Phase II Double-blinded Randomized Controlled Clinical Trial of Chondroitinase ABC by Intraspinal Injection for Treatment of Severe Chronic Spinal Cord Injury in 60 Pet Dogs

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Phase II double-blinded randomized controlled clinical trial of chondroitinase ABC by intraspinal injection for treatment of severe chronic spinal cord injury in 60 pet dogs

by

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A dissertation submitted to the graduate faculty

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ABSTRACT

Traumatic spinal cord injury is a devastating neurologic condition in both veterinary and human medicine and despite research yielding numerous potential interventions with remarkable efficacy demonstrated in rodent models, none has advanced to successful clinical translation. Pet dogs’ predilection for sustaining spinal cord injury, typically due to intervertebral disc herniation or vertebral column fracture, makes them a suitable clinical model in which putative interventions for spinal cord injury can be tested. In recent years, there has been a growing body of experimental evidence that attests to the efficacy of chondroitinase ABC in promoting axonal regeneration and functional recovery after spinal cord injury by reactivating neuroplasticity. Chondroitinase ABC is a commercialized bacterial enzyme that selectively deglycosylates chondroitin sulfate proteoglycans and thereby disrupts the perineuronal nets that limits axonal regeneration. In this 60-dog clinical trial, we examined the therapeutic effect of chondroitinase ABC on the primary outcome measure, pelvic-thoracic limb gait coordination, and several secondary parameters, including motor- and sensory-evoked potentials and urinary bladder compliance. While our study failed to detect a therapeutic effect in chronic, severe thoracolumbar spinal-injured dogs, it has established drug safety in a clinical large animal model. Thus, this study has provided a platform for future investigations in which the dose, route and timing of chondroitinase ABC administration, as well as patient selection, can be adjusted to maximize its potential therapeutic effect and benefit spinal-injured human and veterinary patients, especially if a more treatment-responsive subgroup could be identified.
CHAPTER 1

GENERAL INTRODUCTION

Pathophysiology of Spinal Cord Injury

**Spinal cord injury in human and veterinary medicine**

Traumatic spinal cord injury is a devastating neurologic condition in human medicine that has a current global prevalence of 236 – 1,009 cases per million\(^1\). In the United States, it affects \(\sim 253,000\) people with an annual incidence of \(11,000\); typical causes include violence such as bullet wounds, traffic accidents, and sport- and occupation-related injuries\(^2,3\). The associated annual socioeconomic costs are estimated to exceed \(4\) billion in the United States and \(1.6\) billion in the United Kingdom\(^4\) (www.spinal-research.org). In addition to long-term disabilities caused by motor and sensory impairment, concomitant neurologic dysfunctions, such as urinary incontinence, autonomic dysreflexia and respiratory dysfunction, can drastically impact on quality of life and life expectancy of the affected individuals\(^2,5,6\).

For these compelling reasons, spinal cord injury has attracted intensive research efforts over the past three decades and a large number of interventions of various modalities, including biological, pharmacological and physical, have been developed (Figure 1.1). However, despite research yielding numerous potential interventions with remarkable efficacy demonstrated in experimental rodent models, none has advanced to successful clinical translation.
A desirable translational model would have more comparable spinal cord size, anatomy, physiology and pathophysiology to humans; pet dogs are suitable because of their predilection for sustaining spinal cord injury, typically due to intervertebral disc herniation or vertebral column fracture in T3 – L3 segments, that can lead to severe motor and sensory impairment in the pelvic limbs, and ‘upper motor neuron’ lower urinary tract dysfunction\(^8\).

A subset of spinal-injured pet dogs suffer from injuries of the most severe grade that will result in chronic paraplegia and inability to initiate voluntary urination, which is equivalent to ASIA Grade A\(^a\) in humans\(^8-10\). These comparable features thus render pet dogs a suitable pre-human clinical model for spinal cord injury research and successful clinical translation of potential therapies will benefit human and veterinary patients alike.

\(^a\) ASIA Grade A: in accordance with the classification scheme proposed by the American Spinal Injury Association
Anatomic damage in spinal cord injury

In humans and dogs, the spinal cord begins as a continuation of the medulla oblongata as it exits the foramen magnum and terminates at the level of ~ L1 and ~ L5 – L6 vertebra, respectively\textsuperscript{2,11,12}. The spinal cord of both species consists of cervical, thoracic, lumbar, sacral and coccygeal segments; the human spinal cord comprises 31 segments that are topographically defined by the 31 paired spinal nerves: 8 cervical, 12 thoracic, 5 lumbar, 5 sacral and 1 coccygeal\textsuperscript{2}, while the dog spinal cord is composed of ~ 36 segments: 8 cervical, 13 thoracic, 7 lumbar, 3 sacral and ~ 5 coccygeal\textsuperscript{11,12}.

Within the spinal cord, the centrally located gray matter consists of neurons, neuroglia and nerve bundles entering or exiting the spinal cord at the dorsal or ventral horns; the white matter comprises highly organized, longitudinally arranged axonal bundles that communicate sensory and motor information in ascending and descending tracts between centers in the brain and their somatic and visceral effectors in the periphery\textsuperscript{2,11,12} (Figure 1.2).

Figure 1.2 Spinal Cord Gray Matter. Afferents enter at the dorsal horn and either (1) directly synapse with efferent neurons (red circle) to form a reflex arc (e.g. patellar reflex), or (2) first synapse with the interneurons, which in turn synapse with the efferent neurons (yellow circle) that innervate effector organs, or communicate with centers within the brain (green pathway).
The ascending white matter tracts carry sensory input to the brain and they usually start with the prefix ‘spino-’ and end with the name of the target site within the brain; the descending tracts carry motor impulses from the brain to their effector organs and these tracts usually begin with the prefix that indicate the brain region giving rise to the fibers and end with the suffix ‘-spinal’\textsuperscript{2,11,12} (Figure 1.3).

Figure 1.3 Comparison of Human (a) and Dog (b) White Matter Tracts. Ascending or sensory tracts in red; decending or motor tracts in green\textsuperscript{2,11,12}. 
Because of its structural and functional complexity, a multitude of neurologic dysfunction may ensue should the spinal cord become damaged in the event of traumatic injury. Details of specific functional anatomy, especially those clinically relevant to locomotion and inter-limb coordination, sensory- and motor-evoked potentials, as well as lower urinary tract function, will be discussed in Chapters 3 – 6, in which the main outcome measures used in this clinical trial to assess various aspects of spinal cord function will be independently reported as separate studies.

**Pathophysiology of traumatic spinal cord injury**

Spinal cord injury consists of three chronological phases: (1) primary mechanical injury induced by traumatic forces such as contusion, compression and laceration that directly damage spinal cord parenchyma; (2) secondary injury starting immediately after the primary injury, characterized by intense inflammatory and ischemic processes that exacerbate the primary insult; and (3) chronic phase, occurring days to years post-injury, marked by cavitation and ‘glial scar’ deposition at the lesion epicenter$^{13–18}$ (Figure 1.4).

![Figure 1.4 Phases of Spinal Cord Injury$^{2,7}$](image)
Following the initial mechanical injury that damages neurons, neuroglia, axons, myelin sheath and vascular structures, a series of progressive molecular and cellular events, collectively known as the secondary injury, ensue; these processes include vascular and ischemic changes, free radical formation, ionic imbalances, glutamate excitotoxicity, apoptosis and generalized inflammatory responses, that can result in additional damage including neuronal death and are thus responsible for much of the debilitating effect of traumatic spinal cord injury.\textsuperscript{15-21} 

Trauma-induced blood spinal cord barrier breakdown causes immediate hemorrhage, hypoperfusion and inflammation that is characterized by blood-borne leukocyte infiltration into the injured spinal cord parenchyma; clinically, the lesion epicenter consists predominantly of activated neuroglia and hemorrhagic-leukocytic infiltrates, interposed with debris of macerated cytoarchitecture.\textsuperscript{2,22} While blood extravasation can damage neuronal soma and axons, the hemolysates, especially iron from erythrocyte breakdown, can also be neurotoxic.\textsuperscript{23,24} 

The loss of adequate vascular supply further predisposes the spinal cord to ischemic injury, marked by cell edema, increased endothelial permeability, hemorrhage and infarction; this leads to cell death and triggers inflammatory cascades that recruit and activate leukocytes and neuroglia to release proinflammatory cytokines, such as interleukin-1beta or IL-1β, interleukin-6 or IL-6, tumor necrosis factor or TNF, and leukemia inhibitory factor or LIF, plus reactive free radicals.\textsuperscript{15} 

The levels of reactive free radicals, such as superoxides, hydroxyl radicals, hydrogen peroxides, and nitric oxide, increase significantly after injury and they are primarily synthetized by microglia, macrophages and neutrophils.\textsuperscript{25} Reactive free radicals target polyunsaturated fatty acids, which can in turn devastate neuronal cellular metabolism through means such as altering membrane function by triggering the peroxidation of cell membrane polyunsaturated fatty acid,
or lipid peroxidation, that produces aldehyde products capable of inactivating key metabolic enzymes such as ‘Na\(^+/K^+\)-ATPase’ that maintains neuronal resting membrane potential\(^2,26\).

