Changes in the respiratory system, such as diminished chest wall and ventilatory function, are common following spinal cord injury (SCI) (Brown et al., 2006). Ventilatory patterns have been found to be negatively affected by SCI, with survivors exhibiting varying degrees of reduction in flow rates, lung volumes, and vital capacity (Baydur et al., 2001, Brown et al., 2006). The pathophysiological changes that negatively impact chest wall compliance and the muscles of inhalation and exhalation cause significant reductions in vital capacity with observations of declines up to 50% of normal values in some cases (Brown et al., 2006). We previously found that following hemisection of the C2 spinal cord (C2Hx), in adult rats, arterial pO2 levels were maintained by compensatory increases in respiratory frequency (Goshgarian et al., 1986). Additionally, several studies using whole body plethysmography (WBP) analyses in animals have noted characteristic changes in key respiratory parameters following SCI, including significant increases in lung compliance, diaphragmatic paralysis, lack of activity in the external intercostal muscles, and increased compliance of the abdominal wall (Brown et al., 2006). Ventilatory patterns have been found to be negatively affected by SCI, with survivors exhibiting varying degrees of reduction in flow rates, lung volumes, and vital capacity (Baydur et al., 2001, Brown et al., 2006). The pathophysiological changes that negatively impact chest wall compliance and the muscles of inhalation and exhalation cause significant reductions in vital capacity with observations of declines up to 50% of normal values in some cases (Brown et al., 2006). We previously found that following hemisection of the C2 spinal cord (C2Hx), in adult rats, arterial pO2 levels were maintained by compensatory increases in respiratory frequency (Goshgarian et al., 1986). Additionally, several studies using whole body plethysmography (WBP) analyses in animals have noted characteristic changes in key respiratory parameters following SCI, including significant increases in

1. Introduction

Respiratory dysfunction and related diseases are a major cause of mortality and morbidity following high cervical spinal cord injuries (SCI) (Brown et al., 2006). Clinically, injuries at the level of the second cervical vertebra (C2) often result in a complete paralysis of the respiratory muscles involved in inspiration and expiration, frequently rendering survivors chronically dependent on mechanical ventilator assistance (Choi et al., 2005, Brown et al., 2006). Following SCI resulting in tetraplegia, inefficient ventilation occurs secondary to pathological changes in the respiratory system, such as diminished chest wall and intercostal muscle function, and increased compliance of the abdominal wall. These changes can lead to significant reductions in vital capacity and respiratory frequency, which can negatively impact patient outcomes.

Keywords:
Spinal cord injury
Neurotechnology
Targeted drug delivery
Ventilatory function
Breathing

Nanoconjugate-bound adenosine A1 receptor antagonist enhances recovery of breathing following acute cervical spinal cord injury

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ABSTRACT

Respiratory complications in patients with spinal cord injury (SCI) are common and can have a negative impact on the quality of patients’ lives. Previously, we found that intradiaphragmatic administration of the nanoconjugate-bound A1 adenosine receptor antagonist, 1,3-dipropyl-8-cyclopentylxanthine (DPCPX) induced recovery of diaphragm function following SCI in rats. When administered immediately following the injury, recovery was observed as early as 3 days following SCI and it persisted until the end of the study, 28 days after the drug delivery. The recovery was observed using diaphragmatic electromyography (EMG) as well as phrenic nerve recordings; both of which were conducted under anesthetized conditions. Confounding effects of anesthetic may make data interpretation complex in terms of the impact on overall ventilatory function and clinical relevance. The objective of the present study was to test the hypothesis that intradiaphragmatic administration of nanoconjugate-bound DPCPX, enhances recovery of ventilation following SCI in the unanesthetized rat. To that end, Sprague-Dawley rats underwent C2 spinal cord hemisection (C2Hx) on day 0 and received either: (i) 0.15 μg/kg of nanoconjugate-bound DPCPX or (ii) vehicle control (50 μl distilled water). To assess ventilation, unrestrained whole body plethysmography (WBP) was performed on day 0 (immediately before the surgery) and 3, 7, 14, 21 and 28 days following the SCI. Frequency, tidal volume, and minute ventilation data were analyzed in two minute bins while the animal was calm and awake. We found that a single administration of the nanoconjugate-bound A1 adenosine receptor antagonist facilitated recovery of tidal volume and minute ventilation following SCI. Furthermore, the treatment attenuated SCI-associated increases in respiratory frequency. Taken together, this study suggests that the previously observed DPCPX nanoconjugate-induced recovery in diaphragmatic and phrenic motor outputs may translate to a clinically meaningful improvement in ventilatory function in patients with SCI.

