INTRODUCTION

Traumatic spinal cord injury (TSCI) is an important health problem with long-term disability and devastating human and socio-economic burden.1 The injury mostly affects young males aged 18-32 years.2,3 TSCI is initiated by a mechanical insult to the spinal cord. Common primary mechanisms of TSCI are “compression, contusion, laceration, transection, and traction of the spinal cord”.3 Following the primary injury, a cascade of reactive events transpires within and adjacent to the injury site.4-6 Edema and hemorrhage develop immediately following the TSCI, most notably in the gray matter, followed by alteration of the blood-central nervous system barrier, resulting in infiltration and activation of macrophages and extravasation of neutrophils in the area of tissue damage. A sequence of pathophysiological changes including ischemia, altered ionic homeostasis, free-radical release/lipid peroxidation, glutamate-mediated cytotoxicity, inflammation, and apoptosis, impair the regenerative properties of the injured spinal cord. A cystic cavity may then form as a consequence of invading immune cells and clearing of necrotic tissue.4-6 Additionally, glial scar is formed through a series of complex interactions between astrocytes, oligodendrocyte progenitors, microglia cells, and the infiltrating inflammatory cells.6 The resulting scar acts as mechanical barrier that interferes with the ability of the injured spinal cord to recover. Hence, it is reasonable to hypothesize that anti-fibrotic agents injected at the site of trauma may help the spinal cord to recover.

An interesting phenomenon is noticed in salamanders regarding the ability to regenerate their amputated limbs. This regeneration depends on the expression of a protein called nAG (newt-Anterior Gradient) in the Schwann cells of regenerating axons of the amputated stump.7 The senior author (MMA) hypothesized that the nAG protein may not only be a regenerative protein; but also an anti-fibrotic agent since scarring hinders regeneration. The study centre laboratory designed a nAG plasmid suitable for use in higher vertebrates.7 Following the expression in human fibroblasts, nAG showed a suppressive effect on collagen I / III expression regardless of TGFβ1 (Transforming Growth Factor Beta 1) effect.7 In another study, the authors showed that on-site injection of recombinant nAG reduced scar in the rodent

ABSTRACT

Objective: To evaluate the therapeutic properties of nAG protein during the recovery following acute spinal cord injuries in the rat.

Study Design: An experimental study.

Place and Duration of Study: King Saud University, Riyadh, Saudi Arabia, from September 2014 to September 2015.

Methodology: Eight rats were studied (4 control rats and 4 experimental rats; and hence 50% were controls and 50% were experimental). All rats were subjected to an acute spinal cord injury using the aneurysmal clip injury model. Immediately after the injury, a single intra-dual injection of either normal saline (in the control group) or the nAG protein (in the experimental group) was done. Assessment of both groups was done over a 6-week period with regard to weight maintenance, motor recovery scores, MRI and histopathology of the injury site.

Results: Weight maintenance was seen in the experimental and not in the control rats. Starting at 3 weeks after injury, the motor recovery was significantly (p<0.05) better in the experimental group. MRI assessment at 6 weeks showed better maintenance of cord continuity and less fluid accumulation at the injury site in the nAG-treated group. Just proximal to the injury site, there was less gliosis in the experimental group compared to the control group. At the crush injury site, there was less tissue architecture distortion, less vacuole formation, and less granulation tissue formation in the experimental group.

Conclusion: The local injection nAG protein enhances neuro-restoration, reduces gliosis, and reduces vacuole/granulation tissue formation following acute spinal cord crush injury in the rat aneurysmal clip animal model.

Recombinant nAG protein in spinal cord crush injury in a rat model

ear; with decreased levels of collagen I and III and better of scar maturation in experimental rabbits as compared to controls. These findings may suggest that nAG might enhance the recovery following SCI not only by supporting regenerating axons (as a regenerative protein) but also by reducing gliosis.

In the current study, the aim was to evaluate the therapeutic properties of the nAG protein in the recovery following acute spinal cord injury in the rat aneurysmal clip animal model.

**METHODOLOGY**

This study was conducted following the approval of the Institutional Review Board at the University, and according to the Local Guidelines for Animal Experiments (Project # 13/3833/IRB).

A pilot study was designed; and hence, only eight adult female Sprague-Dawley rats were utilized: 4 control and 4 experimental rats. The injury site of the spinal cord was injected with normal saline in the control group; whereas, the experimental group was injected with the nAG protein in normal saline solution. The study lasted 8 weeks; the initial quarantine took 2 weeks upon receiving the animal subjects, followed by 6 weeks for the actual experiment and motor testing. Before intervention, all rats were housed one per cage under simulated daylight conditions with alternating 12-hour light-dark cycles; they had free access to food and water. Female (rather than male) rats were used due to the ease of managing their bladder emptying manually.