Impairment of normal cellular membrane mechanisms for maintaining the transmembrane balance of ions, especially Na\(^+\), K\(^+\) and Ca\(^{2+}\), raises the resting membrane potential of \(-68\) mV towards 0 mV and thus promote neuronal depolarization and excitability\(^2\). Hyperexcitability is exacerbated by injury-induced release of excitatory neurotransmitters; up-regulation of excitotoxic neurotransmitters, such as glutamate, can be triggered by injury-induced proinflammatory cytokines released by activated resident spinal cord neuroglia and leukocytes that have arrived at the lesion site by infiltration or extravasation\(^2,15,18-21\).

Excitotoxicity is caused by excessive excitation of the glutamate receptors, such as NMDA or N-methyl-D-aspartic acid receptors, AMPA or \(\alpha\)-amino-3-hydroxy-5-methylisoxazole-4-propionate receptors and KA or kainic acid receptors, on neurons and neuroglia, which can lead to ionic imbalances, apoptosis and cell loss\(^16,26\). NMDA receptors activation favors Ca\(^{2+}\) influx through glutamate-gated ion channels on neurons and excess intracellular Ca\(^{2+}\) accumulation can lead to apoptosis and necrosis through mechanisms such as caspase activation and production of nitrogen free radicals\(^26,27\).

Likewise, activation of AMPA receptors can also lead to excess intracellular Ca\(^{2+}\) accumulation by reversing the action of Na\(^+/Ca^{2+}\) exchangers in the neuronal cell membrane, that normally export Ca\(^{2+}\) in exchange for Na\(^+\) import, and thus results in Ca\(^{2+}\) influx, plus the release of Ca\(^{2+}\) from their intracellular stores\(^28\). Furthermore, glutamate-receptor binding can disrupt ionic homeostasis by enabling excessive Na\(^+\) and Cl\(^-\) influx through disruption of transmembrane ionic transport mechanisms such as the ‘Na-K-Cl co-transporter isoform 1’ or NKCC1; excess ions attract water into the cell and causes neurons to swell and degenerate\(^26,29\).
Moreover, spinal cord injury induces activation of various cell populations from several sources: (1) resident neuroglia within the spinal cord parenchyma, such as astrocytes and microglia; (2) peripheral circulation, such as neutrophils, monocytes and macrophages; and (3) adaptive immune response, such as B- and T-lymphocytes\textsuperscript{29}. Neutrophils are the first leukocytes recruited to the site of injury and they release various oxidative and proteolytic enzymes, like myeloperoxidase and matrix metalloproteinase-9, which can intensify the inflammatory response and augment spinal cord parenchymal damage\textsuperscript{30,31}.

Monocyte-derived macrophages from peripheral circulation that are produced in the bone marrow infiltrate the lesion epicenter and phagocytize tissue debris of neurons, axons, neuroglia and myelin\textsuperscript{32}. There are two main subtypes of macrophages found in the lesion epicenter, M1 and M2; M1 macrophages appear soon after injury and persist to exert their phagocytic and pro-inflammatory actions, while M2 macrophages tend to have the opposite effect on injury-induced inflammation as they secrete neuroprotective anti-inflammatory cytokines and chemokines\textsuperscript{33–35}.

The spinal cord resident microglia population undergoes transformation from the quiescent, ramified state to the deramified, amoeboid morphology; like bone marrow-derived macrophages recruited from peripheral circulation, activated resident microglia functionally polarize to adopt one of the two phenotypes: (1) pro-inflammatory M1 that induces the synthesis and release of additional proinflammatory cytokines and excitatory neurotransmitters such as nitric oxide, TNF-\(\alpha\) and glutamate during the secondary injury phase to trigger neurotoxic responses such as apoptosis, necrosis and excitotoxicity, and (2) anti-inflammatory M2 that maintains homeostasis and promotes cell survival and axonal regeneration\textsuperscript{19,20,36}.

Similarly, the astrocyte population consists of functionally diverse subtypes and one of their main roles during secondary injury is to maintain homeostasis; as the lesion epicenter starts
to form cavitation as the result of ischemic injury, inflammatory responses and phagocytic activity of the macrophages and microglia, activated astrocytes migrate to the epicenter and arrange themselves into a cellular network lining the margin, which consolidates to form a dense, chondroitin sulfate proteoglycan-rich ‘glial scar’ surrounding the central cavity, or syrinx. 

**Glial scar formation after spinal cord injury**

The adult mammalian spinal cord has a very limited capacity for functional recovery after severe injury due to a combination of factors: (1) secondary injury that exacerbates the primary insult, (2) release of specific axon-inhibitory molecules like Nogo and myelin-associated glycoprotein from disrupted myelin membranes, and (3) formation of the glial scar that acts as a physical and molecular barrier between the injured and the intact spinal cord parenchyma, hindering axonal regeneration.

Glial scar formation is initiated by blood brain barrier or blood spinal cord barrier disruption and the subsequent exposure of central nervous system parenchyma to blood-borne molecules. Particularly, the blood protein fibrinogen can leak into the parenchyma almost immediately post-injury to trigger glial scar formation through astrocyte activation and subsequent induction of Smad2 phosphorylation in astrocytes via the TGF-β/Smad signaling pathway; conversely, fibrinogen-depleted mice have reduced levels of active TGF-β, mitigated neuroglial activation and reduced chondroitin sulfate proteoglycan deposition post-injury.

Chondroitin sulfate proteoglycan, the main glial scar constituent, is up-regulated after spinal cord injury. Activated astrocytes, macrophages and oligodendrocyte progenitors start depositing chondroitin sulfate proteoglycans such as neurocan and NG2 into the extracellular matrix < 24 hours and peak ~ 1 week, while others such as phosphacan and
brevican peak later at ~ 1 month; levels of up-regulated chondroitin sulfate proteoglycans can persist for months\textsuperscript{37,41,42}. The glial scar, especially the chondroitin sulfate proteoglycans within it, can obstruct axonal extension to synapse with intact neurons, which can lead to axonal growth cone dystrophy or collapse, marked by the formation of bulbous axonal terminals that represent functional stagnation and permanent regeneration failure\textsuperscript{37 – 40}.

Interestingly, collapsed growth cones that have failed to from synapses do not undergo the expected degenerative processes such as synaptic atrophy and elimination; these terminals remain metabolically dynamic with normal mitochondrial and trans-Golgi network functions and sustained synaptic activities including endocytosis and retrograde transportation\textsuperscript{14,43,44}. Recent evidence suggests that the dystrophic axonal growth cones may in fact be the newly formed pre-synaptic terminals synapsing with neuroglia within the glial scar, which express NG2, a prevalent chondroitin sulfate proteoglycan\textsuperscript{45}.

It is hence possible that one mechanism, through which the glial scar inhibits axonal regeneration and synaptogenesis with neurons, is preferential synaptogenesis with chondroitin sulfate proteoglycan-expressing non-neuronal targets in the glial scar. Surprisingly, this apparently undesirable inhibitory effect of chondroitin sulfate proteoglycans is essential during central nervous system development.
Role of extracellular matrix in development

Neuroplasticity, or the central nervous system’s ability to form new synapses or novel neuronal pathways in response to changes in its environment, is largely mediated by molecules within the extracellular matrix that surrounds the developing central nervous system parenchyma and undergoes significant compositional changes as it matures. Structurally, the extracellular matrix provides a framework that maturing cells can migrate within, anchor to and become organized around; while functionally, it supplies a rich source of growth-promoting factors that facilitate neurogenesis, neuritogenesis, axonal pathfinding and synaptogenesis.

For instance, laminin is a very potent substance for guiding embryonic neuronal migration; in vitro, the laminin-coated membrane dramatically augments the migration of cortical neurons; conversely, when the laminin receptor subunits, namely integrin receptor subunits α3 and β1, on the neurons are antigenically blocked with specific antibodies, neuritogenesis is subsequently diminished. In vivo, laminin is an extracellular matrix glycoprotein produced by astrocytes that guides axonal extension during development and regeneration of the corpus callosum in acallosal neonatal mice.

Extracellular matrix molecules also influence corticogenesis; changes in fibronectin expression, for example, influence neuronal migration and axonal extension in the developing cerebral cortex. Fibronectin is abundant in the ‘preplate’ neuropil formed by the pioneer neurons born in the ventricular zone that have migrated radially to just beneath the pial surface; the cerebral cortex precursor or the ‘cortical plate’ then splits the preplate into ‘marginal zone’ and ‘subplate’. Fibronectin, abundant in the preplate and its derivatives, guides the radial...
migration of newborn neurons from the ventricular zone to the marginal zone, subplate and
cortical plate as these layers mature\textsuperscript{49}. Thus, the temporospatial distribution of certain
extracellular matrix molecules can influence development by attracting the migrating neurons
and extending axons towards their intended targets.

Unsurprisingly, equally important are the inhibitory or repulsive cues that prevent
aberrant neuronal migration, axonal projection and synaptogenesis, which could result in the
formation of undesirable or even detrimental neuronal pathways. Through their repulsive action,
chondroitin sulfate proteoglycans, direct neural crest cell migration along their programmed
pathways during development\textsuperscript{53}. After neurulation, neural crest cells detach from the neural tube
to migrate lateroventrally, giving rise to various somatic cell types including osteoblasts,
chondrocytes, melanocytes, as well as cells of the prospective peripheral nervous systems such
as Schwann cells, sensory ganglia and sympathetic and parasympathetic ganglia\textsuperscript{54}.