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frequency and marked reductions in tidal volume (Choi et al., 2005, Fuller et al., 2006, Fuller et al., 2008). Following C2Hx in adult rats, return of diaphragmatic function is facilitated by spontaneous recovery of phrenic motorneurons, which occurs in a time-dependent manner and is mediated by activation of latent, crossed-spinal collaterals that originate from bulbospinal axons in the rostral ventral respiratory group (rVRG), both contralateral and ipsilateral to the injury. These axonal collaterals decussate the spinal cord midline caudal to the site of injury and are referred to as the crossed phrenic pathway (CPP) (Moreno et al., 1992). Previous investigation has shown that the CPP contributes to tidal volume in spinally injured rats, with the ability to generate large inspiratory volumes only during augmented breaths or following vagotomy and this contribution from the CPP is markedly attenuated when crossed phrenic activity was abolished (Golder et al., 2003). While our lab had previously investigated pharmacologic manipulation of the CPP to induce respiratory recovery in rats (Nantwi et al., 1996, Nantwi et al., 2003), the systemic administration of theophylline, an adenosine $A_1$ and $A_2a$ receptor antagonist, in SCI patients caused untoward side effects and thus precluded maximal utility of the therapeutic in a double-blinded, placebo controlled crossover trial (Tzelepis et al., 2006). Since then, we have shown that targeted delivery of a novel tripartite nanoconjugate, composed of a retrograde transynaptic tracer, wheat germ agglutinin conjugated to horse radish peroxidase (WGA-HRP), a nanoparticle carrier, that is, gold nanoparticle (AuNP) and the therapeutic drug, 1,3-dipropyl-8-cyclopentylxantine (DPCPX), is capable of restoring diaphragmatic function following high cervical SCI in rats (Minic et al., 2016). The active pharmacological agent, DPCPX, induces respiratory-related recovery and diaphragmatic contractility through antagonism of $A_1$ adenosine receptors (Lohse et al., 1987). Furthermore, we demonstrated that the nanoconjugate is delivered selectively to the phrenic nucleus and the rVRG (Minic et al., 2016). By acting predominately on the respiratory nuclei, the deleterious side effects caused by the systemic administration of theophylline in humans may be obviated (Minic et al., 2016). By utilizing the method of unrestrained WBP, we seek to determine if targeted administration of this proprietary therapeutic compound (Application number: 14/534,994. Application type: Utility/Design using an application data sheet (37 CFR 1.76), Date Filed: November 6, 2014) will also result in the normalization of ventilatory parameters following high cervical SCI.

2. Materials and methods

All experimental procedures were approved by the Institutional Animal Care and Use Committee at Wayne State University School of Medicine and performed according to the National Institutes of Health guide for the care and use of laboratory animals.

