGUCAACAAUUUGAG-3′
TARGET	5′-CTCAAATTGTTGACTATTAGA-3′

The cocktail of siRNAs used against Casp3 includes four individual siRNAs targeting four different sites in the Casp3 mRNA as given in this table.

Animals received the 4-siRNA cocktail (2 μg per individual siRNA = 8 μg) for *Casp3* and *Sarm1* at two different time points viz. either immediately after SCI or two weeks after injury. The siRNA was stabilized with JetPEI (0.16 μL/μg siRNA) (Polyplus-transfection, France) to improve transfection efficiency *in vivo*. The siRNA cocktail was made up to 20 μL with 5% glucose. The total volume of 20 μL was injected stereotaxically at two sites (10 μL each) which were separated from each other by 4 mm. After a slow injection of 10 μL over a period of 10 min in one site, the capillary was withdrawn after several minutes to prevent any backflow of the injected material through the needle track. As stated earlier, AllStars Negative Control siRNA tagged with Alexa Fluor 555 (Qiagen, Hilden, Germany) was used to verify cellular uptake *in vivo*.

Motor function recovery assessment

Assessment of gross locomotor skills of SCI rats was carried out using the Basso, Beattie, and Bresnahan (BBB) locomotor rating scale.[22] The animals were graded weekly.

Fine motor skills were tested using narrow beam test. Here, animals were allowed to cross two narrow rectangular beams with varying widths (2.5 cm and 1.25 cm) and a cylindrical beam (2.5 cm diameter). Animal performance was scored as described by Merkler.[23] The test assesses the integrity of local spinal networks as well as the supraspinal connections that control fine motor function.[24]

Gene expression analysis

RNA isolation and cDNA synthesis. Animals were euthanized by an overdose of anesthesia (ketamine and xylazine combination). Spinal cords were rapidly dissected out of the vertebral column on ice. A 10 mm segment with injury site as the epicenter was used for total RNA extraction using Trizol reagent (Invitrogen, CA, USA) following the manufacturer's protocol. Purity and integrity of the RNA were determined using NanoDrop spectrophotometer and gel electrophoresis, respectively, before proceeding for downstream application.

The isolated RNA (2 μg) was reverse transcribed using Omniscript RT kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. In brief, 2 μg RNA was incubated in a master mix containing reverse transcriptase enzyme, random hexamers, dNTP mix, buffers, etc. for 1 h at 37 °C in a thermocycler.

Quantitative PCR analyses of mRNA. Proprietary gene specific TaqMan assays and appropriate master mix were obtained from Thermo Fisher Scientific (USA) for analyzing the mRNA levels of caspase 3, sarm1, caspase 8, caspase 9, apoptosis inducing factor, poly(ADP-ribose) polymerase 1, tumor necrosis factor alpha, and toll like receptor 3. The cycling parameters were set as per the manufacturer's recommendation. The changes in mRNA levels were calculated by normalizing the values against each time-matched SCI groups. Gapdh and Hprt1 were used as internal controls. The results were represented as relative fold changes calculated using the log $2^{-\Delta\Delta CT}$ method.[25]

Protein isolation and estimation. Similar to the procedure adopted in sample preparation for PCR procedure, a 10 mm segment with the injury site was collected and homogenized in radioimmunoprecipitation assay (RIPA) buffer with protease inhibitor cocktail (Sigma Aldrich, MO, USA). The homogenate was centrifuged in a high-speed refrigerated centrifuge (Sorvall™ ST 16r, Thermofisher Scientific, MA, USA) at 4°C for 15 min at 12,000 rpm. The supernatant containing the proteins was collected and the protein concentration was estimated using Bradford's protein estimation (Bradford 1976) assay (Biorad, CA, USA).

SDS-PAGE and Western blot analyses. Proteins isolated were used to detect CASP3, SARM1, ED1, NF-H, NF-M, GFAP, and NeuN proteins through standard Western blot procedure. Briefly, 50 μg protein was resolved by SDS-PAGE using a 12% gel and polyvinylidene difluoride membranes transblotted with proteins were probed for the antigen (protein) of interest using appropriate primary antibodies (Abcam, UK). Bands were visualized by incubating membranes with appropriate horseradish peroxidase-conjugated secondary antibodies and by using luminol-based chemiluminescence kit (Thermofisher Scientific, MA, USA). Signals were documented using the G:BOX Chemi XRQ chemdoc from Syngene (India). GAPDH was used as the internal control. The relative protein expression of the proteins probed was calculated by normalizing with the internal control and by using time matched SCI groups as reference.

Histological analyses

Tissue fixation and processing. Animals were euthanized by an overdose of anesthesia, and tissues were fixed in 4% paraformaldehyde through standard transcardial perfusion. After 24 h of postfixation in the same fixative, the spinal cord was dissected out of the vertebral column. A 10 mm piece with the lesion site as the epicenter and 5 mm pieces

Table 4. The details of the antibodies used for immunofluorescence study.

Antigen	Primary antibody	Secondary antibody
ED1 (Macrophages)	Anti-CD68 Antibody [ED1] (Mouse IgG Monoclonal) (Cat# ab31630) 1:500 dilution	Goat Anti-Mouse IgG H&L with Alexa Fluor 488 (Cat# ab150113) 1:1000 dilution
GFAP (Mature astrocytes)	Anti-GFAP Antibody (Rabbit IgG Polyclonal) (Cat# ab7260) 1:1000 dilution	Goat Anti-Rabbit IgG H&L with Alexa Fluor 488 (Cat# ab150077) 1:1000 dilution
NeuN (Neuronal nuclei)	Anti-NeuN Antibody [EPR12763] (Rabbit IgG Monoclonal) (Cat# ab177487) 1:300 dilution	Goat Anti-Rabbit IgG H&L with Alexa Fluor 488 (Cat# ab150077) 1:1000 dilution

Proteins specific to macrophages (ED1), astrocytes (GFAP), and neurons (NeuN) were studied using suitable antibody combination through immunofluorescence as well as Western blot in order to evaluate the status of the respective cell population.

proximal and distal to the lesion segment were taken for histological analysis. The lesion segment after cryoprotective treatment with 30% sucrose was embedded in tissue freezing medium (Leica Biosystems, Germany). Frozen at −20°C, 10-μm-thick sections in the sagittal plane were taken using a cryotome (Leica Biosystems, Wetzler, Germany). The proximal and distal segments were processed and impregnated in paraplast (Leica Biosystems, Wetzler Germany) and 7 μm sections were taken in the transverse plane using a rotary microtome (Leica Biosystems, Wetzler. Germany).

Light microscopy staining and quantification. Sections were subjected to various neurohistological staining procedures as per the standard procedures described in the literature.