Purified nAG protein (SC1259P) was obtained from GenScript Company (USA). Each 100µg of the lyophilized protein was diluted in 25mL of sterile NSS. The solution was then aliquoted into 50 tubes containing 500µL of protein solution with an initial concentration of 150pmol/100 µL and stored at -80°C freezer. Before each experiment, the concentration of nAG protein solution was rechecked by NanoDrop 2000 Spectrophotometer (Thermo Scientific, UK). Utilizing a vacuum concentrator (Eppendorf, Germany), the amount of fluid injected was reduced to 150µL with a final concentration of 92 ng/µL.

All rats were weighed on the day of surgery. They were anesthetized using intra-muscular injection containing 0.2mL Xylazine HCl (20mg/mL, Rompum, Bayer Toronto Ontario) and 0.8mL Ketamine (50mg/mL TEKAM, Hikma Pharmaceuticals, Amman Jordan) at a dose of 0.1mL/100gm body weight.

After the induction of anesthesia, rats were positioned on the operating table with a heating pad in prone position. The hair on the skin on the back of the rat was shaved off. Under aseptic conditions, skin infiltration with 2% Lidocaine HCl (Pharmaceutical Solution Industry, Jeddah, KSA) was done prior to skin incision. Using an operating microscope, a midline skin incision was done.

The para-vertebral muscles were sutured together followed by closing of the skin using simple interrupted sutures. The rats were wrapped with paper blankets and placed in separate cages with food and water under a heat lamp overnight or until full recovery from anesthesia. Administration of prophylactic antibiotic injection, using long acting Amoxicillin (Betamox LA, Norbrook, Northern Ireland) at a daily dose of 0.2 mL, was given postoperatively on day 1, 3 and 5 through intra-muscular route.

After full recovery from anesthesia, the rats were housed again, each in a single cage, in the same pre-injury environment for 6 weeks. Bladders were expressed 2 times daily until spontaneous voiding occurs. Food and water was provided ad libitum.

The animals’ behavior, activity and any important occurrence were monitored and recorded daily. Weight and motor function recovery comparisons between the nAG treated rats and controls were assessed weekly during the whole 6 week period following injury utilizing the Basso-Beattie-Bresnahan (BBB) open field locomotor rating scale, and the modified Tarlov score. One week prior to surgery, all rats were made familiar with the open field environment (91x51 cm flat surface with 10 cm wall height) by our research personnel at 4 different instances by allowing subject rats to walk freely for 3 minutes. Behavioral motor assessments were performed after the injury and weekly thereafter from the 1st to the 6th week. Two independent observers, who were unaware of the treatment groups, made all observations.

Additionally, hind limb function was assessed by a semi quantitative during open field walking, using the modified Tarlov scale (1-7).

After completing 6 weeks from the TSCI, control rat and nAG-treated rats underwent MRI 4.7T Pharmacan...
47/16 Bruker magnet ParaVision 5.1 software equipped with a transmission and reception proton-volume circularly polarized radio frequency coil, with an outer-inner diameter of 89mm/60mm under anesthesia, using the same mixture recommended above. Sequencing includes Sagittal T1 and T2 weighted RARE (Rapid Acquisition with Relaxation Enhancement), scan time 4 minutes and 16 seconds; in plane resolution = 234μm x 234μm; slice thickness = 1 mm; we used 6 slices with 0.5 mm gap. T2-Weighted: TR/TE = 2500/40 ms; RARE factor = 4; Number of excitations = 8; Total scan time = 10 min 40 s; in plane resolution = 234μm x 234μm; slice thickness = 1 mm; we used 6 slices with 0.5 mm gap.

Six weeks following the induced TSCI, rats were deeply anesthetized by placing them individually in an airtight glass jar infiltrated with Sevoflurane (Abbott, USA) vapors until euthanization. A 2 cm segment of the injury site spinal cord was harvested. Staining was performed using injury epicenter sections of formalin fixed, paraffin embedded tissue, which were mounted on Marienfeld histobond and adhesion microscope slides followed by hematoxylin and eosin staining. Immuno-histochemical study of Glial fibrillary acidic protein (GFAP) staining was performed in an automated immunostainer Benchmark-XT (Ventana), according to the manufacturer’s instructions. The tissue sections were de-waxed abord the Benchmark-XT autostainer. The sections were counterstained with haematoxylin, dehydrated, and mounted. Anti-GFAP antibodies (Cell Marque, clone: EP672Y, catalogue code CMC4345002) were used for GFAP staining. The antibody incubation was 16 minutes at 37°C.

Histological assessment was done just proximal to injury site (to evaluate tissue derangement and gliosis formation) and also at the injury site (to evaluate tissue architecture distortion, vacuole formation, inflammation, and granulation tissue formation).