2.1. C2Hx surgery

Fifteen adult male Sprague-Dawley rats (356 ± 11 g); (Charles River Laboratories, Wilmington, Massachusetts) were anesthetized with intraperitoneal injections of ketamine (70 mg/kg) and xylazine (7 mg/kg). The adequacy of anesthesia was assessed by monitoring palpebral reflexes and limb withdrawal periodically throughout the entire duration of the procedure. Supplemental intraperitoneal doses of ketamine (17 mg/kg) and xylazine (2 mg/kg) were administered as needed. At the time of the preparation for surgery, animals were also injected intramuscularly with atropine sulfate (0.04 mg/kg) to promote hemostasis and suppress airway mucus secretions. Left C2Hx was performed as described previously (Moreno et al., 1992). A 4 cm skin incision was made, paravertebral muscles were retracted and laminectomy followed by durotomy at C2 the vertebral segment was performed. C2 dorsal roots were visualized and the cord was cut just caudal to the roots. Note that there are no C1 dorsal roots (Tubbs et al., 2007). The cord was hemisectioned using microscissors which were positioned as medial as possible to the dorsal spinal vein and then moved towards the lateral border. Importantly, the hemisection was considered complete when the ventral surface of the spinal canal was felt by the microscissors and no tissue along the length of the cut was present. Furthermore, a clear separation of the spinal cord stumps approximately 2 mm wide was visualized before proceeding with the physiological verification of the hemisection.

2.2. Electromyography (EMG) analysis and intradiaphragmatic drug administration

Immediately following left C2Hx, all animals underwent a laparotomy and EMG recordings to verify the cessation of muscle contractility in the left hemidiaphragm (LHD), as described previously (Goshgarian and Buttry, 2014; Buttry and Goshgarian, 2014). Only animals that demonstrated a complete paralysis of the LHD were included in the study. We have previously demonstrated that anatomical completeness of the C2Hx corresponds with the complete paralysis of the ipsilateral hemidiaphragm examined via electromyography (Goshgarian, 1981). Therefore, we have historically, verified completeness of the hemisection surgery using the physiological approach only. While the diaphragm was exposed, a 28 gauge Hamilton syringe (Hamilton, Reno, Nevada) was used to inject either a tripartite DPCPX nanoconjugate (50 nl, 0.15 mg of DPCPX/kg), or vehicle control (distilled water) along the entire length of the LHD. The DPCPX nanoconjugate synthesis and dosage determination were described in our previous studies (Minic et al., 2016, Zhang et al., 2016). Thorough in vitro characterization, as well as the validity of the nanoconjugate, have been published in the previous studies. (Minic et al., 2016, Zhang et al., 2016). Animals were randomly assigned and equally divided between control ($n = 8$) and DPCPX ($n = 8$) treatment groups prior to SCI surgery. Postoperative care was provided as described in previous studies from our lab (Buttry and Goshgarian, 2014, Goshgarian and Buttry, 2014, Buttry and Goshgarian, 2015). EMG analysis was performed in two animals to ensure similar efficacy of the nanoconjugate as observed before, i.e. persistence of the nanoconjugate induced recovery for the entire duration of the study (Minic et al., 2016). Once the efficacy was achieved, the same batch of the nanoconjugate was used in all the experimental animals.

2.3. Monitoring of respiratory parameters via plethysmography in unanesthetized rats

Respiratory parameters were quantified in conscious, unrestrained, spontaneously breathing rats using a WBP system designed for rodents (Data Sciences International, St. Paul, MN). A differential pressure transducer was calibrated with known airflow and pressure signals prior to data collection. Boyle’s law was used to correlate box flow, (the air exchanged in and out of the chamber due to the rat’s respiration), to the animal’s respiratory flow. A transducer was used to capture pressure deflections in the plethysmography chamber, and an integrated software program was used to translate pressure changes into expired volumes (Ponemah Analysis Software, Data Sciences International, St. Paul, MN). A negative bias flow regulator set to 2 l/min was used to supply animals with fresh room air and to prevent CO$_2$ accumulation from expired breaths during plethysmography recordings. All recordings were conducted under room air conditions. Rectal temperatures were taken immediately before and following all plethysmography sessions since changes in body temperature can theoretically produce changes in tidal volume (Drorbaugh and Fenn, 1955, Farrell Epstein and Epstein, 1978). If body temperature dropped by $\sim$0.3 °C, the Drorbaugh and Fenn equation would have been employed to calculate a correction factor; however, in no instance was a change in body temperature this significant was observed. During each plethysmography session, respiratory data were acquired for 30 min. Animals were allowed to acclimate to the plethysmography chamber, and periods when rats were ambulating, grooming or otherwise moving were excluded from
analysis. Baseline preoperative recordings were obtained for each animal no earlier than 24 h prior to C2Hx surgery. Postoperative plethysmography studies were conducted at 3 days following C2Hx, and then at 1, 2, 3, and 4 weeks after. Measurements of respiratory parameters including frequency, tidal volume and minute ventilation were acquired during each plethysmography session.