Luxol fast blue (LFB) stain (Sigma Aldrich, MO, USA) was used to demonstrate the status of myelination in the spinal cord.[26] In transverse sections from proximal and distal segments stained with LFB, the percentage of the myelinated area was calculated by the following formula

$$\text{Percentage of Myelination} = \frac{\text{Area of blue pixels}}{\text{Total white matter area}} \times 100$$

Total white matter area and the area of blue pixels in the white matter were calculated using the threshold option in the ImageJ software (National Institutes of Health, Bethesda, MD, USA).

The apoptotic cells in the spinal cord were demonstrated by using the modification in Crossmon's trichrome method proposed by Anton (1999).[27] The cells stained red were counted as apoptotic cells. Masson's trichrome stain was used to demonstrate general tissue architecture.[28] Glial fibers and connective tissue (collagen) would appear as dark blue/violet against a background of other structures stained in tones of pink color.

Immunohistochemistry and fluorescence microscopy. Tissue sections from animals with scrambled siRNA injections were probed for ED1, NeuN, and GFAP antigens using appropriate primary and secondary antibodies tagged with Alexa Fluor 488 fluorophore. Given that scrambled siRNA was tagged with Alexa Fluor 555, rhodamine filter set (red channel) was used to visualize signals from siRNA, and the antigens' presence were screened using an fluorescein isothiocyanate (FITC) filter set (green channel). DAPI was used to label nuclei. Individual channel images were acquired through a microscope with epi-fluorescence attachments (Nikon 2E, Japan) and were merged using the Nikon (NIS-Elements) software to localize the scrambled siRNA signals with respective antigens probed for. The details of the antibodies used are given in Table 4.

Quantification, numerical data representation, and statistical analysis. Semi-quantitative statements regarding immunofluorescence results were made based on the observations of two independent assessors who were blind to the experimental design and animal groups. For histological quantification of myelination area, spared tissue area and number of apoptotic cells, following standard stereological principles, ImageJ software Version 1.51j8 (a freeware from National Institute of Health (NIH), USA—official website http://imagej.nih.gov/ij) was used.

All the numerical data were represented through appropriate graphs and tables. Each value represents the mean of the group concerned ± standard error of the mean. Statistical analyses were carried out using one-way analysis of variance method using GraphPad Prism Version 7.0 Software, and P value ≤ 0.05 was considered as significant.

Results

Safety and efficacy of siRNA usage

The siRNA cocktails were found to effectively reduce their respective mRNA levels in cultured cells subjected to oxidative stress without eliciting any evident toxicity to the cells (data not shown). The mRNA levels of *Tnf-α* and *Tlr-3* did not increase in siRNA-treated animals when compared with SCI group animals. In fact, on day 18, siRNA-treated animals (both immediate and delayed injection groups) had lower mRNA levels for these genes when compared with matching injury group animals (Figure 2(a) and (b)).

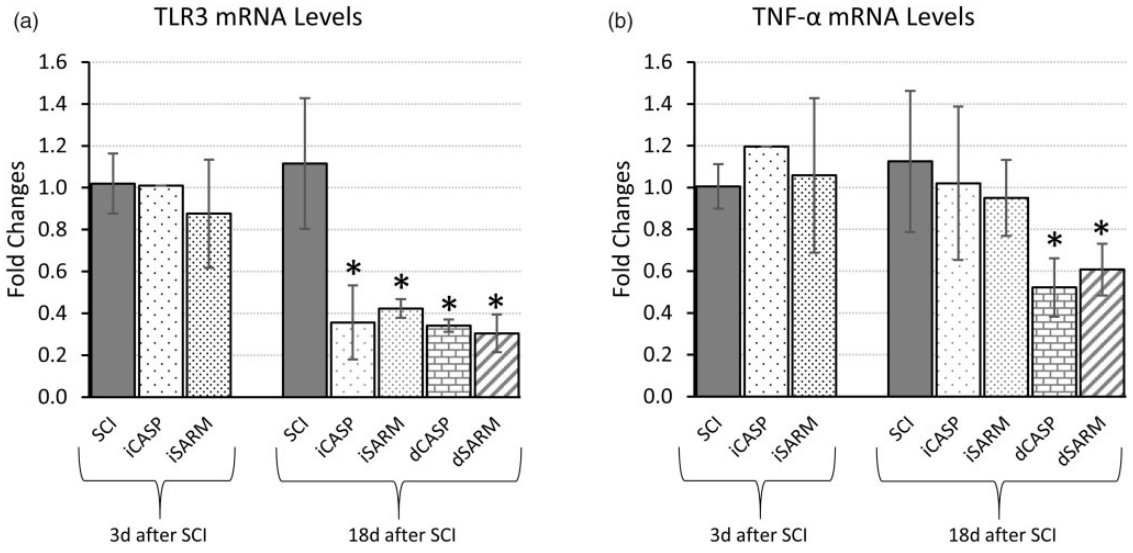

Figure 2. Absence of immune/inflammatory reaction triggered by siRNAs. Intraspinal injections of siCASP3 or siSARM1 did not increase the mRNA levels of TLR3 (a) or TNF-α (b) either in the short term (3 days) or in the long term (18 days). This could possibly indicate the absence of any immune/inflammatory reaction triggered by the siRNAs. Data represented as mean ± standard error of mean. Asterisk (*) represents significance when compared with SCI group at $P \leq 0.05$. SCI: spinal cord injury.

