Effects of C2 hemisection on respiratory and cardiovascular functions in rats

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Abstract
High cervical spinal cord injuries induce permanent neuromotor and autonomic deficits. These injuries impact both central respiratory and cardiovascular functions through modulation of the sympathetic nervous system. So far, cardiovascular studies have focused on models of complete contusion or transection at the lower cervical and thoracic levels and diaphragm activity evaluations using invasive methods. The present study aimed to evaluate the impact of C2 hemisection on different parameters representing vital functions (i.e., respiratory function, cardiovascular, and renal filtration parameters) at the moment of injury and 7 days post-injury in rats. No ventilatory parameters evaluated by plethysmography were impacted during quiet breathing after 7 days post-injury, whereas permanent diaphragm hemiplegia was observed by ultrasound and confirmed by diaphragmatic electromyography in anesthetized rats. Interestingly, the mean arterial pressure was reduced immediately after C2 hemisection, with complete compensation at 7 days post-injury. Renal filtration was unaffected at 7 days post-injury; however, remnant systolic dysfunction characterized by a reduced left ventricular ejection fraction persisted at 7 days post-injury. Taken together, these results demonstrated that following C2 hemisection, diaphragm activity and systolic function are impacted up to 7 days post-injury, whereas the respiratory and cardiovascular systems display vast adaptation to maintain ventilatory parameters and blood pressure homeostasis, with the latter likely sustained by the remaining descending sympathetic inputs spared by the initial injury. A better broad characterization of the pathophysiology of high cervical spinal cord injuries covering a longer time period post-injury could be beneficial for understanding evaluations of putative therapeutics to further increase cardiorespiratory recovery.

Key Words: C2 spinal cord injury; cardiovascular; diaphragm activity; heart function; hemiplegia; rat model; respiratory; ultrasound

Introduction
High cervical spinal cord injuries (SCI) induce permanent neuromotor and autonomic deficits in vital functions, such as respiratory, cardiovascular, and renal functions (Eckert and Martin, 2017). People living with high SCI are frequently mechanically ventilated and often present cardiac dysfunction (Ahuja et al., 2017; Eli et al., 2021). However, compensatory mechanisms can occur spontaneously to allow the survival of these patients and some functional recovery (Chay and Kirshblum, 2020). To study the pathophysiological processes and subsequent compensatory mechanisms following a high cervical injury, a reliable preclinical animal model is required since human studies are limited. Among preclinical models of high SCI, the rat C2 hemisection (C2HS) model is the most documented, and this injury impacts both the central respiratory and cardiovascular systems (Keomani et al., 2014; Navarrete-Opazo et al., 2015; Vinit et al., 2016; Bezudnymaya et al., 2018; Cheng et al., 2021a; Jesus et al., 2021; Michel-Flutot et al., 2021, 2022; Rana et al., 2021).