3. Data analysis

Frequency, tidal volume and minute ventilation data were collected as absolute values and were expressed as a (i) change (Δ), (ii) percent (%) of the control levels obtained on day 0 (before C2Hx). Data distribution passed the Shapiro-Wilk normality and the Brown-Forsythe test for equal variance, which allowed an ANOVA analyses to be performed.

Data were analyzed using a two-way repeated measures design, followed by the Holm-Sidak test for multiple comparisons. The analyses revealed that the effect of one factor (i.e. treatment) depends on the level of the other factor (i.e. time), thus the present study is limited in reporting main effects of each factor independently and is focused on reports of statistically significant interactions. The post hoc test was performed only in cases when ANOVA revealed significant interaction between the two factors: the effect of drug and the effect of time. A p value < 0.05 was considered statistically significant.

4. Results

The effect of DPCPX nanoconjugate treatment on diaphragmatic function following C2Hx is shown in Fig. 1. The figure shows representative examples of diaphragmatic EMG recordings taken from the left hemidiaphragm (LHD, ipsilateral to hemisection) and right hemidiaphragm (RHD, contralateral to hemisection) following the administration of (i) 50 μl of distilled water vehicle control (left panels) or (ii) 0.15 μg/kg of DPCPX nanoconjugate (right panels) into the LHD. The nanoconjugate and vehicle injections were performed on day 0 (immediately after C2Hx) with recordings being shown from the same animal on day 0, and 28 days after the injections. Recordings are shown from the posterior areas of the diaphragm. Note the absence of LHD activity at all time points in the vehicle control group, while the RHD activity was apparently unaffected by hemisection in these animals. In the DPCPX nanoconjugate-treated group, LHD paralysis is observed immediately following the C2Hx similarly as in the control animals. However, 28 days following the treatment, activity within the LHD is reinstated. The restored activity is respiratory-related as it is synchronized with the activity in the RHD. Effects of the DPCPX nanoconjugate on inducing recovery of the hemidiaphragm paralyzed by the hemisection have been thoroughly examined and published recently therefore, the purpose of using EMG analysis in the present study was only to ensure similar physiological efficacy of the nanoconjugate. Of note is the fact that in our hands spontaneous recovery does not occur until 6 weeks following the injury therefore, the interpretation of EMG recordings is limited to a simple “yes” or “no” answer with regards to the presence of any respiratory related activity in the previously paralyzed LHD. Furthermore, the EMG electrodes are placed in the equivalent sites of LHD and RHD during each recording. Twenty-eight days following treatment with 0.15 μg/kg DPCPX nanoconjugate, activity of the hemidiaphragm ipsilateral to the hemisection was detected.