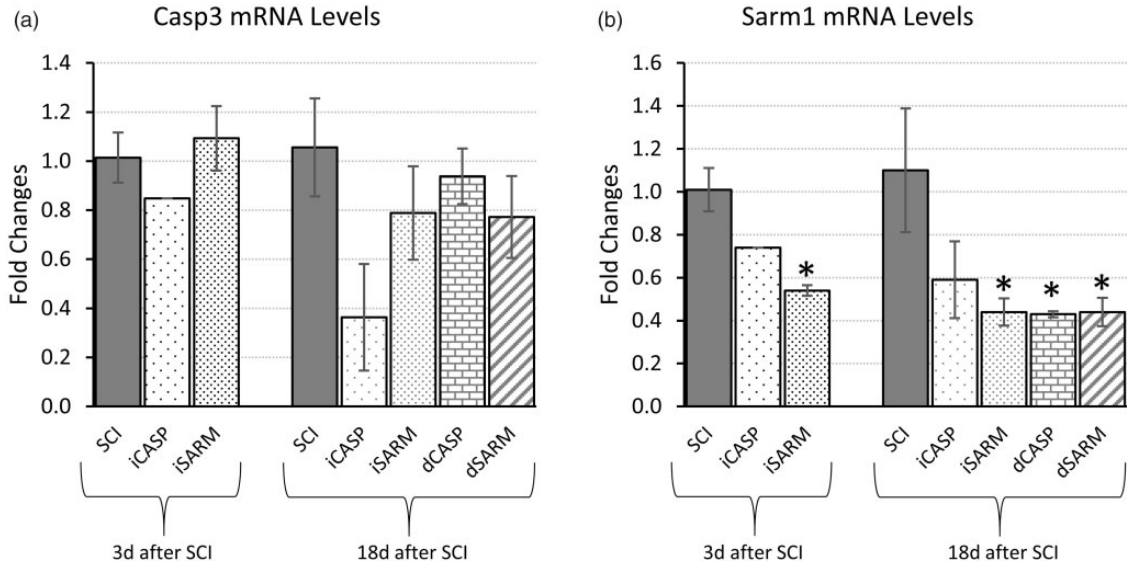

Figure 3. Effect of siRNAs on mRNA levels of target genes and their collateral effects. siRNA for casp3 effectively reduced the levels of casp3 mRNA when given immediately after injury but not very effective when given 15 days after SCI (a). Interestingly, siCASP, irrespective of the time of administration, reduced the Sarm1 mRNA levels (b). siSARM contrarily reduced the Sarm1 mRNA levels but failed to show significant reduction in Casp3 mRNA levels. Such effects of siRNAs in reducing the mRNA levels of genes which were not targeted by the siRNA may not always be a case of "off-target" effect rather a resultant of indirect effect due to gene interactions (refer to text for details). Data represented as mean ± standard error of mean. Asterisk (*) represents significance when compared with SCI group at $P \leq 0.05$.

The siRNA for Casp3 and Sarm1 effectively reduced their respective mRNA levels (Figure 3(a) and (b)). siCasp3 reduced Casp3 protein levels when given either immediately or two weeks after injury (Figure 4(a)). However, siSarm1 reduced Sarm1 protein levels only when it was given 15 days after injury (Figure 4(b)). Importantly, in animals injected with both siRNAs, reduction in mRNA levels were similar to their stand-alone counterparts (Figure 5).

The vehicle and stabilizer for siRNA, i.e. JetPEI, when injected alone, did not cause changes in mRNA expressions. Negative control siRNA (i.e. scrambled siRNA) injection caused subtle changes in the mRNA levels of various genes. They were, however, insignificant. In addition, the changes appeared to be random and did not present any logical pattern. Therefore, any nonspecific effects due to siRNA transfection for the observed changes (especially for the genes of interest viz. Casp3 and Sarm1) was unlikely.

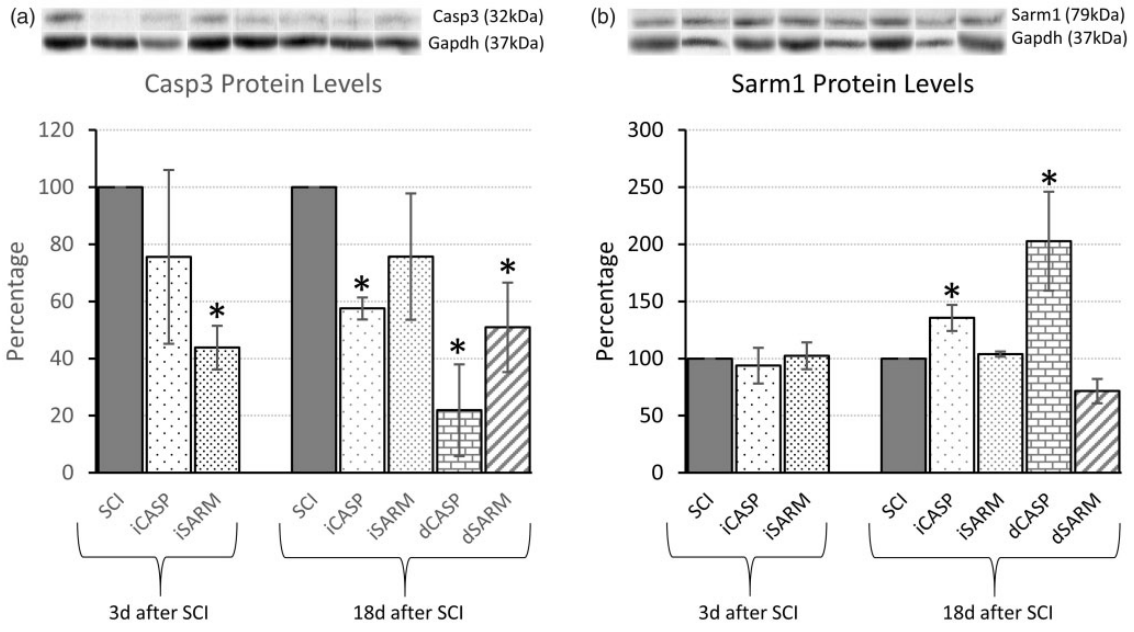

Figure 4. Efficiency of gene silencing by siRNA on target and collateral genes. Casp3 protein levels were reduced in siCASP groups and Sarm1 protein levels were reduced in delayed administration of siSARM. While siCASP exhibited gene silencing irrespective of the time of administration, siSARM was effective only when given 15 days after SCI, indicating the efficacy of siRNAs could differ according to the temporal changes in gene expressions. While siSARM reduced the Casp3 protein expression, the reverse was not observed. Refer to text for a plausible speculation for this effect. Thus, siRNAs could exhibit "cluster effects" on other genes which get influenced by the downregulation of siRNA targeted gene. Blots are representative images from triplicates. SCI: spinal cord injury.

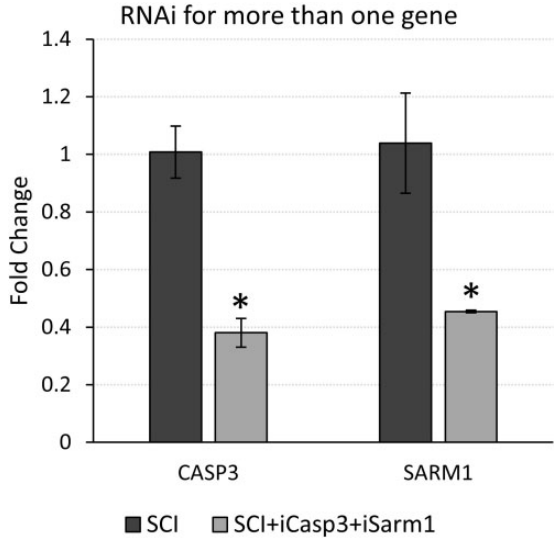

Figure 5. Possibility of RNAi for more than one gene. Simultaneous injection of siCasp and siSarm siRNA suppresses the expression of both target genes effectively. This indicates the feasibility of combining siRNA to target multiple genes simultaneously. Data represented as mean ± standard error of mean. Asterisk (*) represents significance when compared with SCI at $P \leq 0.05$. SCI: spinal cord injury.