Weight maintenance data and locomotor scores were expressed as standard deviation from the mean for the control group and experimental group. Statistical analysis was performed using SPSS software (version 22.0.0.0) with a confidence level of 95%. Results are expressed as means and standard deviation (SD). A value of p < 0.05 was considered statistically significant using the paired t-test for comparison of weight maintenance values; and using the unpaired t-test for comparison of loco-motor (BBB and Tarlov) scores. Normality of distribution assumption was checked using Q-Q plot.

**RESULTS**

At baseline, the mean weight of the animals was 244.75 ±12.7 grams for control group and 276.75 ±23.27 for nAG group, without statistical significances (p=0.0523). Animals were weighed again at the end of the experiment at 6 weeks after injury. There was a significant drop of weight in the saline treated group (p=0.026). The weight of the nAG-treated group was maintained 6 weeks post-injury with no significant difference noticed when compared to their weight prior to injury (p=0.059), as shown in Table I.

The comparison of the motor recovery between the nAG-treated and control rats is summarized in Tables II and III. At baseline, the BBB score (Table II) showed an initial flaccid hind limb paralysis (score of zero) in both nAG-treated and saline controls. Assessment during the 2nd week showed no significant differences between the tested groups. However, assessment at 3 weeks (mid-experiment) showed better BBB scores in nAG treated rats (9.5 ±1.91) compared to controls (1.25 ±0.5); and the difference was significant (p < 0.0001). Subsequent weekly evaluation of the BBB score also showed significantly better motor recovery (p < 0.0001) in the nAG group compared to control group at the end of the study at 6 weeks with mean BBB score of 17.5 ±1 compared to control with mean of 5.75 ±3.3 (Table II).

Using the modified Tarlov score, motor assessment at 1 and 2 weeks post-injury showed better mean scores in the nAG-treated rats compared to controls; but the difference was not significant. However, the modified Tarlov scores in the nAG-treated rats were significantly better than controls at 3, 4, 5, and 6 weeks, as shown in Table III.

### Table I: Weight maintenance assessment.

<table>
<thead>
<tr>
<th>Group</th>
<th>Weight prior to SCI</th>
<th>Weight 6 weeks after SCI</th>
<th>Significance (paired t-test)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>244.75 ±12.71</td>
<td>212.87 ±3.409</td>
<td>p = 0.026</td>
</tr>
<tr>
<td>nAG</td>
<td>276.75 ±23.27</td>
<td>287.5 ±30.49</td>
<td>p = 0.059</td>
</tr>
</tbody>
</table>

### Table II: BBB scores (mean ±SD) in control (n=4) and nAG-treated (n=4) rats.

<table>
<thead>
<tr>
<th>Weeks</th>
<th>Control</th>
<th>nAG</th>
<th>Significance (unpaired t-test)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0 ±0</td>
<td>0 ±0</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>0.75 ±0.5</td>
<td>4.25 ±3.4</td>
<td>p = 0.08</td>
</tr>
<tr>
<td>3</td>
<td>1.25 ±0.5</td>
<td>9.5 ±1.91</td>
<td>p &lt; 0.0001</td>
</tr>
<tr>
<td>4</td>
<td>1 ±0.816</td>
<td>12.75 ±1.89</td>
<td>p &lt; 0.0001</td>
</tr>
<tr>
<td>5</td>
<td>1.75 ±0.5</td>
<td>15.5 ±1.73</td>
<td>p &lt; 0.0001</td>
</tr>
<tr>
<td>6</td>
<td>5.75 ±3.3</td>
<td>17.5 ±1</td>
<td>p &lt; 0.0001</td>
</tr>
</tbody>
</table>

### Table III: The modified Tarlov scores in control (n=4) and nAG-treated (n=4) rats.

<table>
<thead>
<tr>
<th>Weeks</th>
<th>Control</th>
<th>nAG</th>
<th>Significance (unpaired t-test)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.25 ±0.5</td>
<td>0.5 ±0.577</td>
<td>p = 0.537</td>
</tr>
<tr>
<td>2</td>
<td>1.00 ±0.0</td>
<td>2.5 ±1.29</td>
<td>p = 0.059</td>
</tr>
<tr>
<td>3</td>
<td>1.25 ±0.25</td>
<td>4.0 ±0.816</td>
<td>p = 0.001</td>
</tr>
<tr>
<td>4</td>
<td>1.75 ±0.5</td>
<td>3.75 ±0.957</td>
<td>p = 0.01</td>
</tr>
<tr>
<td>5</td>
<td>2.00 ±0.0</td>
<td>4.5 ±0.577</td>
<td>p &lt; 0.0001</td>
</tr>
<tr>
<td>6</td>
<td>2.25 ±0.5</td>
<td>4.750 ±0.5</td>
<td>p &lt; 0.0001</td>
</tr>
</tbody>
</table>
MRI assessment was done at 6 weeks after injury using both T1 and T2 weighted imaging. Sagittal sections showed better maintenance of cord continuity in the nAG group compared to control group (Figure 1). There was also less fluid accumulation around the lesion site in the nAG group (Figure 1).