Fig. 2 shows representative tracings of respiratory flow measured in awake, freely moving animals spontaneously breathing room air. The top panel shows tracings measured immediately before C2Hx (day 0), while the middle and bottom panels show tracings obtained 28 days after, from vehicle control (50 μl distilled water) and DPCPX nanoconjugate (0.15 μg/kg) treated animals, respectively. In the control animal (middle panel) ventilatory flow is reduced compared to pre-injury levels (day 0). Nanoconjugate treatment (bottom panel) increased the respiratory flow as well as frequency when compared to the control treated animals. Frequency increased from 102 bpm before C2Hx, to 126 bpm and 144 bpm following administration of the distilled water

![Fig. 1. Representative electromyographic (EMG) recordings of the left and right hemidiaphragm (LHD and RHD, respectively) taken on day 0 (immediately following C2Hx, top two recordings) and 28 days after (bottom two recordings) administration of (i) distilled water vehicle control (left), (ii) intradaphragmatic administration of 0.15 μg/kg of DPCPX nanoconjugate (right). Amplitude of the EMG recordings, y-axis, is expressed in Volts. x-axis represents time, measured in seconds. Calibration bar: 1 s. Note the absence of LHD muscle activity in the vehicle treated control animals on day 0 and day 28 (top and bottom left, respectively) and presence of LHD activity 28 days following the nanoconjugate treatment (bottom right).](Image)
and DPCPX nanoconjugate, respectively. Table 1 shows raw ventilatory data obtained in vehicle control treated animals and in animals treated with 0.15 μg/kg of DPCPX nanoconjugate. Of note is that data are expressed as a change (Δ) in absolute values thus, multiplication of frequency and tidal volume data should not yield minute ventilation values. ANOVA analysis followed by the post hoc test revealed a statistically significant difference between the experimental groups (p < 0.05). Specific time point comparisons revealed that DPCPX nanoconjugate treatment attenuated the rise in respiratory frequency 14 days following the C2Hx (p = 0.042). These observations are similar to what we reported recently (Minic et al., 2016). Administration of the DPCPX nanoconjugate increased frequency 2 weeks following the injury, however, by week 4 the effects diminished and the control and the nanoconjugate increased frequency when compared to the pre C2Hx levels. Frequency continued increasing until day 14 and then displayed a gradual decrease towards the pre surgery levels. Nevertheless, on day 28, frequency remained elevated compared to the pre hemisection baseline (p = 0.024). DPCPX nanoconjugate treated animals exhibited an increase in frequency 3 days following C2Hx, however that increase occurred to a lesser extent than in control animals. Frequency remained elevated to a similar extent throughout the duration of the study. ANOVA analysis followed by the post hoc test revealed a statistically significant attenuation of the rise in respiratory frequency in DPCPX nanoconjugate treated animals on days 14 and 21 with respect to the control group (p < 0.05). These data follow the similar trend described in Table 1 and demonstrate the effect of the experimental treatment on respiratory frequency with respect to time elapsed following spinal cord injury. The data suggest that the DPCPX nanoconjugate is efficient in reducing the rise in frequency only at specific time points, i.e.14 and 21 days, following the surgery. Similar to the top panel, the bottom panel of Fig. 2 shows tidal volume average data. Vehicle treated animals (open circles) exhibited decrease in tidal volume 3 days post C2Hx and the decrease persisted for the entire duration of the study (until day 28). DPCPX nanoconjugate treated animals (black circles) exhibited a decrease in tidal volume acutely, that is 3 and 7 days post C2Hx. By day 14, the tidal volume returned to the pre C2Hx levels and continued increasing until the end of the study. The statistical analysis revealed significant increase in tidal volume in DPCPX nanoconjugate vs. vehicle control treated animals 21, and 28 days following C2Hx (p < 0.008). In summary, these data and the data presented in Table 1 suggest that the DPCPX nanoconjugate treatment has more pronounced effects on tidal volume and these effects are significant when at least two weeks of the injury have passed.