In the mouse embryonic sclerotome that will later give rise to adult skeletal tissue,
regions of chondroitin sulfate proteoglycan distribution and neural crest cell migration pathways
are mutually exclusive; changes in chondroitin sulfate proteoglycan distribution in the
sclerotome during development chaperone the migrating neural crest cells to their stereotypic
destinations\textsuperscript{53}. Conversely, disruption of embryonic chondroitin sulfate proteoglycans, with
sodium chlorate or $\beta$-D-xyloside, enables neural crest cell to migrate along novel pathways
within the dorsal portion of the somite that deviate from their natural lateroventral routes\textsuperscript{53}.

Hence, extracellular matrix molecules shape central nervous system development by
influencing neuronal migration and axonal growth through both attractive and repulsive cues.
Part of the reason that the immature central nervous system is capable of responding to these
cues is their inherent plasticity, which becomes especially pronounced during the ‘critical
period’, a period during which exposure to the appropriate experiences governs the formation and organization of the correct neuronal networks\textsuperscript{55}.

**Experiences shape development during the ‘critical period’**

The immature central nervous system is particularly malleable during the critical period. For instance, postnatal development of the visual cortical circuitry in kittens rely on exposure to visual stimuli 4 weeks – 3 months of age; as such, if vision is monocularly deprived during this period, the visual cortical layer IV territories that are normally innervated by the deprived eye via the geniculocortical afferents will shrink considerably, while areas that are usually innervated by the contralateral eye will reciprocally expand\textsuperscript{56,57}. Simultaneously, there will be a shift in the pattern of visual cortical neurons’ responsiveness to stimuli, or ocular dominance, in favor of the visual input received by the non-deprived eye\textsuperscript{56,57}. The same occurs in immature monkeys\textsuperscript{58}. These findings suggest that visual cortical development is shaped by experience during the critical period. Interestingly, ocular dominance shift cannot be elicited in older animals\textsuperscript{58}, implying that the critical period is perishable and once it has ended, plasticity diminishes and further experiences only have very limited impact on the established neuronal connections.

**Loss of plasticity during transition to adulthood**

As it matures, the mammalian central nervous system becomes increasingly refractory to experience-dependent reorganization; this cements function acquired during development, preserves established neuronal pathways, stabilizes functional synapses and prevents anomalous synaptic remodeling in response to subsequent experiences\textsuperscript{47,48,59,60}. This change in plasticity is largely driven by events that occur within the extracellular matrix; of particular significance is
deposition of the ‘perineuronal nets’ around neurons, whose main constituent is the ‘hyaluronan – lectican – tenascin-R’ ternary complex, organized around the chondroitin sulfate proteoglycan ‘lectican’ (Figure 1.5) \(^{55,59,61,62}\).

![Figure 1.5 Perineuronal Net Structure.](image)

Chondroitin sulfate proteoglycans are a family of proteoglycans consisting a protein core (single solid lines) and covalently linked chondroitin sulfate sugar or glycosaminoglycan side chains (blue hexagons), each consists of repeating disaccharide units. The lectican family (aggrecan, brevican, neurocan and versican) are a major component of the perineuronal nets. Lecticans have three regions: (1) a N-terminal G1 domain (pink triangles) that binds with the backbone of hyaluronan (double lines), an abundant extracellular matrix glycosaminoglycan, through link proteins (e.g. Bral-1 or HAPLN 2); (2) a side chain attachment region; and (3) a C-terminal G3 domain (green squares) that binds with tenascin-R (Tn-R), one of the five extracellular matrix proteins in the tenascin family. This ‘hyaluronan – lectican – tenascin-R’ ternary complex forms the perineuronal nets that surround mature neurons within the adult central nervous system.

Perineuronal nets emerge within the extracellular matrix relatively late during development towards the end of the critical period. Hamster spinal cord motor neurons, for instance, begin to lose their experience-dependent plasticity, induced by sciatic nerve lesion and thoracic spinal cord hemisection, when Cat-301-postive neurons appear around postnatal Day 7 – 10; Cat-301 is a monoclonal antibody with specific affinity for the key perineuronal net constituent chondroitin sulfate proteoglycans\(^{55,61 – 63}\).
Quantitative immunostaining of rat visual cortex layers II – IV has revealed temporal changes in the expression levels and relative proportions of the main chondroitin sulfate proteoglycans between postnatal Day 7 and adulthood; while aggrecan, brevican and versican experience increases of variable magnitude, neurocan levels concurrently decline\textsuperscript{59}. Accompanying changes in their levels during development are alterations in the disaccharide sulfation pattern of their side chains, with a remarkable shift from the 6-sulfated and 0-sulfated glycosaminoglycan to 4-sulfated glycosaminoglycan between postnatal Day 0 and adulthood\textsuperscript{59}.

Thus, it is possible that perineuronal net deposition towards the end of the critical period is driven by changes in the structure and composition of chondroitin sulfate proteoglycans during development, possibly in favor of the subtypes that are more plasticity-restricting. Other extracellular molecules, such as the ‘link proteins’ or Crtl1 that stabilizes intermolecular bonds within the extracellular matrix, could also influence the formation of perineuronal nets.

Crtl1 mRNA levels rise dramatically around postnatal Day 14 in the mouse visual cortex layers II – III, coinciding with emergence of the perineuronal nets; conversely, genetically modified Crtl1-knockout adult mice develop disorganized, attenuated perineuronal nets and retain central nervous system plasticity beyond the natural critical period, such that a remarkable decline in visual acuity and a shift in ocular dominance can be induced by 3-day monocular deprivation, which would not ordinarily be possible after the critical period\textsuperscript{59}.

Similarly, plasticity persists beyond the natural critical period in the spinal cord of Crtl1-knockout adult mice. One week following C6 – C7 unilateral dorsal hemisection that severs ascending sensory axons innervating the medial two digits of the ipsilateral forepaw, transganglionic cholera toxin B labeling reveals significant axonal sprouting into the lesioned area in the Crtl1-knockouts, but not in the controls\textsuperscript{59}. Retention of plasticity well beyond the end
of the critical period in the absence of properly formed perineuronal nets thus raises the question: through which molecular mechanism(s) do perineuronal nets restrict plasticity?

Interestingly, the effect of Crtl1-knockout on neuroplasticity is reminiscent of that of the bacterial enzyme ‘chondroitinase ABC’, which revives plasticity by digesting the chondroitin sulfate proteoglycan sugar side chains. Early in vitro experiments suggest that Neu7, a neurite growth-inhibitory astrocyte cell line, secretes a heat stable, highly inhibitory extracellular matrix proteoglycan that is particularly sensitive to chondroitinase ABC-mediated side chain digestion. When the proteoglycan-producing Neu7 astrocytes are treated with a proteoglycan synthesis inhibitor that results in under-sulfation of the proteoglycan and thereby reduces its side chain charge density, dorsal root ganglion axonal growth is noticeably enhanced. These findings hence suggest that the side chains could be a key effector of axonal growth inhibition, albeit further investigations are warranted to establish this speculative causal relationship.

In summary, the loss of central nervous system plasticity during development is likely due to changes within the extracellular matrix such as perineuronal net deposition; these changes hinder axonal regeneration after injury. Chondroitin sulfate proteoglycans are a family of inhibitory substances that impair axonal regeneration and functional recovery after spinal cord injury, they are thus a plausible therapeutic target for interventions that are designed to promote axonal regeneration and functional recovery by reactivating neuroplasticity.
Experimental evidence in laboratory animal models

Chondroitinase ABC is a commercialized bacterial enzyme, purified from *Proteus vulgaris*, that selectively deglycosylates chondroitin sulfate proteoglycans and removes the glycosaminoglycan side chains from its protein core, thereby disrupting perineuronal nets \(^{60,61,72}\) (Figure 1.6). There is a growing body of experimental evidence that attests to the efficacy of chondroitinase ABC in promoting axonal regeneration and functional recovery after traumatic spinal cord injury by reactivating neuroplasticity.

A 2002 study reports that through chondroitin sulfate proteoglycan degradation at the lesion site, chondroitinase ABC improves locomotor and proprioceptive functions, assessed by outcome measures like narrow metal beam crossing, wire grid test and footprint analysis, in experimental rats after C4 dorsal column crush injury; these functional improvements parallel the partial restoration of electrophysiological conductivity across the lesion as while the vehicle-treated rats have no recordable cortical evoked cord dorsum potentials, they were present in the chondroitinase ABC-treated rats, albeit with a 60% decline in latency or conduction velocity compared to the sham-treated rats \(^{74}\).
The benefit of chondroitinase ABC is more pronounced when used as an adjunct treatment in a multimodal therapeutic approach. A 60-rat study suggests that while chondroitinase ABC on its own can enhance corticospinal axonal sprouting, remarkable improvement in manual dexterity only occurs when specific rehabilitation regimens are concurrently reinforced after C4 cut injury, measured by the number and accuracy of pellets retrieved, which doubles in the ‘chondroitinase + rehabilitation’ group compared to either intervention given alone. This suggests that chondroitinase ABC-mediated plasticity reactivation and subsequent axonal sprouting work synergistically with rehabilitation training that reinforces and refines the regenerated synaptic pathways; the net result of this approach that simultaneously target multiple therapeutic pathways is augmented functional improvement.