H&E staining of the injured area of the spinal cord was only done at 6 weeks after injury; and hence, it was not possible to assess the degree of the initial inflammatory process. Just proximal to the injury site, there was general preservation of the structure of the gray-white matter with no cavitation (vacuole) formation in both the experimental and control groups (Figures 2A and 2B). However, GFAP immune staining of the same area (just proximal to the injury site) showed a denser gliosis in controls compared to experimental rats (Figures 3A and 3B).

At the crush injury site, control animals showed significant loss of tissue architecture, features of infarction, significant distortion with vacuole formation and organized granulation tissue (Figure 4A). In contrast, the crush injury site of experimental animals showed less tissue architecture distortion, less vacuoles, and less granulation tissue formation (Figure 4B).

**DISCUSSION**

The current study is novel since it represents the first attempt in the literature to test the nAG protein in TSCI. Although this was a pilot study and the number of tested animals was small, the difference in motor recovery was highly significant in experimental rats compared to controls. Hence, we plan to proceed with large animal models.

In salamanders (which are lower vertebrates), the nAG protein works mainly as a regenerative protein to induce the formation of a new limb following amputation. Our laboratory has also shown that the nAG protein induces better regeneration of distal digital tips in mice (which are higher vertebrates). We have also demonstrated that the nAG protein is a strong anti-fibrotic agent in both human fibroblasts and rabbit ear scar models. The current experiment shows a lesser degree of gliosis, less tissue distortion, and less granulation tissue formation with the use of nAG (Figures 3 and 4). This may explain the enhanced motor recovery of the nAG group compared to controls. However, a direct nAG effect on...
the axonal growth cones is still a possibility and requires further studies.

The pathophysiology of glial scar formation at the site of TSCI is well described in the literature. Hemorrhage and hypoxia at the injury site result in the hypertrophy and proliferation of astrocytes, which induces gliosis. It is important to note that nAG did not completely abolish gliosis (Figure 3). The complete ablation of reactive astrocytes (and gliosis) in TSCI models is harmful, because astrocytes are known to have several beneficial functions such as the reduction of toxic level of glutamate, the assistance in the re-formation of the blood-central nervous system-barrier at the injury site, and the production of anti-oxidants.

In the skin, the nAG protein is known to induce degradation of ground substance proteins and glycoproteins of the extra cellular matrix via increasing the expression of matrix metalloproteinases. The effect of nAG on ground substance proteins and glycoproteins of the injured spinal cord has not been studied; although it is reasonable to suppose that it may have a similar enhanced degradation effect. Following TSCI, several cells (astrocytes, macrophages, and microglial cells) produce large amounts of proteoglycans, which are harmful to the neuro-restoration process. These proteoglycans inhibit oligodendrocytes progenitor cells (via the stimulation of a surface receptor protein tyrosine phosphatase-sigma, PTP-sigma), inhibit neuron regeneration (via interaction with the neural-cell adhesion molecule known as N-Cam), and induce growth cone collapse (via the activation of RhoA-protein kinase C signals). Different modulations of the abnormal extracellular matrix following TSCI are known to improve the motor recovery in experimental animals with TSCI. Examples of these modulations include the suppression of collagen synthesis (via hydroxylase inhibitors), the suppression of proteoglycans (via administration of Decorin), the neutralization of proteoglycans (via the administration of chondroitinase enzyme), and the suppression of inhibitory pathways of proteoglycans (via blocking PTP-sigma receptors or blocking RhoA signals).

Another notable finding of the study was the decreased fluid accumulation around the lesion site (Figure 1), which is an indication of a decreased inflammatory response. Another indication of reduced inflammation in the nAG-treated group is the reduced granulation tissue (Figure 4). The suppression of the inflammatory phase in TSCI is known to improve the functional recovery. nAG has also been shown to suppress the inflammatory phase and decrease edema in digital amputation injury model.

The acute clip compression model in rats is a standard model of TSCI, and is known for its consistency and reliability. Although nAG was shown to induce better recovery using this model, its effectiveness in larger animals should be established prior to human trials.

**CONCLUSION**

nAG appears to be an attractive protein that enhances motor recovery following TSCI in the rat. The pathophysiology of this enhanced recovery may involve possible nAG effects including suppression of gliosis, enhancement of degradation of proteoglycans, maintenance of tissue architecture, and suppression of the inflammatory response/edema; however, this needs focused research.

**REFERENCES**