In the central nervous system, the descending respiratory pathway originates from the rostral ventral respiratory group and is located in the rostral ventral medulla (Feldman et al., 2013). It bilaterally and monosynaptically connects to the phrenic motoneuron pool (at the C3–C6 spinal segments of the spinal cord) that bilaterally innervates the diaphragm (which is the main inspiratory muscle) via the phrenic nerve (Feldman et al., 1985; Lipski et al., 1986; Vandeweerd et al., 2018). Lateral hemisection at the C2 spinal cord segment induces deafferentation of the corresponding phrenic motoneuron pool below the lesion, leading to immediate hemidiaphragm paralysis (Vinit et al., 2006). With this injury, the presence of a contralateral intact spinal cord allows animal survival (Fuller et al., 2008; Keomani et al., 2014; Bezudynaya et al., 2018; Cheng et al., 2021a; Rana et al., 2021). In this model, modest respiratory recovery can be observed on the injured side and is mainly sustained by silent pathways connected to the deafferented phrenic motoneurons that cross the spinal cord midline at the C3–C6 level. This recovery is termed the crossed phrenic phenomenon (Goshgarian, 2003; Fuller et al., 2008; Vinit and Kastner, 2009; Ghali, 2017; Bezudynaya et al., 2018). C2HS also induces the loss of half of the descending vasomotor axons that innervate the cardiovascular system. Anatomically, sympathetic regulation of the heart and vessels arises from the metameric thoracic T1-L2 spinal cord levels (Hou and Rabchevsky, 2014). In upper spinal cord injury, following the cervical injury, the parasympathetic activities remain intact and will overcome the sympathetic influence, producing a de facto impact on sympathetic/parasympathetic homeostasis and causing autonomic dysfunction, including cardiovascular dysregulation and renal function alteration (Hou and Rabchevsky, 2014; Biering-Sørensen et al., 2018). In humans living with chronic high SCI, orthostatic hypotension with episodes of
immediately following SCI, patients experience life-threatening conditions (Elkayam et al., 2021). The recommended initial treatment is to immobilize and stabilize the patient, maintaining the mean arterial pressure (MAP) between 85–90 mmHg (Elkayam et al., 2021). Functional research evaluations in acute SCI studies are complex and impossible in humans. Therefore, preclinical models are necessary to understand the cardiovascular physiopathology following acute and subacute SCI stages, but unfortunately, no data are available from preclinical models of high cervical spinal cord injury. Transection or contusion of the rat spinal cord at the thoracic level (T1–T3) weeks post-injury (Poorsamjedi-Meibod et al., 2019). Nevertheless, few studies have used lower-cervical models of SCI, and these studies always applied complete transection, with bradycardia, hypotension, and a reduced heart rate and sympathetic tone observed weeks after injury (Jo et al., 2020). These diverse preclinical studies used invasive and terminal experiments to assess the pathophysiology of respiratory insufficiency (i.e., diaphragm) dysfunction by thiocurcas (DeVeau et al., 2018; Squair et al., 2018a, b). Although no studies have evaluated the cardiovascular effect of incomplete cervical SCI in a preclinical animal model, most patients living with SCI suffer from high-level (cervical) and often incomplete spinal injury.

In the present study, we investigated several vital physiological parameters (respiratory function, cardiovascular function, and renal filtration) 7 days post-C2 spinal cord hemisection in Sprague-Dawley rats.

Methods

Ethics statement

All experiments reported in this manuscript conform to policies set by the National Institutes of Health (USA) in the Guide for the Care and Use of Laboratory Animals and EU Directive 2010/63/EU for animal experiments. All experiments were performed on 44 male Sprague-Dawley rats (7-week-old, body weight 350 g, Janvier, Le Genest-Saint-Ise, France). We did not use female rats to avoid non-reproducible data due to hormonal variation. Hormonal cycle change is used to respiratory changes (Beheur and Kinkead, 2011). The animals were housed in individually ventilated cages in a state-of-the-art animal care facility (2CARE animal facility, accreditation A78-322-3, France) with access to food and water ad libitum and a 12-hour light/dark cycle. These experiments were approved by the Ethics Committee of the University of Versailles-Saint-Quentin and complied with French and European laws regarding animal experimentation (Apafis #2017111516297308, approval date August 9, 2021). The experimental design is provided in Figure 1 for a better clarity in the experimental design.

Chronic C2 spinal cord hemisection

Before anesthesia, the rats were subcutaneously injected with the pre-anesthetic drugs buprenorphine (0.03 mg/kg; Buprécare, Axience, Pantin, France), medetomidine (Bogal 24%, 30 mg/kg; Virbac, Carros, France), medetomidine (0.1 mg/kg; Mèdétor, Virbac), and carpofen (Rimadyl, Zoetics, Malakoff, France, 5 mg/kg). Approximately 10 minutes after injection, anesthesia was induced in a closed chamber (5% isoflurane in 100% O2). The rats were intubated and ventilated with a rodent ventilator (model 683, Harvard Apparatus, South Natick, MA, USA), and anesthesia was maintained with isoflurane (2.5% in 100% O2) throughout the surgical procedure. After the skin and muscles were retracted, laminectomy and durotomy were performed at the C2 level. The spinal cord was then sectioned unilaterally (left side) with microscissors. A microscalpel was used immediately after the microscissors to ensure the existence of a potential section of remaining fibers as previously described (Keomani et al., 2014). The muscles and skin

Figure 1 | Experimental design.