Fig. 4 represents minute ventilation average data. Three days following C2Hx, control animals demonstrated a decrease in minute ventilation, which may likely be due to the profound decrease in tidal volume (see Fig. 2). By day 14, minute ventilation returned to the pre surgery levels. Furthermore, continuing until day 28, minute ventilation exhibited an incremental decline and the decline was temporally correlated with a decrease in frequency and reduction in tidal volume (see Fig. 2). Three days following C2Hx, the DPCPX nanoconjugate treated animals exhibited a slight increase in minute ventilation compared to the pre-surgery levels. The increase in minute ventilation was likely due to an increase in respiratory frequency accompanied by minimal changes in tidal volume (see Fig. 2). Starting on day 7, minute ventilation reached pre-surgery levels and from then on continued increasing until the end of the study. Statistical analysis showed a significant

### Table 1

<table>
<thead>
<tr>
<th>Day</th>
<th>Frequency (Δbpm)</th>
<th>Tidal Volume (Δml)</th>
<th>MV (Δml/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>DPCPX nanoconjugate</td>
<td>Control</td>
</tr>
<tr>
<td>Day 3</td>
<td>19.3 ± 6.3</td>
<td>19.8 ± 8.7</td>
<td>12.4 ± 3.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Day 7</td>
<td>21.9 ± 3.0</td>
<td>22.1 ± 9.8</td>
<td>0.36 ± 7.8</td>
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<tr>
<td></td>
<td></td>
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<tr>
<td>Day 14</td>
<td>37.5 ± 4.6</td>
<td>19.6 ± 4.8*</td>
<td>20.4 ± 7.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 21</td>
<td>29.1 ± 4.8</td>
<td>17.8 ± 5.7</td>
<td>20.4 ± 7.6</td>
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<td></td>
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<td></td>
</tr>
<tr>
<td>Day 28</td>
<td>22.3 ± 5.1</td>
<td>19.6 ± 6.6</td>
<td>4.31 ± 6.8</td>
</tr>
</tbody>
</table>

Data are expressed as changes with respect to baseline obtained immediately before C2Hx.

* p < 0.05 vs. control.
difference in minute ventilation between DPCPX treated vs. control animals 21 and 28 days post injury ($p < 0.05$) (see Fig. 3).

5. Discussion

The major finding of the study is that following C2Hx, a single intradiaphragmatic administration of DPCPX nanoconjugate has an effect on ventilation. The nanoconjugate delivery attenuated a decline in tidal volume observed in vehicle control treated animals. By week 2, tidal volume returned to pre-injury levels and continued increasing until the end of the study. Furthermore, the treatment attenuated a rise in respiratory frequency 2 and 3 weeks following the injury.

Previously we have observed reinstatement of diaphragmatic function measured using electromyography as early as 3 days following DPCPX nanoconjugate administration. We need to stress that the present study shows that the nanoconjugate injection had a more delayed effect on ventilation. The earliest time point at which significant changes were noted, was two weeks following the injury. Attenuation of the rise in respiratory frequency was noted 14 days following administration of the nanoconjugate, while the earliest time point that increases in tidal volume and minute ventilation were observed was 21 days post injection. Similarly as observed previously, the changes persisted until the end of the study. Differences in lag time may be due to differences in levels of respiratory system studied. Given that the diaphragm muscle receives direct projections from the rVRG and the phrenic nucleus, which are sites of action for the nanoconjugate, the effects produced can likely be observed earlier than when respiration is measured globally using WBP. The explanation for the delay may be two fold: (i) diaphragmatic recovery needs to reach a certain strength in order to result in measurable changes in ventilation, (ii) there may exist competing mechanisms that mask the nanoconjugate-induced changes in ventilation early on (i.e. 0–14 days). For example, some reports suggest a contributing role of intercostal nerves towards the inspiratory component of respiration (Agostoni et al., 1965, Remmers and Marttila, 1975, DiMarco et al., 1987, DiMarco, 2005, Navarrete-Opazo and Mitchell, 2014). Since C2Hx leads to weakening of the ipsilateral intercostal nerves, in order to maintain ventilation, accessory respiratory muscles may work to increase the respiratory rate and preclude the nanoconjugate-mediated increases in tidal volume to be observed early on (Remmers and Marttila, 1975, Kirkwood et al., 1984, Warren, 2002). Finally, competing mechanisms operating acutely following SCI are indicated through changes in tidal volume. Trends towards the improvement in tidal volume are observed as early as three days following nanoconjugate treatment although these changes did not reach statistical significance ($p = 0.090$). Similar competing mechanisms involved in mediating respiratory plasticity have been described in the literature (Nichols et al., 2012) where interplay between adenosine and serotonin systems had an effect on plastic changes observed following SCI. Adenosine A$_2$ receptors had an inhibitory action on serotonin-mediated respiratory plasticity. In light of the observations presented herein, A$_2$ receptors may constrain actions of adenosine A$_1$ receptor subtype on ventilation in the first two weeks following SCI.