Ancillary gene expression changes induced by RNAi

It was observed that siSarm1 influenced the expression of *Casp3* at both mRNA and protein levels (Figures 3(a) and 4 (a)). Contrarily, siCasp3 did not result in the reduction of *Sarm1* protein but reduced mRNA levels (Figures 3(b) and 4 (b)). Interestingly, both siCasp3 and siSarm1 were found to influence the mRNA levels of related genes such as *Casp8, Casp9, Aif, Parp1, Nmnat2, Gfap, Gap43*, and *Mag*. Table 5 represents an efficient perturbation of the death signal because of casp3 and/or sarm1 knockdown. In addition, mRNA levels of related genes in death pathways were lowered due to siCasp3/siSarm1 administration. This indicates the indirect action of the siRNAs on other related genes due to gene interactions.

Long-term tissue level changes caused by siCasp3 and siSarm1

During the autopsy, it was observed that injured spinal cords present evidence of atrophy/necrosis at the lesion site with reduced cross-sectional area. Masson's trichrome stain showed the presence of necrotic cavity at the lesion epicenter. Comparatively, siRNA-injected groups exhibited better tissue morphology. Histological changes were insignificant upon qualitative analysis (Figure 6(a) and (b)). Quantitation of spared tissue indicated a mild reduction of cavity area in groups injected with siRNAs immediately after SCI. Better preservation of tissue was evident in delayed siRNA injection groups (Figure 6(c)).

Analyses of myelination using LFB stain indicated that the corticospinal tract was better preserved in the distal segments of the siRNA-treated groups when compared to the lesion (Figure 7(a)). However, total myelination area of proximal and distal segments when quantified did not show any significant increase in siRNA groups (Figure 7 (c)). The number of apoptotic cells as indicated by the modified trichrome stain significantly increased in the SCI group. siRNA treatments reduced the apoptotic cell population very effectively in the distal segments but not in proximal segments (Figure 7(b) and (d)).

Table 5. Effect of siCASP and siSARM on the mRNA levels of the related genes.

	3 days after SCI			18 days after SCI				
Gene	**SCI**	**iCasp3**	**iSarm1**	**SCI**	**iCasp3**	**dCasp3**	**iSarm1**	**dSarm1**
Casp8	1.02 ± 0.12	0.20 ± 0.0*	0.96 ± 0.26	1.20 ± 0.41	0.67 ± 0.33	0.68 ± 0.05*	0.73 ± 0.14	0.83 ± 0.12
Casp9	1.04 ± 0.21	0.73 ± 0.0*	1.12 ± 0.34	1.21 ± 0.47	0.60 ± 0.07	0.29 ± 0.09*	0.55 ± 0.09*	0.43 ± 0.08*
Aif	1.01 ± 0.10	1.22 ± 0.0	0.59 ± 0.17*	1.26 ± 0.52	0.40 ± 0.07*	0.25 ± 0.05*	0.45 ± 0.04*	0.32 ± 0.08*
Parp1	1.06 ± 0.21	1.02 ± 0.0	1.03 ± 0.06	1.45 ± 0.08	0.79 ± 0.14*	0.88 ± 0.16*	0.99 ± 0.09*	1.18 ± 0.33

Injection of siRNAs against Casp3 (siCASP) and Sarm1 (siSARM) resulted in the gene expression changes in the associated genes of cell death pathways as given here. Both immediate injections after SCI (iCasp3 and iSarm1) as well as delayed ones—2 weeks after SCI (dCasp3 and dSarm1) resulted in the reduced expression of Casp8, Casp9, Aif, and Parp1 mRNA levels. Values represent group mean ± SEM. SCI: spinal cord injury.
*Statistically significant difference when compared with the SCI group at $P < 0.05$ level.

Figure 6. Morphological changes and tissue necrosis in the spinal cords. Representative images of control, SCI, and siRNA-treated group spinal cords as seen during autopsy and after sagittal sections stained with Masson's trichrome. Typical necrotic thinning out of the spinal cord at the injury epicenter (white arrow) seen in lesion group animals. Comparatively, in siRNA-treated animals, tissue damage (yellow arrow) at lesion site appears mitigated (a). However, histology revealed that the necrotic cavity was filled with infiltrated cells and scar tissue (b). Quantification indicated meagre tissue sparring evident in dCASP and dSARM groups when compared with SCI group (c). SCI: spinal cord injury. (A color version of this figure is available in the online journal.)

Macrophage infiltration, gliosis, and neuronal status

ED1 protein, the marker for macrophages increased in all siRNA injected groups, especially on 21st day in the siCASP3 group and by 6th day in the siSARM1 group (Table 6). Likewise, the proteins GFAP, NF-M, NF-H, and NeuN were all found to be increased in the siRNA groups (Table 6).

Quantification of cell-specific proteins through Western blot indicated increased protein levels of ED1 (Macrophages), NF-H, NF-M, NeuN (Neurons and Neurofilaments), and GFAP (Astrocytes). Prevention of death in the cells by the siRNAs might have resulted in the increased levels of cell-specific proteins.

Cellular uptake of siRNA in vivo

The spread of siRNA in the tissue planes and cellular uptake of siRNA injected intraparenchymally was assessed using scrambled siRNA tagged with Alex Fluor 555. siRNA diffusion in the rostrocaudal axis was higher in an injured cord when compared to an uninjured cord. In the uninjured cord, the dye was confined to 1–2 mm from the injection site, whereas in the injured cord, the dye spread was seen extended to about 4–5 mm from the site of injection. The quantity of dye uptake was more pronounced in macrophages, followed by astrocytes, and to the least extent in neurons (Figure 8).

Functional outcome of RNAi in SCI

At the end of eight weeks, gross motor skills evaluated using the BBB score did not show any significant improvement in siSarm1 groups. However, in both immediate and delayed injection groups of siCasp3, up to a two-point improvement was observed when compared to the SCI group (Figure 9(a)). Fine motor skills evaluated through the narrow beam presented similar findings, with the siCasp3 groups' average better than the other groups (Figure 9(b)).

Discussion

The results obtained in the present study are very encouraging, as they are affirmative of the previous studies on siRNA usage, which reported tissue preservation and recovery of function.[17,29] The concerns that arose after introspection of the methodology adopted and results obtained are presented herewith.