(A) Timeline for experiments done at 7 days (7 D) following surgery for injured and sham animals, i.e., whole-body plethysmography, ultrasound, mean arterial pressure (MAP) recording, and diaphragm electromyography (EMG) recording. (B) Timeline for experiments done before and until 1 hour post-injury (PI) for mean arterial pressure recording. (C) Timeline for experiments done at 7 D for PI for renal function evaluation.

were then sutured. To reverse the effect of medetomidine, atipamezole (0.5 mg/kg; Revertor, Virbac) was intramuscularly injected. The isoflurane anesthesia was then turned off, and when the rats showed signs of waking up, the endotracheal tube was removed. Sham rats received the same surgery without C2 spinal cord hemisection. In this study, a total of 18 rats received a C2 spinal cord hemisection, and a total of 9 rats received a sham surgery.

Whole-body plethysmography

In total, 15 rats were used for whole-body plethysmography recordings. The rats were randomly divided into two groups: sham (n = 7) and 7 days post-injury (7 D, n = 8).

Whole-body constant flow (2 L/min) plethysmography (EMKA, France) was used to assess global ventilatory function (e.g., the tidal volume, minute ventilation, and breathing frequency) in our rats, as previously described (Fayssoil et al., 2021). The rats were weighed, and the plethysmography system was calibrated before the animals were placed in the chambers. After a 30-minute acclimation period, recording began under normoxic conditions (room air). The minute ventilation and tidal volume were reported according to the bodyweight (per 100 g) for each animal. Data obtained from one of the sham rats were corrupted and were not analyzed, meaning the number of plethysmography recordings used for this group is n = 6.

Echography data acquisition

In total, 15 rats from the 15 rats used for whole-body plethysmography recordings were also used for ultrasound data acquisition. The rats were randomly divided into two different groups: sham (n = 7) and 7 D (n = 8). One of the rats from the 7 D group used for whole-body plethysmography recordings was not used for ultrasound recordings due to technical event.

To evaluate diaphragm activity and cardiac function, a high-resolution ultrasound system (LOGIQ E9; GE, Solingen, Germany) and a high-frequency (18 MHz) linear L8-181 probe (GE) were used. Anesthesia was induced by isoflurane (5% at 500 mL/min in 100% O2) in a closed chamber and maintained through a nose cone (isoflurane balanced at 1.5–2% in 100% O2). Each rat’s anterior thorax and upper abdomen were shaved before exploration. The rats were placed in the supine position on the imaging platform, and their rectal temperature was monitored and maintained at 37.5 ± 0.5°C throughout the experiment. Ultrasound gel was applied to the chest skin before measurement.

After visualization of the diaphragm, M-mode ultrasound was used to assess the diaphragm motion (diaphragmatic inspiratory motion (Figure 2) and inspiratory time and expiratory time (I:E)) in a closed chamber and maintained through a nose cone (isoflurane balanced at 1.5–2% in 100% O2). Each rat’s anterior thorax and upper abdomen were shaved before exploration. The rats were placed in the supine position on the imaging platform, and their rectal temperature was monitored and maintained at 37.5 ± 0.5°C throughout the experiment. Ultrasound gel was applied to the chest skin before measurement.