With advancement in biomedical technologies, chondroitinase ABC, thermo-stabilized with trehalose, can now be delivered in a temporally and spatially controlled manner using a hydrogel-microtube-based system. This new chondroitinase ABC delivery system extends the \textit{in vivo} chondroitin sulfate proteoglycan-suppressive effect of a single dose of chondroitinase ABC to up to 6 weeks post-treatment, which is associated with improved locomotor function in experimental rats measured by gait analysis 6 weeks after T10 hemisection. This improved design eliminates the need for sustained chondroitinase ABC delivery through suboptimal conventional means such as repeated intrathecal injections or placement of indwelling catheters, thus rendering its administration more practical and its clinical translation more likely.

More recently, a 2014 study reports a novel, extended delivery approach using gene therapy and a lentiviral vector to transduce the host cells to express chondroitinase ABC in experimental rats. Compared with the conventional chondroitinase ABC-treated group, the lentiviral vector-chondroitinase ABC-treated group demonstrates more pronounced chondroitin
sulfate proteoglycan digestion and tissue bridge formation across the lesion epicenter 8 weeks after impactor-induced T10 – T11 contusion injury, and considerably reduced cavity formation and higher neuron count adjacent to the lesion epicenter\textsuperscript{76}. Functionally, lentiviral vector-chondroitinase ABC-treated rats have fewer horizontal ladder foot-slips at all weekly time-points up to 10 weeks post-injury; this parallels the concurrent improvement in cord dorsal column electrophysiological conductivity\textsuperscript{76}.

Surprisingly, new experimental evidence from a 2016 study suggests that the glial scar may aid axonal regeneration\textsuperscript{77}. In this study, glial scar-forming astrocytes are genetically manipulated such that they would either (1) lose their ability to produce a normal glial scar in response to injury, or (2) form a glial scar that would only persistent for 5 weeks post-injury; in both scenarios, glial scar obliteration are associated with reduced axonal growth and more extensive tissue degeneration after T10 crush injury in mice\textsuperscript{77}. How can we reconcile this novel finding with our long-held belief that the glial scar is axon-inhibitory?

One of the reasons that generalized glial scar ablation seems to produce worsened outcomes could be that the ablation techniques utilized in this study lack functional selectivity. Concurrent RNA sequencing reveals that both astrocytic and non-astrocytic populations in the glial scar can produce growth-promoting molecules that aid axonal regeneration post-injury\textsuperscript{77}. Hence, mass disruption of the glial scar may be counterproductive for axonal regeneration, as it could non-selectively eliminate both its growth-inhibitory and growth-promoting effects.

It is also possible that several sub-types of astrocytes that reside within the glial scar, possessing reciprocal abilities of growth-promotion and growth-inhibition, may be concomitantly destroyed by generalized glial scar ablation and thus undesirably shifting the balance towards net axon inhibition. Thus, interventions that target specific molecular mechanisms, rather than
blanket-treat an entire cell population, may be more likely to produce desirable and predictable therapeutic effects and, perhaps even more importantly, fewer undesirable and unpredictable side effects, and subsequently undergo fruitful clinical translation.

**Challenges in clinical translation of chondroitinase ABC**

Clinical trials investigate the efficacy of putative interventions and indirectly assess the quality of the animal models from which the treatment response data have been derived. Encouragingly, numerous rodent studies have demonstrated chondroitinase ABC’s efficacy in promoting axonal regeneration and functional recovery after spinal cord injury. However, upon closer examination, there are some concerning elements, associated with both the animal models and the data generated from them, which may have distorted the true therapeutic effect of chondroitinase ABC and thus may impact on its clinical translatable.

At least some aspects of functional improvement after chondroitinase ABC administration can plateau\(^{67,68,78}\), presumably when a certain critical point is reached. This raises the question of whether an adequate level of functional recovery could be achieved in patients before onset of this therapeutic plateau, after which further improvement would be improbable. There is also considerable inter-individual and inter-study variability in the magnitude of treatment response\(^{64,66,67,74,75,78}\), which warrants skepticism concerning the reproducibility of chondroitinase ABC’s reported therapeutic effect and thus its estimated translational value.

Another related issue is the large number of outcome measures that are often used relative to study group sample size, which triggers concerns regarding statistical power and the possible occurrence of Type I error, or false positives arising from random probability. This is especially alarming when numerous hypotheses are tested using many outcome measures in a
study that contains multiple small groups. For instance, the well-cited and perhaps one of the better-powered studies, reporting the effect of chondroitinase ABC on forelimb function in spinal-injured rats with or without rehabilitation therapy, comprises four groups of 10 – 13 subjects; while for motor function alone, seven outcome measures are reported in graphs and checked for significance\textsuperscript{68}. Treatment effect data derived from inadequately powered studies could be misleading as the apparent statistical significance may more likely be the result of random probability rather than real therapeutic effect.

Clinically, severe injuries, typically caused by spinal column fracture in people and intervertebral disc herniation in dogs, have elements of both contusion and compression, which can cause extensive primary mechanical damage and secondary injury (Figure 1.7). In contrast, experimental models, especially the earlier ones, often involve relatively mild, focal crushing or incisional injuries affecting $< \frac{1}{4} – \frac{1}{2}$ spinal cord cross-sectional area\textsuperscript{64,66 – 68,75,78}, both primary and secondary injuries are thus considerably subdued compared to their clinical counterparts.

Another consideration is that there may be significant delays, possibly days to weeks, before an intervention that requires intrathecal administration could be practically delivered, presumably by a trained specialist; while in animal models, chondroitinase ABC is often administered immediately or shortly after injury\textsuperscript{65 – 68,75}. Since treatment effect data on chronic injuries are limited, it is possible that chondroitinase ABC’s clinical therapeutic effect, if any, may only benefit the subgroup with acute injuries. Further, disparity in the timing of drug delivery renders its clinical therapeutic window difficult to determine.
Figure 1.7 Magnetic Resonance Imaging of a 4-year-old Dachshund with Intervertebral Disc Herniation. Lesion epicenter is directly over L1 – L2 intervertebral disc space (middle dotted line in top left image) and the herniated disc material causes moderate to severe spinal cord compression (white arrow in top right image) at this level, markedly distorting the spinal cord outline, compared to one spinal cord segment cranial to the lesion epicenter (bottom images).

**Role of pet dogs as translational models in pre-human clinical trials**

Discrepancies between our current models and their clinical counterparts raise uncertainty regarding the generalizability of chondroitinase ABC’s treatment effect to clinical injuries, which could vary considerably due to injury and patient heterogeneity\(^79\). Like human patients, spinal-injured pet dogs vary substantially in age, size, genetics, lifestyle, concurrent medical conditions, injury chronicity and severity, plus their caretaker’s ability and commitment.

Investigations conducted on pet dogs, through statistical means such as multivariate analysis, may hence help identify subgroups that are most likely to respond to the treatment and/or most susceptible to its potential side effects and assist with the development of personalized treatment strategies that are more likely to lead to successful clinical translation.

A recent UK clinical trial in paralyzed pet dogs using autologous olfactory mucosal cell transplants\(^80\) highlights their practical utility as disease models in pre-human clinical trials, in addition to the more immediate veterinary application, should the potential therapy be proven effective. Additionally, the remarkable cost differential renders the clinical canine model
economically desirable. While the current costs of clinical trial enrollment in the United States and Europe are $50,000 – 100,000 per person, those of the veterinary trials are only fractional by comparison, unlikely to exceed several thousand dollars per animal.

Clinical trial phases

Clinical trials are designed to evaluate the efficacy and safety of potential interventions and they are categorized into four phases: Phase I studies are typically open-label safety or pilot trials that are intended to uncover any adverse reactions of the interventions, especially those with narrow therapeutic indices; Phase II are blinded, controlled therapeutic exploratory studies that are designed to detect therapeutic effects in a narrow spectrum of patients using specific outcome measures; Phase III are therapeutic confirmatory studies, typically blinded, randomized and controlled, that definitively confirm Phase II results in a broader spectrum of patients; Phase IV studies involve long-term evaluation of benefit and harm in clinical settings following marketing approval by regulatory authorities (Figure 1.8).

Figure 1.8 Clinical Trial Phases.
Conclusion

The disappointing long-term functional outcomes of severe, traumatic spinal cord injury in human and veterinary medicine can be attributed to a combination of factors including: (1) secondary injuries that exacerbate the primary mechanical insult; (2) formation of the axon-inhibitory glial scar; (3) loss of inherent neuroplasticity after the critical period; and (4) lack of effective therapies for assisted functional recovery. Chondroitinase ABC is currently one of the most promising treatments because of its ability to reactivate neuroplasticity through chondroitin sulfate proteoglycan degradation and subsequently promote axonal regeneration and functional recovery in rodent models. As chondroitinase ABC enters the early phase of clinical translation, emphasis should be placed on selecting suitable animal models, such as spinal-injured pet dogs, that are both economical and representative of the natural disease processes, in which its treatment effect and pharmacodynamics can be thoroughly investigated.
Pre-study Planning

Pre-study animal ethics and safety approval

This study was approved by Institutional Animal Care and Use Committee and Institution Biosafety Committee (IBC) at Iowa State University to be conducted at Iowa State University College of Veterinary Medicine. Written consent was obtained from the owner of each participant before commencement of any procedures related to the clinical trial. The owner was informed regarding the investigational nature of the study, requirements concerning hospitalization and recheck visits, randomization and blinding, and the procedures used as outcome measures and the potential risks that they carried.