Nature of siRNA, dosage, and route of administration

In most siRNA studies, a single locus on the mRNA is targeted by the antisense strand of the siRNA[30–33] and

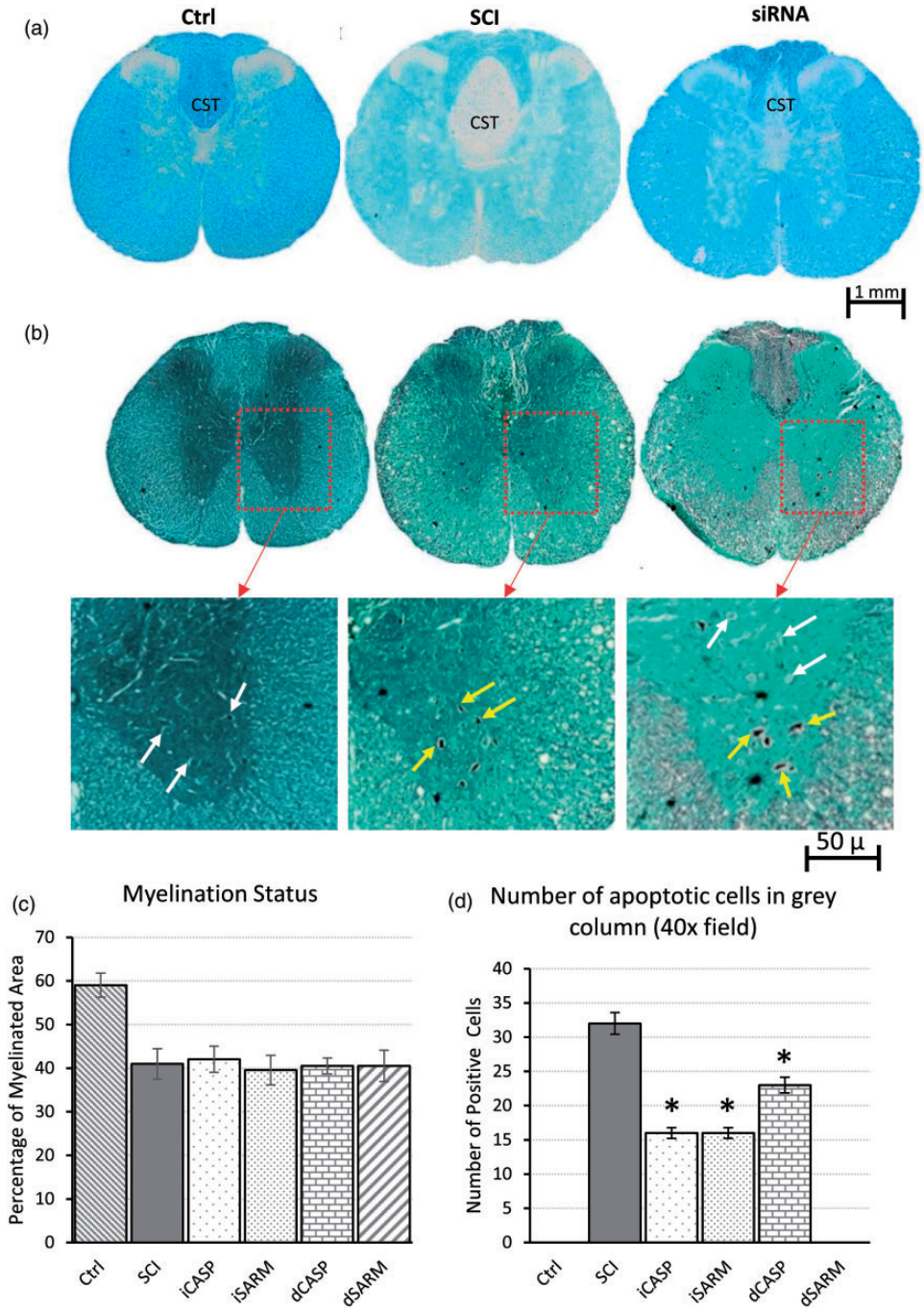

Figure 7. Status of myelination and apoptosis in neurons 8 weeks after SCI and siRNA injection. Representative LFB images (a) in which myelinated area appears in blue and non-myelinated regions have creamy white color. In a few instances, myelination, especially in the CST area of distal segment was slightly better in siRNA groups (as shown here). However, groups' mean calculated after quantification did not show any significant difference when compared with SCI group (c). Representative images of modified trichrome staining (b) illustrating green colored normal cells (white arrows) and red-colored apoptotic cells (yellow arrows). Quantification showed significant reduction of apoptotic cells in all the siRNA groups. It is not known whether absence of any positive cell in dSARM group was due to the inappropriate stage of cell death to be detected by the methodology adopted. Asterisk (*) represents statistical significance at $P \leq 0.05$ level when compared with SCI group. SCI: spinal cord injury. (A color version of this figure is available in the online journal.)

often delivered systemically through intravenous, intrathecal, intraventricular, or transnasal routes.[34,35] As naked siRNAs are unstable, they were chemically modified to withstand enzymatic degradation and immune recognition.[36] Cytosolic uptake was improved using strategies like encapsulation, conjugation to nanoparticles, or exposure to photomechanical waves.[10,37–39]

Being noninvasive/minimally invasive, systemic administration offers a lucrative choice for the administration of siRNAs in humans through multiple dosages. However, it necessitates infusion of large quantities of siRNA in order to achieve effective concentration at the site of injury. Excessive siRNA could lead to an interferon response in the body[40] and might exhaust the

Table 6. Quantification of proteins specific to the distinct cell population.

	6 days after SCI			21 days after SCI				
Protein	**SCI**	**iCasp3**	**iSarm1**	**SCI**	**iCasp3**	**dCasp3**	**iSarm1**	**dSarm1**
ED1	100	81.39 ± 11.17	173.15 ± 20.66*	100	141.10 ± 32.60*	142.56 ± 35.95*	139.98 ± 65.23	152.282 ± 29.24*
NF-H	100	96.99 ± 9.35	104.76 ± 12.38	100	121.58 ± 21.35*	111.69 ± 23.73	132.2 ± 9.44*	113.87 ± 12.14
NF-M	100	72.02 ± 9.82*	107.43 ± 14.62	100	82.58 ± 20.89	67.44 ± 9.98*	110.33 ± 7.61	106.9 ± 30.51
GFAP	100	371.74 ± 98.22*	450.05 ± 29.16*	100	165.07 ± 35.65*	117.35 ± 72.82	959.65 ± 35.72*	505.14 ± 41.53*
NEUN	100	159.23 ± 39.31*	52.64 ± 17.45*	100	133.34 ± 48.66	148.39 ± 1.85*	81.1 ± 5.9*	91.66 ± 3.08

Injection of siRNAs against Casp3 (siCASP) and Sarm1 (siSARM) resulted in the changed levels of proteins specific to distinct cell populations viz. neurons, astrocytes and macrophages. Using Western blotting, the proteins were quantified at two different time points viz. 6 days and 21 days after SCI. Administration of respective siRNAs were either immediately after SCI (iCasp3 and iSarm1) or 15 days after SCI (dCasp3 and dSarm1). Protein levels were quantified as percentage by normalizing against the levels in the lesion group, i.e. SCI group (considered as 100%) at corresponding post-operative periods. Values represent group mean ± SEM. SCI: spinal cord injury.