Table 1 | Whole body plethysmography in unanesthetized eupneic rats

| Mean ± SD (minimum–maximum) |
|-----------------------------|

| Parameters | 7 D Sham | 7 D PI |
|------------|----------|--------|
| T(ms)      | 186 ± 26 (161–230) | 208 ± 34 (163–279) |
| T(lms)     | 350 ± 77 (266–448) | 307 ± 53 (215–392) |
| Vl(mL/min/100 g) | 43.80 ± 7.00 (35.83–54.19) | 42.02 ± 6.71 (31.65–53.23) |
| Vl(mL/100 g) | 0.37 ± 0.03 (0.32–0.40) | 0.34 ± 0.03 (0.29–0.37) |
| Respiratory rate (breaths/min) | 120 ± 17 (102–146) | 125 ± 20 (92–159) |
| PI: Post-injury; T_e: expiratory time; T_i: inspiratory time; V_l: minute ventilation; V_l: tidal volume. | 7 d Sham (n = 6) and 7 D (n = 8). |
Renal function was evaluated in 17 rats by determining the glomerular filtration rate (GFR) and renal threshold for glucose excretion. Anesthesia was induced using isoflurane (5% balanced in 100% O2) in an anesthesia chamber and intracardially perfused with heparinized 0.9% NaCl (10 mL) followed by 0.9% NaCl (10 mL) followed by Antigensin solution (DIAPATH, Martinsen, Germany). After perfusion, the C2 spinal cord was carefully removed and stored in fixative for 24 hours. After post-fixation, tissues were cryoprotected for 48 hours in 30% sucrose (in 0.9% NaCl), and stored at −80°C. Frozen longitudinal sections 10 cm thick were cut using a cryostat (MICROM, Waltham, MA, USA). Every fifth section was used for lesion reconstruction and to examine the extent of C2 injury.

Histological reconstruction of the extent of C2 injury
Longitudinal sections from C1–C3 spinal cord were used to assess the doro-ventral and medio-lateral extent of injury in all animals. Brightfield microscopy (Aperio AT2, Leica, Nanterre, France) was used to examine the sections stained with cresyl violet histochemistry (Figure 2C). Images were analyzed using previously measured BG and UGE (mg/min) = GFR (dL/min) × (BG (mg/dL) – RTG (mg/dL)). The RTG values were calculated using the Khi2 confidence interval (95%). SigmaPlot 12.5 software (Systat Software, San Jose, CA, USA) was used to compare the MAP and heart rate analysis results obtained from the different groups. The amplitudes of the EMG recordings were double-integrated (Time to threshold relationship in which UGE is low). Scatter plots were created with SigmaPlot 12.5 software (Systat Software, San Jose, CA, USA) and XLStat Premium 2016.1.1 (Addinsoft, Paris, France; https://www.xlstat.com) were used for all statistical analyses. The results showed that the 7 d sham, 1 h PI, and 7 d PI groups had significantly different levels of BG and UGE in comparison to the control group. This indicates that the C2 spinal cord injury had a significant impact on renal function.

Renal function evaluation
Renal function was evaluated in 17 rats by determining the glomerular filtration rate (GFR) and renal threshold for glucose excretion. The rats were randomly divided into three groups: control (n = 6), 1 h PI (n = 7), and 7 d PI (n = 6). The animals were anesthetized by intraperitoneal injection of ketamine (100 mg/kg; Ketamine, Vircalb + xylazine (10 mg/kg; Rompun, Bayer, La Garenne-Colombes, France) and then placed in the supine position. The body temperature was continuously monitored using a rectal probe and maintained at 37.5 ± 0.5°C. After a laparotomy, a catheter was inserted into the bladder for urine collection. The rats were divided into three groups, with 1) one group placed in the right jugular vein for intravenous injection of the insulin solution (10 mg/kg + mannitol 1 g/kg bodyweight at 0.1 mL/min) and 2) one group placed in the left carotid artery for blood sampling. After an observation period of 10 minutes, insulin solution was injected (0.1 mL/min at 37°C) for 20 minutes. Urine output was collected during the last 10 minutes of insulin injection. Afterward, 250 µL of blood was collected from the arterial catheter.