The effect of the injury on ventilation is similar to what others have reported in the literature: decreases in tidal volume, increases in respiratory frequency, which persist up to several weeks following the injury with accompanying increases in minute ventilation (Teng et al., 1999, Golder et al., 2003, Teng et al., 2003, Choi et al., 2005, Fuller et al., 2006). However, some differences in ventilation have been reported across different groups. For example, Fuller et al. observed progressive increases in respiratory frequency up to 5 weeks following the injury (Fuller et al., 2006). The present study demonstrates return of respiratory frequency towards the pre-C2Hx levels 4 weeks following SCI, similarly to what Teng et al. reported (Teng et al., 1999). Correlated with the return in respiratory frequency, Teng et al. observed a return in tidal volume towards the pre-C2Hx level. In the present study tidal volume remained reduced for the entire duration of the study. Interestingly, Nicaise et al. (2013) investigated effects of cervical contusion injury acutely (up to 2 weeks) following SCI and observed statistically significant changes, i.e. decreases in tidal volume and increases in respiratory frequency no later than 24 h after the injury. Ventilation was different from in sham animals on any subsequent day of testing. The observed differences likely have little to do with the upcoming spontaneous recovery since Fuller et al. showed that spontaneous recovery of phrenic nerve and diaphragm muscle exerts minimal contributions to overall ventilation during normoxic conditions (Fuller et al., 2008). Additionally, spontaneous recovery in our laboratory is usually observed no earlier than 6 weeks following SCI (Nantwi et al., 1999). The described changes may reflect subtle differences in how SCI surgery is performed (i.e. suction of the spinal cord to create a gap vs. creating a gap using the microscrews), differences in levels at which SCI is induced and differences in animal strains (Teng et al., 1999, Fuller et al., 2006).

Previously we have reported respiratory-related recovery of diaphragm muscle and phrenic nerve activity following single intradiaphragmatic administration of DPCPX nanoconjugate (Minic et al., 2016). The objective of the present study was to test the effects of a similar dose of the nanoconjugate on ventilation using unrestrained

![Fig. 3. Comparison of ventilatory parameters before (day 0) and 3, 7, 14, 21, and 28 days after C2Hx in animals treated with: (i) vehicle control, distilled water (black circles) and (ii) 0.15 µg/kg DPCPX nanoconjugate (white circles). Tidal volume (%), and frequency (F, %) were quantified during wakefulness and quiet breathing. The data are expressed as a percent change from absolute values obtained before C2Hx which are set to 100%. DPCPX nanoconjugate treatment attenuated a rise in F and improved tidal volume following C2Hx. $p < 0.05$ vs. control.](image-url)

![Fig. 4. Effect of DPCPX nanoconjugate treatment on minute ventilation following C2Hx. The data were obtained on day 0 (immediately before C2Hx) and 3, 7, 14, 21, and 28 days after. Black bars represent control animals treated with distilled water while striped bars represent animals treated with 0.15 µg/kg of DPCPX nanoconjugate (white hashed bars) animals. The recordings were obtained during wakefulness and quiet breathing in unrestrained and unanesthetized animals. The data are expressed as a percent change from absolute values obtained before C2Hx (day 0). Minute ventilation was improved 21 and 28 days following the treatment with DPCPX nanoconjugate. $p < 0.05$.](image-url)
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