*Statistically significant difference when compared with the SCI group at $P < 0.05$ level.

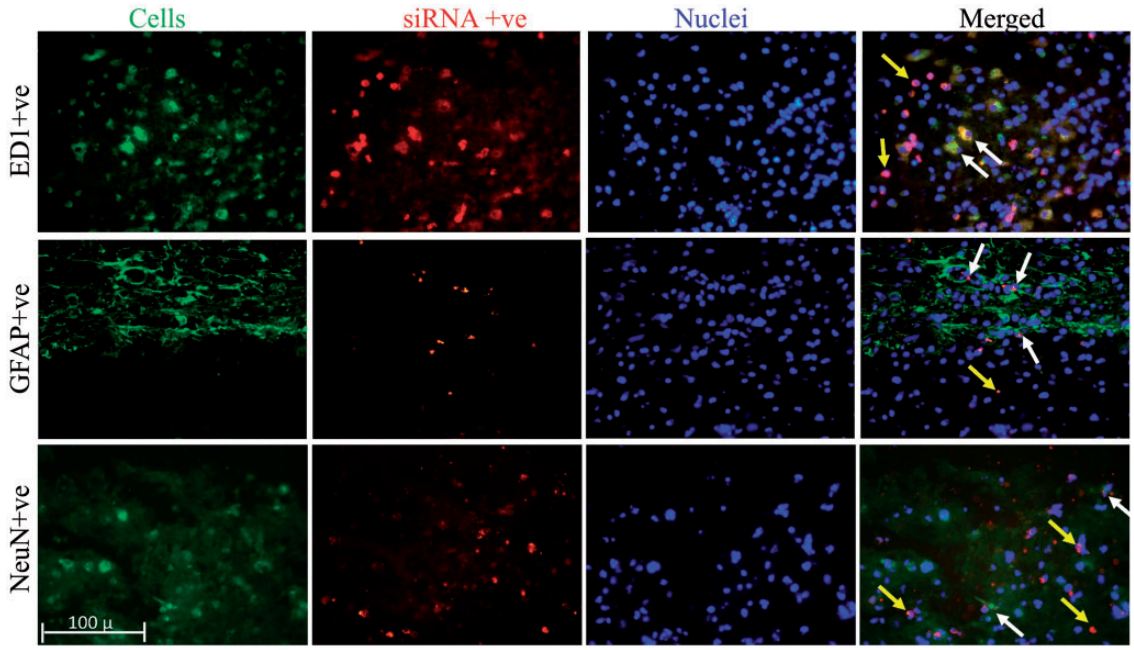

Figure 8. siRNA uptake by different cell types. Scrambled siRNA with red fluorescence tag (alexor 555) was used in IHC to study the siRNA uptake by different cell types. Top row—ED1+ve macrophages; Middle row—GFAP+ve astrocytes; Bottom row—NeuN+ve neurons. White arrows indicate co-localization of siRNA in cells probed for. Yellow arrows indicate siRNA present in other cells than the one probed. In general, macrophages were the most prominent cell population with siRNA followed by astrocytes and neurons were the ones with least uptake. Scale bar = 100 µ. (A color version of this figure is available in the online journal.)

RNA-induced silencing complex (RISC).[41] Due to lack of RISC, free siRNA might undergo rapid degeneration and elimination.[42] In addition, dysregulation of the innate functions of miRNAs due to nonavailability of RISC could lead to cytotoxicity.[42] Likewise, chemical modifications and/or conjugations can present additional problems of complexity in design, unpredictability of dissociation upon entry into the cells and retention of potency.[43]

Parsons *et al.*[44] opined that targeting multiple sites can be more effective than just targeting a single site in a mRNA during RNAi. Accordingly, in the present study, usage of a cocktail of four siRNAs resulted in effective gene silencing with the minimal concentration as recommended by the jetPEI manufacturers based on intracerebral applications. This along with the direct intraparenchymal route of administration could have avoided the undesirable immune responses (associated with large doses administered systemically) as evident from the lack of upregulation in Tnf-α (a marker of inflammation) and Tlr-3 (a marker for immune response) mRNA levels. PEI (polyethyleneimine) is known to be a stabilizer for siRNAs.[45] The use of PEI containing vehicle (jetPEI) was found to be efficient in delivering the siRNA and ensuring its stability as evident from the persistence of effective gene silencing 21 days postinjection.

Toyooka *et al.*[16] applied an atelocollagen containing a cocktail of siRNAs against GFAP and Vimentin on the surface of the injured spinal cord. While a reduction in the targeted gene expression was reported, no significant functional recovery was documented. No other paper describes the use of siRNA cocktails *in vivo*. In this context, the present study's approach of using siRNA cocktails by direct intraparenchymal delivery after stabilizing with PEI appears to be a simple yet effective option especially