Pre-study Phase I safety trial for chondroitinase ABC injection

An open-label Phase I trial had been completed prior to the commencement this current study in which the safety of intrathecal injection of heat-stabilized chondroitinase ABC in pet dogs with chronic, severe spinal cord injury was assessed. Five dogs were recruited within ~ 3 weeks through an alert placed on a website for owners of paralyzed pet dogs (http://www.dodgerslist.com/). All five dogs met the inclusion and exclusion criteria that would later be used for this current study (Figure 2.3). Each dog received chondroitinase ABC

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b IACUC log number 3-13-7526-K
intrathecally under fluoroscopy guidance; the total dose of 625 mU was equally divided between two spinal cord sites, the lesion epicenter and the presumed cranial margin of the central pattern generator in the lumbosacral intumescence at the L3 – L4 interspace$^{11,83–85}$ (Figure 2.6).

The main goal of the safety trial was to determine if there was any evidence of neuropathic pain, such as hypersensitivity or allodynia, at or near the injection site, which can be associated with glial scar digestion and aberrant axonal sprouting. Outcome measures used were: (1) weekly physical and neurological examination, including von Frey sensory testing of skin over the dorsum until post-injection Week 8, and (2) owner questioning regarding possible adverse effects. No dogs exhibited evidence of adverse effects and several showed improved mobility, although this was not formally quantified in the study.

**Pre-study sample size calculation**

Enrolled patients were randomized at a ratio of 1:1 to (1) Intervention to receive intrathecal chondroitinase ABC with physical therapy or (2) Control to receive transcutaneous sham hypodermic needle insertion with physical therapy. To detect attainment of a certain level of performance, defined as 25% dogs in the Intervention group and 0% dogs in the Control group recovering thoracic-pelvic limb gait coordination measured by phase shift by Month 6$^c$, a minimum of 48 dogs were required for the study$^{86}$:

\[
N = \frac{P1(1 - P1) + P2(1 - P2)}{(P1 - P2)^2} \times C(p, power)
\]

In this formula, $N$ represents the minimum number of subjects in each experimental group; $P1$ and $P2$ represent the proportion of detectable treatment effect in each of the two

$^c$ Both Intervention and Control groups had 0% thoracic-pelvic limb gait coordination prior to intervention at the time of baseline data collection or Day 0.
equally sized groups, or Intervention and Control in this case; and \( C(p, \text{power}) \) is equal to 7.9 when \( \alpha = 0.05 \) and \( \text{power} = 80\% \). Thus, a minimum of 24 were required in each arm:

\[
24 = \frac{0.25(1 - 0.25) + 0(1 - 0)}{(0.25 - 0)^2} \times 7.9
\]

However, a target of 60 dogs was set to (1) allow for a small proportion of dropout due to owner decision and/or unforeseen euthanasia or death; and (2) permit detection and improve estimation of more subtle treatment effects, especially those on spinal cord functions that would be less likely to recover after severe traumatic injury, such as lower urinary tract function.

**Pre-specified outcome measures**

Four pre-specified outcome measures were used in this study to assess various aspects of spinal cord function, including motor, sensory and autonomic. The primary outcome measure was thoracic-pelvic limb gait coordination measured by phase shift: Chapter 3. The secondary outcome measures were: (1) presence of transcranial magnetic motor-evoked potentials in pelvic limbs: Chapter 4; (2) the most cranial level of somatosensory-evoked potentials recordable from the spinal cord: Chapter 4; and (3) urinary bladder compliance: Chapter 5.

\[\text{d} \] Actually analysis deviated from this; details outlined in Chapter 3, Materials and Methods.
Patient Selection and Management

Patient recruitment

Client-owned pet dogs were recruited January 2014 – March 2016 via four main channels: (1) Lloyd Veterinary Medical Center’s and other websites (Figure 2.1); (2) social media including Facebook and Twitter; (3) television and press interviews; and (4) referral by veterinarians and rehabilitation centers. Study information and contact details were available on the websites and social media sites, and in the clinical trial brochure (Appendix Item A).

**Figure 2.1 List of Websites Advertising Clinical Trial**

<table>
<thead>
<tr>
<th>Website Name</th>
<th>Website URL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dodger’s List</td>
<td><a href="http://www.dodgerslist.com/">http://www.dodgerslist.com/</a></td>
</tr>
<tr>
<td>Iowa State University Lloyd Veterinary Medical Center</td>
<td><a href="http://vetmed.iastate.edu/vmc/small-animal/clinical-trials/chondroitinase-clinical-trial-0">http://vetmed.iastate.edu/vmc/small-animal/clinical-trials/chondroitinase-clinical-trial-0</a></td>
</tr>
<tr>
<td>Spinal Cord Injury Research and Advocacy</td>
<td><a href="https://spinalcordresearchandadvocacy.wordpress.com/">https://spinalcordresearchandadvocacy.wordpress.com/</a></td>
</tr>
<tr>
<td>Spinal Research</td>
<td><a href="http://www.spinal-research.org/">http://www.spinal-research.org/</a></td>
</tr>
<tr>
<td>VETgirl</td>
<td><a href="http://vetgirlontherun.com/">http://vetgirlontherun.com/</a></td>
</tr>
<tr>
<td>About.com</td>
<td><a href="http://vetmedicine.about.com/fl/chondroitinase-trial-for-spinal-injury-part-1.htm">http://vetmedicine.about.com/fl/chondroitinase-trial-for-spinal-injury-part-1.htm</a></td>
</tr>
</tbody>
</table>
Patient selection

Patient selection was carried out in four sequential steps (Figure 2.2). If a potential candidate had suitable signalment and clinical history such as traumatic spinal cord injury secondary to intervertebral disc herniation or vertebral column fracture based on the medical records provided by their regular veterinarian, including diagnostic imaging report or images, surgery and neurologic examination report, an in-person evaluation was offered for further assessment of eligibility (Figure 2.3). Neurologic examination and general physical examination were independently performed by a board-certified veterinary neurologist\(^e\) and a veterinary neurology resident\(^f\). In addition to excluding any concurrent systemic or neurologic conditions that might confound data collection or interpretation, specific neurologic tests were performed to assess the severity and extent of the spinal cord injury.

In particular, spinal cord segmental reflexes in the thoracic limbs, pelvic limbs and perineum were tested to ensure that the injury was confined to T3 – L3 segments. The reflexes

\(^e\) Nick Jeffery BVSc, PhD, MSc, DipECVS, DipECVN, CertSAO, DipSAS, FRCVS
\(^f\) Hilary Hu BSc BVSc
tested included withdrawal reflexes, patellar reflexes, anal tone and reflex; panniculus reflex or ‘cutaneous trunci cut-off’ was also determined, by pinching the paraspinal skin bilaterally starting from the level of L5 – L6 dorsal spinous processes using a pair of forceps to elicit bilateral contractions of the cutaneous trunci muscles, to further localize the level of spinal cord injury. Injury severity was determined by assessing the dog’s ability to ambulate\(^8\) and the presence of conscious pain perception or ‘deep pain’\(^9\) in the pelvic limbs and tail by crushing the nail bed, digit and tail with forceps in small dogs or pliers in larger dogs\(^11,87 – 89\).

![Figure 2.3 Clinical Trial Inclusion & Exclusion Criteria.](image)

Dogs that met all inclusion criteria and none of the exclusion criteria (Figure 2.3) were consecutively enrolled with written consent from their owners, with knowledge and understanding of various aspects of the clinical trial, including randomization, blinding,

\(^8\) A dog was considered ‘ambulatory’ if able to walk ten consecutive steps unaided without falling or the lateral aspect of any part of the foot or metatarsals touching the ground surface.  
\(^9\) Deep pain response was considered present if the animal vocalized, turned its head to the source of the stimulus, or attempted to bite in response to the stimulus.
assessments and procedures that would be performed during the clinical trial, owner
commitment, potential sources of risks and costs. The clinical trial involved four week-long
visits Monday – Friday at Months 0, 1, 3 and 6, during which neurologic assessments were
performed to detect treatment effects and possible adverse effects by comparing between
Intervention and Control (Figure 2.4).
Figure 2.4 Clinical Trial Schedule.

NJ = unblinded investigator Nick Jeffery; HH = blinded investigator Hilary Hu.
Participants were housed in Canine Wards at Lloyd Veterinary Medical Center with regular in-hospital patients. Daily treatment and assessment were performed by a qualified veterinarian\(^i\) with trained veterinary students. Physical therapy was supervised by a certified canine rehabilitation technician\(^i\) with trained veterinary students at Canine Rehabilitation Center, which involved twice-daily sessions with patient-tailored exercises depending on the rehabilitation technician’s assessment and recommendation. The sessions typically involved cart exercise, passive range of motion maneuvers, weight shifting, balancing exercise, standing-to-sitting-to-standing exercise, pool exercises and underwater treadmill exercise (Figure 2.5).