Tissue processing
At the end of the experiments, all animals were euthanized by intracardiac perfusion of pentobarbital (EUGENON, Axience, Pantin, France, 0.2 mL/kg), intracardially perfused with heparinized 0.9% NaCl (10 mL) followed by Antigensin solution (DIAPATH, Martinsen, Germany). After perfusion, the C1–C3 spinal cord was removed and stored in fixative for 24 hours. After post-fixation, tissues were cryoprotected for 48 hours in 30% sucrose (in 0.9% NaCl), and stored at −80°C. Frozen longitudinal sections (10 cm thick) were cut using a cryostat (MICROM, Waltham, MA, USA). Every fifth section was used for lesion reconstruction to examine the extent of C2 injury.

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A deeper diaphragm activity evaluation was also performed, in which the activity was first assessed non-invasively by ultrasound through inspiratory course evaluation (during diaphragm contraction) (Figure 3A and B). At 7 d PI, the intact side presented a similar inspiratory course to that of the 7 d sham group (P = 0.708), and a significantly higher value compared to the injured side (P < 0.001) (Figure 3C). Diaphragm activity was invasively evaluated in the same animals by diaphragm electromyography reflecting the neural drive to the diaphragm (Figure 3D). Similar to the inspiratory course, the integrated diaphragm amplitude in the intact side (7 d PI group) showed no difference compared to the 7 d sham group (P = 0.081) but was significantly higher than that in the injured side (P < 0.001) (Figure 3E). The relationship between the inspiratory course and integrated diaphragm amplitude was then explored. A positive relationship was observed, and the injured side of rats in the 7 d PI group was significantly different compared to rats in the 7 d sham group and the intact side of rats in the 7 d PI group (Figure 3F). Confidence ellipses were calculated using the Khi2 confidence interval (95%).

**Effects of C2 spinal cord hemisection on renal function**

Renal function, which relies on the MAP, was evaluated following C2 SCI. There were no differences between the control, 7 d sham, and 7 d PI groups. No difference in the glomerular filtration rate, renal threshold for glucose, and water reabsorption was observed following C2HS at 7 days PI (Table 3).

**Effects of C2 spinal cord hemisection on cardiac function**

Cardiac function was evaluated by ultrasound (Figure 5A) in rats in the 7 d sham and 7 d PI groups. There was no difference between the two groups in anatomical parameters, including the diastolic LV diameter (P = 0.277) (Figure 5B), systolic LV diameter (P = 0.15) (Figure 5C) and aortic diameter (Table 4). The rats in the 7 d PI group presented a lower LV ejection fraction compared with the 7 d sham group (P = 0.036; Figure 5D). The rats in the 7 d sham and 7 d PI groups showed no difference in the E/A ratio (diastolic function) (P = 0.620; Figure 5E), isovolumetric relaxation time (Table 4) and aortic velocity time integral (P = 0.186; Figure 5F).

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Discussion

To date, this study is the first to analyze several vital physiological parameters (respiratory and cardiovascular functions) using high-resolution ultrasound to investigate cervical C2 partial injury in rats. Surprisingly, at 7 days post-C2HS, the rats did not show any respiratory deficit under quiet breathing conditions when the respiratory parameters were evaluated by plethysmography. Several studies have shown that the level of injury is associated with a significant decrease in VT and VM associated with an increase in the respiratory frequency (Goldner et al., 2001b; Lovett-Barr et al., 2012; Navarrete-Opazo et al., 2017) and progressive spontaneous restoration of these parameters over time (Navarrete-Opazo et al., 2017). This discrepancy can be explained by the development of compensatory mechanisms involving respiratory-related muscles not directly impacted by the initial injury (i.e., intercostal and abdominal muscles) (Katagi et al., 1994). Following such an injury, in the resting state under eupneic conditions, these animals can fully maintain respiratory gas exchange homeostasis and respiratory function similar to those of pre-injury animals (Beth Zimmer et al., 2015; Navarrete-Opazo et al., 2015), leading to our observations in the injured rats. Interestingly, in the present study, despite the lack of a deficit according to plethysmography, the diaphragmatic activity measured by ultrasound (diaphragm displacement) and direct electromyography on the muscle (EMGdia) revealed diaphragmatic hyperactivity under eupneic conditions. Both the ultrasound and electromyography evaluations were realized on the same animals and presented a positive relationship, demonstrating the reliability of these evaluations. Respiratory deficits after cervical partial injury have been well described in many studies, mostly using invasive methods such as diaphragm electromyography using telemetry (Navarrete-Opazo et al., 2017; Bezudnyaya et al., 2018; Urban et al., 2019; Rana et al., 2021), terminal diaphragmatic recordings (Gophan et al., 1979; Vinit et al., 2006; Keomani et al., 2014; Manni et al., 2017; Bezudnyaya et al., 2018; Cheng et al., 2021a, b), and even terminal phrenic nerve recordings (Fuller et al., 2003; Vinit and Kastner, 2009; Lovett-Barr et al., 2012; Lee et al., 2015). However, this diaphragmatic deficit can be partially investigated using non-invasive ultrasound, resulting in a robust correlation with invasive diaphragmatic EMG activities. This correlation between diaphragmatic EMG activities and respiratory ultrasound parameters has been previously described in naive rats (Fayssoil et al., 2021), and these data evince the growing interest in using this non-invasive evaluation to monitor spontaneous respiratory reorganization following C2 injury in individual animals over time.