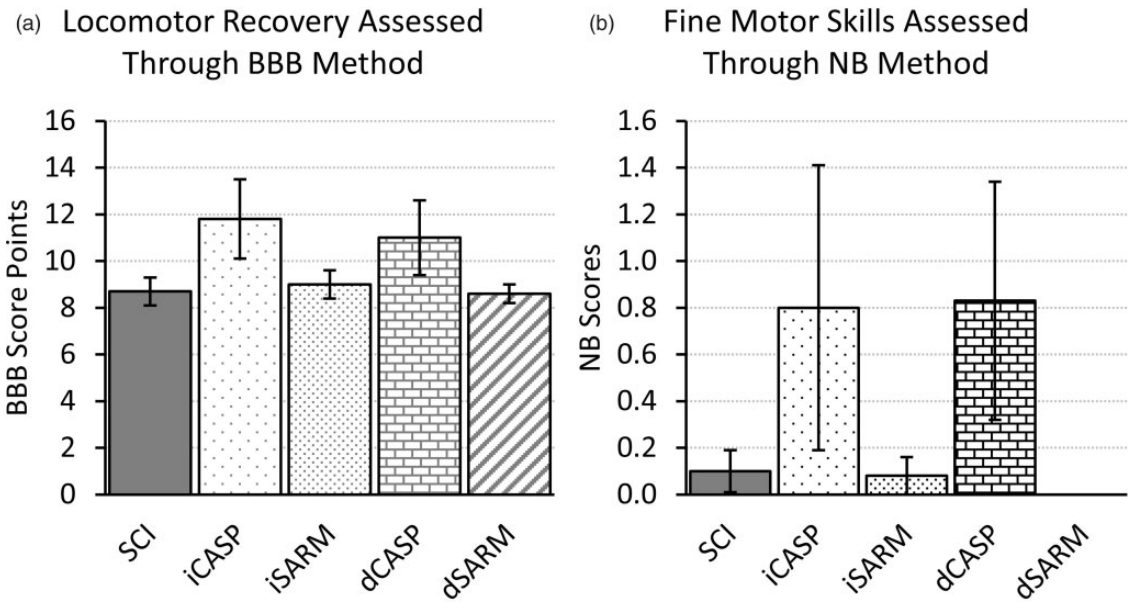

Figure 9. Effect of siRNA on locomotor recovery. At eight weeks post SCI, BBB scores reached 8.7 in the scale of 0 (totally paraplegic) to 21 (normal walking) points. There was no improvement in siSARM1 groups. Although statistically insignificant, siCASP3 groups were comparatively better than SCI group (a). In a scale of 0 (paraplegic) to 6 (normal) points in narrow beam, group mean in SCI animals barely reach 0.1 point. No improvement noted in siSARM1 groups. Similar to BBB scores, siCASP3 group average was high albeit lack of significance due to the high degree of variation observed (b). Although scoring was carried out on weekly basis, only the scores at the end of eight weeks were shown here rather than as trends over the entire period. SCI: spinal cord injury.

without any evidence of harmful effects. Given that the usage of pulled glass capillaries (with a tip diameter of ~20 to 30 microns) and stereotaxic method of injection cause negligible trauma to the spinal cord, direct intraparenchymal delivery can be a viable option for human application also.

The effectiveness of RNAi on target genes and collateral effects on ancillary genes

Apoptosis is triggered immediately after SCI and persists throughout the pathogenesis. Thereby, it would result in the upregulation of Casp3. Consequently, both immediate and delayed administration of siCasp3 effectively silenced Casp3 expression. Wallerian degeneration of axons typically reaches its peak around two weeks after axonal injury and so the expression levels of the associated gene viz. Sarm1 might follow a similar pattern. Coincidentally, siSarm1 downregulated gene expression, only when administered two weeks after injury. Since there is no previous report of siRNA administration at different time points, the observation in the present study is unique and could indicate that maximum efficiency of siRNAs can be realized only when administered at the target mRNA's expression peak. Implying that the time of intervention with siRNA may be a critical factor.

In most siRNA studies, only the expression pattern of the targeted gene was monitored, while other genes in the spectrum were seldom addressed.[30,31,46] Yet, there were some reports on the cluster effects of siRNA on the mRNA levels of nontargeted but related genes.[47] In the present study, both siCasp3 and siSarm1 were found to influence the mRNA levels of each other and some related genes involved in the cell death pathway. siSarm1 was found to reduce the protein levels of Casp3 indicating a strong interaction between the two genes.

Wallerian degeneration of axons induces apoptosis in the oligodendrocytes which myelinate those axons (Beattie *et al.*, 2016).[48] Mitigation of Wallerian degeneration by siSarm1 might have reduced apoptosis in oligodendrocytes, thereby resulting in the reduction of Casp3 expression as observed in the study. Thus, silencing effects of a siRNA on another nontargeted gene cannot simply be brushed aside as nonspecific/off-target effect. A thorough understanding of gene interactions and secondary effects of gene silencing is required to precisely predict the outcome of siRNA therapy.

The cluster effects of siRNAs may be advantageous; through careful selection of siRNA, multiple effects can be achieved simultaneously. At the same time, potential undesirable effects might ensue due to unforeseen gene interactions.

Differential cellular uptake of siRNA and its relevance

Both the siRNAs used in the study, especially, siCasp3 appears to have prevented death in the cells which might have taken up the siRNA. Accordingly, macrophages, astrocytes, and neurons might have been rescued from death as evident from the increased protein levels of ED1, GFAP, and NeuN respectively. Bright field microscopy indeed indicated better-preserved myelin and less apoptotic cells in the siRNA-treated groups. Semi-quantitative immunohistochemistry (IHC) results confirm the increased presence of macrophages and glia but failed to establish neuronal preservation satisfactorily.

Infiltrating macrophages adopt M1 or M2 lineage depending upon the induction received from Tnf-α or

Tgf-β, respectively.[49,50] *Tnf-α* increases immediately after the injury, while *Tgf-β* requires more time to upregulate its level.[51] siRNA given two weeks after SCI could have prevented death in the freshly infiltrating macrophages during that time. These macrophages would adopt M2 lineage due to the presence of TGF-b whose level also raised concomitantly. Given that M2 macrophages are beneficial,[52] this could explain the better tissue preservation observed in the delayed siRNA groups. Increased GFAP protein and supportive IHC results indicate increased gliosis. Astrocytes' role in recovery after SCI is debatable. While they assist neurons metabolically and are a source of growth factors,[53] their role in axon growth inhibition and mechanical blockage for axon pathfinding[54] needs attention. Despite the increase in NeuN protein levels, IHC failed to show increased neuron number.

Consolidating the histology results, it appears that the tissue preservation observed might be due to the preservation of non-neuronal tissue components comprising of connective tissue, astrocytes, and oligodendrocytes rather than the preservation of neurons. Semi-quantitative IHC-based assessment in animals injected with fluorescence-tagged siRNA adds support to this troublesome conclusion as uptake of siRNA was primarily by macrophages and astrocytes and not neurons.

In general, neurons are difficult to transfect when compared to astrocytes and macrophages,[55] through intraparenchymal delivery of siRNA or lentiviral vectors.[38,56] Some studies recommend viral vector-mediated delivery of shRNA to achieve RNAi in neurons rather than using siRNAs.[57] Thus, differential uptake of siRNA by the cells was not surprising; however, it raises certain concerns.

With no control over the cells which take up siRNA, complications may brew-up if a gene in an unintended cell gets silenced. This differential and indiscriminative uptake of siRNA could present additional trouble in designing the stability of siRNA. For example, silencing casp3 in neurons for several weeks may be beneficial; the same effect in M1 macrophages could prolong inflammation, which is detrimental.

Some hope is around the corner to target selective cell population for the delivery of siRNA. This innovative strategy advocates the conjugation of siRNA to an antibody raised against the surface antigen specific to a cell type. By this, the siRNA would be taken close to the cells of interest thereby, aiding in selective transfection.[58] This approach is very promising but not enough studies have validated the approach.