Figure 2.5 Physical Therapy. Clinical trial patient, 4-year-old Miniature Dachshund, doing pool exercises in Canine Rehabilitation Center.

Patient Randomization

A sealed, opaque envelope, containing a piece of paper labeled ‘Intervention’ or ‘Control’, was opened during each patient’s Clinical Trial Visit 1 after all baseline data had been collected and immediately before the scheduled injection, such that each patient had 50% chance

\(^i\) Hilary Hu BSc BVSc
\(^j\) Joanna Hildreth CVT, CCRP
of being allocated to either Intervention or Control group. The envelopes were made up in three blocks of 20, each containing 10 of either Intervention or Control. Both the patient’s owner and the investigator responsible for data collection remained blinded to experimental group allocation until the end of the 6-month clinical trial enrollment period and did not know the nature of the randomized block sizes until the trial had been completed.

Chondroitinase ABC Preparation and Delivery

**Chondroitinase ABC injection preparation**

Trehalose and microtubes used for preparing the injections were supplied by the Bellamkonda laboratory at Georgia Institute of Technology and Emory University (R102). Chondroitinase ABC, freeze-dried upon arrival and stored at -80°C, was thawed ~ 15 minutes prior to reconstitution. In a laminar flow flood hood, 800 µL of 38% trehalose buffer was added to a 15 U chondroitinase ABC bottle and mixed thoroughly by shaking and turning until fully dissolved. The solution was divided into two equal 7.5 U, or 400 µL, aliquots and stored in a -80 °C freezer until use. Since the microtubes begin releasing chondroitinase ABC immediately after loading, they were combined the night before injection; one 7.5 U chondroitinase ABC aliquot was removed from the -80 °C freezer, thawed and mixed with a 10 mg tube of dry lipid microtubes using micropipettes in a sterile laminar flood hood and mixed gently with a micropipette before stored overnight at 4 °C. Each 400 µL aliquot contained 7.5 U.

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1 Hilary Hu BSc BVSc
2 Chondroitinase ABC (*Proteus vulgaris*), produce code: AMS-E1028-10, AMSBIO, online order from [http://www.amsbio.com](http://www.amsbio.com)
chondroitinase ABC per 10 mg microtubes; this was the total dose for each dog and was stored for a maximum of two weeks at 4 °C before use.

**Patient preparation for injections**

The entire anesthesia protocol, from pre-anesthesia assessment and pre-medication to monitoring and finally to post-general anesthesia recovery, was performed by the Anesthesia Service at Lloyd Veterinary Medical Center, comprising board-certified veterinary anesthetists, veterinary anesthesia residents and technicians. Prior to scheduled injections, the patient was pre-medicated with intramuscular dexmedetomidine at 0.015 – 0.025 mg/kg and intramuscular butorphanol at 0.1 – 0.4 mg/kg. General anesthesia induction was achieved with a slow intravenous bolus of propofol at 5.5 mg/kg. The patient was intubated and maintained on 1 – 2.5 % isoflurane with oxygen during the entire procedure with close monitoring. Once fully anaesthetized, the patient was transported to the Fluoroscopy Procedure Room and placed in left lateral recumbency and surgically clipped, between the levels of mid-thorax and wings of the ilium, before surgically scrubbed with alternating chlorhexidine and povidone iodine.

**Chondroitinase ABC delivery**

Following preparation, patient was positioned on the fluoroscopy table in right lateral recumbency. A dog that had been assigned to Intervention to receive chondroitinase ABC had percutaneous placement of 20 or 22 gauge spinal needles through the interarcuate ligaments at (1) the lesion epicenter and (2) the L3 – L4 interspace, where the cranial margin of the central pattern generator in the lumbosacral intumescence in dogs is presumed to be located11,83 – 85.
Local delivery of chondroitinase ABC was designed to revive functional neuronal sprouting or plasticity of the damaged axons and denervated target neurons.

The spinal needles were advanced until they reached the vertebral canal ventral floor, which were guided by fluoroscopy and confirmed by observing cerebrospinal fluid dripping from the needle hub. Once the needles had reached the ventral floor, they were retracted a few millimeters to ensure intra-parenchymal placement; needle tip positions were confirmed on fluoroscopy (Figure 2.6). A total of 200 µL chondroitinase ABC solution was injected into each site via the spinal needles; at each site, 100 µL solution was injected with the bevel of the spinal needle facing cranially and 100 µL injected with the bevel facing caudally.

![Figure 2.6 Fluroscopy-guided Intrathecal Spinal Needle Placement.](image)

In Control patients, hypodermic needles were inserted at the same two locations transcutaneously to mimic the intrathecal chondroitinase ABC injections in order to maintain blinding of the owner and the blinded investigator. After either procedure, each patient was transported to Anesthesia Recovery for extubation and close monitoring until all vital parameters returned to normal, after which the patient was transported back to Canine Wards.
A total of 196 potential candidates were evaluated, of which, 111 were excluded because their injury history was either unavailable or did not meet all inclusion criteria (Figure 2.7). Of the 85 that had eligible medical history and were thus offered in-person evaluations, a further 12 were excluded because their owners either declined or failed to respond\textsuperscript{m}. Seventy-three were evaluated in person and 13 were excluded because they did not meet all inclusion and exclusion criteria. The 60 dogs that fulfilled all criteria were consecutively enrolled in the study.

\textsuperscript{m} Each owner was contacted at least three times by phone or email before exclusion.
\textsuperscript{n} Lower motor neuron signs (e.g. reduced or absent limb reflexes) would suggest that the spinal cord injury was extensive and thus unlikely to respond to focal chondroitinase ABC injection.
The 60-dog cohort consisted 29 females and 31 males, of which 31 were Dachshunds while the rest were small to medium breeds < 20 kg (Table 2.1). Intervertebral disc herniation was the cause of spinal cord injury in 48 and the remaining 12 had sustained traumatic vertebral column luxation or subluxation. Surgical intervention was implemented in 42 and 18 were managed conservatively with medication for cage-rest. At the time of baseline data collection at Day 0, 51 dogs were non-ambulatory and 54 had absent deep pain in their pelvic limbs and tail.

<table>
<thead>
<tr>
<th>Table 2.1 General Summary of 60 Participants.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex</td>
</tr>
<tr>
<td>Breed</td>
</tr>
<tr>
<td>Cause</td>
</tr>
<tr>
<td>Treatment</td>
</tr>
<tr>
<td>Ambulation&lt;sup&gt;o&lt;/sup&gt;</td>
</tr>
<tr>
<td>Deep pain&lt;sup&gt;p&lt;/sup&gt;</td>
</tr>
<tr>
<td>Age (year)</td>
</tr>
<tr>
<td>Weight (Kg)</td>
</tr>
<tr>
<td>Chronicity (month)</td>
</tr>
</tbody>
</table>

<sup>o</sup> A dog was considered ‘ambulatory’ if he/she was able to walk ten consecutive steps unaided without falling or the lateral aspect of any part of the foot or metatarsals touching the ground.

<sup>p</sup> Deep pain response was considered present if the animal vocalized, turned its head to the source of the stimulus, or attempted to bite in response to the painful stimulus applied to the pelvic limb digits and/or tail.
Dogs that were classified as ‘ambulatory’ at Day 0 all had absent deep pain, suggesting that their spinal cord injuries were severe or complete, such that their pelvic limb ambulation was most likely mediated by injury-induced central pattern generator activation, or ‘spinal-walking’, rather than true recovery of locomotion; this is discussed in Chapter 3. These dogs typically shows poor inter-limb coordination, which can serve as a surrogate for change in neurologic function after receiving Intervention. Following baseline data collection, the participants were equally randomized to Intervention or Control with 30 in each group (Table 2.2).

Table 2.2 General Summary of Intervention and Control.

<table>
<thead>
<tr>
<th></th>
<th><strong>Intervention</strong></th>
<th><strong>Control</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Sex</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>15 (50%)</td>
<td>14 (47%)</td>
</tr>
<tr>
<td>Male</td>
<td>15 (50%)</td>
<td>16 (53%)</td>
</tr>
<tr>
<td><strong>Breed</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dachshund</td>
<td>13 (43%)</td>
<td>18 (60%)</td>
</tr>
<tr>
<td>Others</td>
<td>17 (57%)</td>
<td>12 (40%)</td>
</tr>
<tr>
<td><strong>Cause</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Disc herniation</td>
<td>22 (73%)</td>
<td>26 (87%)</td>
</tr>
<tr>
<td>Vertebral column fracture</td>
<td>8 (27%)</td>
<td>4 (13%)</td>
</tr>
<tr>
<td><strong>Treatment</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Surgery</td>
<td>21 (70%)</td>
<td>21 (70%)</td>
</tr>
<tr>
<td>Conservative</td>
<td>9 (30%)</td>
<td>9 (30%)</td>
</tr>
<tr>
<td><strong>Ambulation</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Non-ambulatory</td>
<td>25 (83%)</td>
<td>26 (87%)</td>
</tr>
<tr>
<td>Ambulatory</td>
<td>5 (17%)</td>
<td>4 (13%)</td>
</tr>
<tr>
<td><strong>Deep pain</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Absent</td>
<td>26 (87%)</td>
<td>28 (93%)</td>
</tr>
<tr>
<td>Present</td>
<td>4 (13%)</td>
<td>2 (7%)</td>
</tr>
<tr>
<td><strong>Age (year)</strong></td>
<td>6.2/6.0/0.5 – 14</td>
<td>6.1/6.0/1 – 13</td>
</tr>
<tr>
<td><strong>Weight (Kg)</strong></td>
<td>Mean/median/range</td>
<td>8.2/6.3/2.7 – 20</td>
</tr>
<tr>
<td><strong>Chronicity (month)</strong></td>
<td>19.5/10.5/3 – 75</td>
<td>26.5/17/3 – 89</td>
</tr>
</tbody>
</table>
CHAPTER 3  
GAIT EVALUATION