The C2HS that we performed was a complete hemisection, which impacted the bulbar spinal respiratory pathway and part of the cardiovascular descending pathways, particularly the sympathetic part (Fig. 1). After C2HS, an abrupt reduction of the MAP was observed due to the transaction of half of the sympathetic innervation responsible for maintaining blood pressure homeostasis. However, this reduction was not permanent, since the MAP returned to pre-injury values on days 1–5 following C2HS, as previously been observed in several publications using similar injury models but has never been further explored. Cardiovascular parameters were used in these previous studies as a control for analyzing respiratory parameters because the animals were not exposed to external mechanical ventilation (Lee et al., 2016; Vinit et al., 2016; Lee and Gonzalez-Rothi, 2017). However, following total transection or contusion at the cervical or thoracic level, the MAP remained modified for weeks (Bell et al., 2017; Lujan et al., 2018; Squair et al., 2018b; Lujan and DiCarlo, 2020). In this study, the spontaneous recovery of the MAP could have been due to vascular compensation (i.e., vascular vasocostriction) that occurred between the moment of trauma and 7 days PI. For instance, reorganization of the sympathetic neural rewiring (i.e., around preganglionic neurons) (Kraaijouk and Weaver, 1996). This neural plasticity could be sustained by oversensitization or an increase in the putative sympathetic intraspinal neuronal rewiring following C2HS. This study however had some limitations. We were not able to evaluate the cardiac function immediately following the spinal cord injury due to experimental and technical feasibility. Although we observed a cardiac systolic dysfunction at 7 days PI, we only hypothesize the C2HS induced it right after injury. We also did not reveal the potential mechanism responsible for the compensation in MAP observed at 7 days PI. Further investigation will be required to elucidate the specific mechanisms involved in this phenomenon.

In conclusion, this study demonstrated that C2HS in a rat model leads to diverse vital pathophysiological defects that can, in some cases, undergo spontaneous restoration. A better broad characterization of the physiopathology of high SCI would benefit the understanding of evaluation of putative therapeutics to further increase observed cardiorespiratory recovery.

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Additional file: Additional Figure 1: Schematic of the impact of C2 spinal cord injuries on respiratory and cardiovascular descending pathways. References

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Additional Figure 1 Schematic of the impact of C2 cervical spinal cord injuries on respiratory and cardiovascular descending pathways.

Schematic representing the impact of a C2 spinal cord hemisection on the respiratory descending pathways (black arrow), the parasympathetic descending pathways (green arrow) and the sympathetic descending pathways (red arrow). The C2 hemisection induces an interruption of the descending pathways below the injured site for the descending respiratory (black dotted line) and sympathetic (red dotted line) pathways. rVRG: Rostral ventral respiratory group.