To selectively target M1 macrophages, Gao and Li[11] used an antibody-tagged siRNA, and this conjugated siRNA was recognized by M1 macrophages and was readily phagocytosed. It is yet to be confirmed that antibody conjugated siRNA would not lead to any adverse immune reactions in the body and until such time, targeting a specific cell population in RNAi will remain elusive.

Functional recovery due to siCASP3 and siSARM1

As neurons were found to be the cells with the least uptake of siRNA, the observed downregulation effects assessed by molecular methods might be resultant from other cells. siCasp3 would rescue any cell from undergoing *Casp3* dependent apoptosis as the role of Casp3 (executing the cell death) is common in all the cells. Casp3 downregulation in cells other than neurons such as M2 macrophages, astrocytes, and oligodendrocytes can also be beneficial due to covert beneficial effects offered by these cells. On the contrary, *Sarm1* controls axonal degeneration in neurons and inhibition of sarm1 using siRNA in kainic acid-induced excitotoxic lesions in retinal ganglionic cells show that it plays a critical role in mediating Wallerian degeneration,[59] but its role in cells like macrophages and astrocytes are unclear. However, with three different protein–protein interaction domains viz. an N-terminal Armadillo motif (ARM), two sterile α motif (SAM) domains and a C-terminal TIR domain,[60] *Sarm1* could play a role in multiple signaling pathways. Downregulation of Sarm1 in cells other than neurons may not have any beneficial effect in terms of preventing axonal degeneration. This might explain the relatively better recovery in siCasp3 groups, but not in siSarm1 groups, despite effective knockdown of gene expression and tissue preservation seen in siSarm1 groups.

A mere 5% sparing of corticospinal tract was able to preserve foot placing responses after injury in rats.[61] Therefore, in immediate injection siCasp3 group, in addition to the collateral benefits arising out of preservation of other cells, a mildly higher preservation of neurons in comparison to the other groups might have resulted in improved motor recovery. Neurons in the central nervous system (CNS) perform heterogeneous roles due to the differences in their connections. Because of this, statistically insignificant preservation of cells could result in better functional recovery and *vice versa*. Therefore, the extent of tissue preservation and the magnitude of functional recovery after SCI may not be directly proportional to each other.

Repeated systemic administrations of siRNA using non-invasive or minimally invasive approaches to enhance the quantum of recovery may be counterproductive for the reasons discussed earlier. In addition, the action of siRNA in the unintended cell(s) elsewhere in the body can be catastrophic. For example, while silencing casp3 at the injury site in the spinal cord is beneficial, executing the same in a precancerous cell can lead to tumorigenesis. Therefore, transient RNAi against different genes needs to be tested to get an unambiguous view of the potentials of siRNA-based therapies in producing functional recovery after SCI.

The present study also highlights the following issues to be addressed to qualify RNAi-based approaches for SCI:

1. Temporal changes in gene expression are proportional to the severity of the injury. In experimental studies, the severity of the injury is predictable and consistent. On the contrary, in human victims varying severity of the injury along with differences in age, level of injury, ethnicity, comorbidity, lifestyle, etc. can potentially influence temporal changes in gene expression; making the task of identifying the right time to intervene a difficult one.

2. The diffusion of siRNA in the spinal cord was found to vary between injured and uninjured spinal cord indicating the role played by tissue integrity. With no yardstick for prediction, diffusion of siRNA from the injection site to unintended regions due to the wired nature of the cells can result in unpredictable outcomes.
3. Fixing the stability of siRNAs *in vivo* can be technically challenging as neither inordinately short-lived nor perpetually stable siRNA are desirable.
4. Gene interactions (gene network) are highly complicated and need to be studied in detail, as cluster effects of siRNA on other related genes can potentially result in undesirable consequences.
5. Limitations in current technology can be a major obstacle in the evaluation of siRNA efficacy. Given that estimations using PCR and Western blot cannot discriminate between cell types, gene silencing effects observed cannot be reliably attributed to having occurred in neurons. Gene silencing effects in the other cells like glia or macrophages in the vicinity can lead to erroneous conclusions. Alternative assessments for gene silencing in a cell-specific manner such as *in situ* hybridization cannot match with the sensitivity of PCR or Western blot for quantification of silencing effects. This along with differential uptake of siRNA by different cell population can make the interpretations prone for errors.
6. Gene therapy in CNS tissue can present an additional issue due to the differential function among its cells (heterogeneous functions of neurons). Because of this, despite molecular and cellular level benefits, functional recovery may vary; questioning the veracity of conclusions reached purely on molecular parameters.

Summary and conclusion

Transient RNAi through a single injection of siRNA can be very effective with a prolonged reduction in gene expression as observed. This may be better than chemical inhibitors whose silencing efficiency is not as persistent.[62] Permanent silencing using vector delivered shRNA can help in understanding mechanisms but may not be suitable for human applications for the reasons outlined earlier.

The present study corroborates previous siRNA studies in SCI with regard to the feasibility of intraparenchymal delivery of siRNAs, the efficacy of gene silencing by siRNAs *in vivo* and siRNA induced beneficial effects like tissue preservation and functional improvements.

The unique observations of the present study include the feasibility of simultaneous RNAi for more than one gene, improved silencing efficiency of siRNA cocktails, competence of a single injection of siRNA in effective gene silencing, and differential effects of siRNAs based on the time of intervention and role of the targeted gene in the cells.

To conclude, transient RNAi through direct injection of siRNAs appears to have potentials for human applications. However, several important questions are yet to be addressed through basic science research. Gene therapy going awry can be more debilitating than the primary injury as we need to target the right gene at the right time in the right cell to achieve success.

Authors' contributions: The study concept was derived by SV and was designed by SV and FMM. Animal surgeries were done by SV. Animal maintenance and data collection were done by FMM, PC, KC, KI, and KJ. Bioinformatics was carried out by FMM and RS. SV and FMM made the critical analyses of data, wrote the final manuscript, and take the responsibility for the accuracy of the data.

DECLARATION OF CONFLICTING INTERESTS

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

FUNDING

The author(s) disclosed receipt of the following financial support for the research, authorship, and/or publication of this article: This study was supported by a research grant from Department of Biotechnology (DBT), Ministry of Science and Technology, Government of India awarded to SV (Grant number: BT/PR8678/AGR/36/758/2013). FMM was supported by senior research fellowship from Department of Science and Technology (DST), Government of India through INSPIRE program. PC received junior research fellowship from the above referred DBT sponsored project. KC received university research fellowship from University of Madras.

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(Received June 16, 2019, Accepted August 2, 2019)