Introduction

Traumatic thoracolumbar spinal cord injury in pet dogs, typically secondary to intervertebral disc herniation and vertebral column fracture, can cause varying degrees of locomotor impairment in the pelvic limbs, ranging from mild ataxia to paraplegia. Assessment of the gait, or synchronous limb locomotion in different stereotypic patterns including walk, trot, pace, amble and gallop, is an established functional parameter for investigating motor function impairment in thoracolumbar spinal-injured veterinary patients\textsuperscript{90}–\textsuperscript{94}. Common gait assessment techniques in both human and veterinary medicine include: (1) temporal analysis to measure gait parameters, such as velocity, symmetry and relative stance and swing phase duration, by recording foot placement with apparatuses including force plates; (2) kinematics to study the relative movement of body or limb segments by means such as high-speed motion picture analysis and electrogoniometry; (3) electromyography to examine individual skeletal muscle activity; and (4) kinetics to evaluate the forces generated by a limb or within specific joints during locomotion\textsuperscript{90,95–100}. Normal locomotion relies on continuous communication between central neural circuitries within the brain and spinal cord, and their sensory input including proprioceptive, vestibular, visual and auditory; the dynamic sensorimotor interaction confers flexibility and adaptability to locomotion in response to the evolving intrinsic and extrinsic stimuli\textsuperscript{101}. Voluntary locomotion is initiated in the cerebral motor cortex and modulated in several brain
locomotor areas before the information descends in pyramidal and extrapyramidal tracts to reach spinal cord motor neurons.

Three pyramidal tracts arise from the motor cortex of the dog: (1) corticonuclear tract that synapses with the ipsilateral cranial nerve nuclei as it descends in the crus cerebri, pons and pyramid; (2) corticopontine tract that synapses with ipsilateral pontine nucleus, which gives rise to the pontocerebellar fibers that decussate to reach the contralateral cerebellum; and (3) corticospinal tract that descends in the ipsilateral internal capsule, crus cerebri and pons before reaching the pyramid, where ~ 75% fibers decussate and ~ 25% remain ipsilateral to reach spinal cord motor nuclei that in turn trigger skeletal muscles to execute a certain effect via peripheral nerves\textsuperscript{102,103} (Figure 3.1).

Extrapyramidal tracts, especially rubrospinal and reticulospinal tracts, whose nuclei originate in the red nucleus in midbrain and the reticular formation respectively, spanning from midbrain to medulla oblongata, modulate motor function initiated by the motor cortex\textsuperscript{102,103}. The red nucleus communicates with forebrain and cerebellum via the olivary nucleus and mediates movement initiated by the motor cortex via the rubrospinal tract; while the reticular formation, through simultaneous stimulation and inhibition of spinal cord motor neurons that innervate limb extensors and flexors via pontine and medullary reticulospinal tract, respectively, coordinates stereotyped motor patterns such as spinal cord reflexes and gait patterns\textsuperscript{102,103}.

Two additional supraspinal centers are involved in locomotion: (1) the ‘mesencephalic locomotor area’ in the dorsal reticular formation at the mesopontine junction; and (2) the ‘central pattern generator’, which is a diffuse neural network in the thoracolumbar spinal cord\textsuperscript{103–106}. Further, the hypothalamus may also influence locomotion, possibly by augmenting mesencephalic locomotor area stimulation through summation, as electrical stimulation of the hypothalamus can elicit stepping in anesthetized rats; on the other hand,
stimulation of other brain areas, such as ventral layers of the dorsal colliculus, ventromedial midbrain and lateral tegmentum, can inhibit locomotion\textsuperscript{107,108}.

Figure 3.1 Motor Tracts in the Dog. Corticospinal tract (blue) arises from the motor cortex and descends ipsilaterally until reaching the pyramid, where fibers either continue ipsilaterally in the ventral corticospinal tract or contralaterally in the lateral corticospinal tract. Extrapyramidal tracts (red): rubrospinal and reticulospinal tracts originate in the red nucleus and reticular formation\textsuperscript{103}.

Quadrupedal gaits that require thoracic-pelvic limb coordination are executed by spinal cord central pattern generators under the direction of supraspinal centers; stimulation of the mesencephalic locomotor area, for instance, activates the reticular formation, which in turn influences the central pattern generator via reticulospinal tract to produce rhythmic, repetitive alternation between flexor excitation during ‘swing phase’ when the foot is mid-air and extensor excitation during ‘stance phase’ when the foot contacts the ground\textsuperscript{103,109,110}.
Interestingly, the central pattern generator can produce locomotor patterns or ‘fictive patterns’ independent of supraspinal input following spinal cord injury by relying on peripheral sensory stimulation elicited by means such as tail crimping and perineal or pelvic limb stimulation\textsuperscript{111–114}. While flexor afferent stimulation and transmission in one limb elicits discharge in ipsilateral spinal cord flexor motoneurons with simultaneous contralateral extensor motoneuron excitation, contralateral transmission of flexor afferent is concurrently inhibited\textsuperscript{114}.

Based on earlier experimental evidence, the reciprocal action of the motoneurons controlling the flexors and extensors in each limb has been postulated to be modulated by the segmental spinal cord interneurons that mediate mutual inhibition between motoneurons with reciprocal actions, or two ‘half-centers’, such that only half-center is active at a time; while inhibition of one half-center on the other is released when the former gradually becomes fatigued, triggering a ‘phase switch’ associated with the excitation of the latter\textsuperscript{114,115}.

However, this simplistic model of binary limb movement driven by dichotomous motoneuron activation fails to explain: (1) more complex locomotor patterns involving the sequential activation of various muscle groups in a limb during a step cycle, which are much more complicated and temporally stratified than the simple flexion-extension phase switch; and (2) activation of a subset of motoneurons in both swing and stance phases of a step cycle\textsuperscript{101,116}. More sophisticated theories, attempting to explain the role of central pattern generator in orchestrating gait patterns, have since been proposed, however, the exact mechanisms remain elusive\textsuperscript{102,110,117,118}.

Experimentally, dogs and cats deprived of normal supraspinal locomotor control can be trained and/or stimulated to repetitively step and ambulate on a moving treadmill; these animals exhibit alternating pelvic limb flexion and extension resembling normal ambulation; however, they can only ambulate at lower speeds with a longer swing phase and weaker
stepping force in the pelvic limbs compared to normal animals, and show gait abnormalities like foot dragging and reduced angular stifle excursion\textsuperscript{111 – 113,119 – 125}.

The ability to regain locomotion appears to be particularly striking in first time-injured neonates and repeat-injured adults such that the operates begin to show locomotion as early as 24 hours following complete caudal thoracic spinal cord transection\textsuperscript{98,121,124,126}; this suggests that neuroplasticity, either inherent or injury-induced, activates spinal cord locomotor control in the absence of normal supraspinal input. Further, the spinal cord central pattern generator becomes more functionally complex and excitable over time, producing more diverse, complicated fictive patterns such as paw-shaking, stepping and strong rhythmic limb flexion when the paw is squeezed, in chronically spinal-injured animals\textsuperscript{112}.

Clinically, a subset of chronic, severe thoracolumbar spinal-injured pet dogs, typically with absent deep pain\textsuperscript{q}, can ambulate or ‘spinal-walk’ on their pelvic limbs; these dogs manifest distinctive pelvic limb locomotor characteristics that, from our direct clinical observation, resemble those described in experimental studies, including poor inter-pelvic and pelvic-thoracic limb coordination, high step cycle variability, foot dragging, lateral instability, reduced range of motion that is most noticeable in proximal joints like the stifle and hip, and speed limitation.

These gait abnormalities arise as the central pattern generator has a limited ability to precisely execute complicated locomotor patterns by relying solely on peripheral sensory input; the deficiency becomes more pronounced at higher ambulation speeds that normally require the recruitment of larger, faster pelvic limb motor units by supraspinal locomotor centers\textsuperscript{111,113,125,127,128}. Gait parameters in spinal-injured dogs that manifest varying degrees of locomotor impairment, presumably dependant on the spinal cord tracts affected and extent of

\textsuperscript{q} Deep pain response, usually tested by squeezing the pelvic limb digits and tail with forceps or pliers, is a clinical neurologic test for determining the severity of spinal cord injury and its absence is associated with severe to complete spinal cord injury.
the damage, can thus be used as functional outcome measures to assess the magnitude of treatment effect of potential therapies for spinal cord injury\textsuperscript{129–131}.

Relatively simple, reproducible and cost-effective means of gait evaluation, such as treadmill gait analysis, can be very useful in clinical and translational spinal cord injury research as it allows the investigator to quantify the extent of neurologic deficits. Treadmill gait assessment can be done with a speed-adjustable treadmill and a number of cameras arranged around the treadmill for recording and relaying motion data while the test subject ambulates on the treadmill; both spinal-injured and spinal-intact dogs can learn to do so with little or no training\textsuperscript{80,91–94}. Treadmill gait assessment has relatively high inter- and intra-observer agreeability, rendering it a valuable tool, either used independently or in conjunction with other locomotor tests such as open field score, in multi-center clinical studies\textsuperscript{91}.

Specific gait analysis outcome measures can be utilized to evaluate the treatment effect of potential therapies on locomotor function by detecting functional changes in thoracolumbar spinal-injured dogs over time. These outcome measures include metrics for assessing inter-limb coordination such as the ‘stepping score’ or pelvic- to thoracic-limb step ratio, the ‘coordination score’ or the proportion of pelvic limb steps that are coordinated and the ‘mean diagonal coupling interval’ that measures the temporal variability in pelvic limb placement responsible for thoracic-pelvic limb incoordination; other established gait metrics include the variability in step cycle duration, the temporal ratio of the swing to stance phase of a step cycle, and the lateral stability of pelvic limb paw placement\textsuperscript{80,91–94}.

We herein report the treatment effect of chondroitinase ABC on locomotion in severely spinal-injured\textsuperscript{r} pet dogs by comparing between Intervention and Control at baseline Day 0, followed by rechecks at Months 1, 3 and 6. Pelvic-thoracic limb coordination

\textsuperscript{r} 0\% of the cohort showing pelvic-thoracic limb coordination when pre-intervention data was collected from Intervention and Control groups at Day 0.
measured by inverse of coefficient of variation (1/CV)$^8$ of the pelvic-thoracic limb phase shift while ambulating on a treadmill was used as the outcome.

Materials and Methods

Study approval, written consent, patient selection and chondroitintase ABC administration are outlined in Chapter 2. Eight infrared motion capture cameras$^1$ mounted on adjustable tripods were arranged around and directed at the treadmill, four on either side. The recording field on the treadmill was calibrated with a T-shaped wand in accordance with instructions outlined in the Vicon Nexus user manual$^u$ before each treadmill gait recording session.

The cameras recorded limb motion of the dog while ambulating on the treadmill by detecting the 10 mm reflective markers attached to ten pre-specified anatomic landmarks: (1) elbow joint at the lateral aspect of the proximal olecranon, (2) front paw at the lateral aspect of 5th digit, (3) stifle joint at the lateral aspect of proximal tibial tuberosity, (4) hock at the lateral aspect of proximal calcaneal tuberosity, and (5) lateral aspect of 5th digit of hind paw (Figure 3.2(a)).

---

$^8$ CV = standard deviation/mean, 1/CV = mean/standard deviation; a larger value means smaller data variability. Since variability is very small in spinal-intact dogs, 1/CV should increase in Intervention.

$^1$ Motion capture cameras: 8-camera Bonita Vicon Motion Systems Ltd. UK.

$^u$ Vicon Nexus user manual: Vicon Nexus 1.8.5, Vicon Motion Systems Ltd. UK.
Figure 3.2 Gait Recording. (a) Reflective markers taped to specific limb landmarks; (b) motion capture cameras mounted to tripods record limb movement of a marker-labeled dog walking on a moving treadmill with sling support; (c) camera input can be viewed in real-time, along with camera positions labeled 1, 2 and 3; (d) markers can be manually labeled and joined to represent individual limb segments.

Since most dogs were unable to ambulate and/or stand on their pelvic limbs, a sling, secured to the treadmill railing on either side, was placed underneath their caudal abdomen to provide weight support and as a safety measure to prevent injury in case the dog slipped and fell while ambulating; height of the sling was adjusted such that long axis of the dog’s back would be roughly parallel to the ground, as one would expect in a spinal-intact dog while standing (Figure 3.2(b)).

Speed of the treadmill was adjusted such that the dog was able to leash-walk or harness-walk comfortably with the guidance and encouragement of an assistant. Speed selection for final gait analysis was based on two criteria: (1) the thoracic limbs must be advancing at an even pace with minimal speed variation during the recording; (2) the same speed must be used at all four time-points for the same dog.
At least one 1 – 2 minute recording(s) that qualified containing ~100 consecutive steps of each limb, or 6,000 – 12,000 frames, was obtained. When a dog ambulated, the cameras captured motion at a frequency of 100 frames per second and relayed this input via cable connections to a computer (Figure 3.2(c)). The input was viewed in real-time in Vicon Nexus as a two dimensional representation of limb motion in a three-dimensional space that could be deconstructed into three planes or axes for analysis: (1) x-axis represented forward-backward motion; (2) y-axis represented lateral motion; and (3) z-axis represented vertical motion.

Data cleaning was done in accordance with Vicon Nexus user manual. Briefly, markers at each pre-specified landmark were manually color-labeled and joined to form segments: elbow to fore paw, stifle to hock, hock to hind paw, and stifle to hind paw (Figure 3.2(d)). Gaps in motion trajectories caused by unlabeled or missing markers were filled using the software-recommended trajectories, which were extrapolated from labeled trajectories of the same markers and their temporospatial relationships with other markers. After gap-filling, any additional unlabeled markers and trajectories were deleted before the recording was saved in several default formats; of which, the ‘.c3d’ format was exported to MATLAB for analysis.

For coordination, z-axis or vertical motion was used. A ‘step’ was defined as when the paw height exceeded 10% of elbow height; timing of each step peak or when the paw was maximally raised was used for comparing the timing of the pelvic limb paw step peak in relation to the thoracic limb paw step peak that immediately preceded it, or ‘phase shift’. To minimize noise, exclusions were imposed such that any z-axis paw motion was excluded if:

---

v Vicon Nexus 1.8.5 motion analysis software, Vicon Motion Systems Ltd. UK.
w Vicon Nexus user manual: Vicon Nexus 1.8.5, Vicon Motion Systems Ltd. UK.
x MATLAB R2015a (8.5.0.197613) 64-bit, The MathWorks Inc.
(1) it was < 10% elbow/stifle height; (2) its minimum peak prominence was < 0.75 cm; or (3) inter-peak time was < 0.25 second.

Step cycle uniformity was used for selecting the reference limb such that whichever thoracic limb had a smaller step cycle variance was chosen, which was then matched with the contralateral pelvic limb. Thoracic-pelvic limb phase shift analysis was done by extracting, for each step within a 1 – 2 minute recording, the time difference or ‘phase shift’ between two time-points: (1) when the reference thoracic paw had its maximum z-axis value (i.e. maximally raised in the air) and (2) when the corresponding pelvic paw had its maximum z-axis value.

The phase shift of every step was extracted for each recording that contained a sufficient number of pelvic limb steps, or > 20% thoracic steps in the same recording. The mean and standard deviation of the phase shifts of each recording was used to calculate the inverse of coefficient of variation (1/CV)\(^\text{y}\) that quantified data variability in relation to the population mean (i.e. larger 1/CV = smaller variability)\(^\text{z}\).

Statistical analysis\(^\text{aa}\) for pelvic-thoracic limb phase shift was done by using cross-sectional time series regression with inclusion of baseline values as a covariate to allow for differences when the participants were enrolled. This method of analysis deviated from the original design outlined in Chapter 2 as the pre-study sample size calculation was based on a categorical outcome (i.e. 25% Intervention animals would regain coordination), while the actual outcome of phase shift (1/CV) was continuous. A similar previous study using continuous outcome measures had used a similar sample size to that calculated here\(^\text{80}\).

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\(^\text{y}\) Coefficient of variation = standard deviation/mean, 1/CV = mean/std. deviation.

\(^\text{z}\) Since variability is expected to be very small in spinal-intact dogs, 1/CV should increase over time in Intervention group as phase shift becomes more uniform.

\(^\text{aa}\) Statistical analysis was performed by Dr. Nick Jeffery using Stata 11 Data Analysis & Statistical Software, StataCorp LP, College Station, TX.
Results

Sixty dogs were recruited but data were not available from all at all time-points due to patient dropout and missed rechecks (Table 3.1). Pre-intervention baseline was collected at Day 0 from 60/60 patients in Intervention and Control; at Month 1, data were available from 58/60; at Month 3, from 55/60; and at Month 6, from 52/60. General summary of Intervention and Control are reported in Chapter 2.

Table 3.1 Data Availability for Gait Evaluation.

<table>
<thead>
<tr>
<th>Time/Group</th>
<th>Intervention</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline</td>
<td>30</td>
<td>30</td>
</tr>
<tr>
<td>Recheck 1</td>
<td>29</td>
<td>29</td>
</tr>
<tr>
<td>Recheck 2</td>
<td>27</td>
<td>28</td>
</tr>
<tr>
<td>Recheck 3</td>
<td>27</td>
<td>25</td>
</tr>
</tbody>
</table>

Gait coordination was assessed at the four time-points and compared between Intervention and Control, resulting in eight groups (Table 3.2, Figure 3.3). After excluding recordings in which < 20% thoracic limb steps were matched by pelvic limb steps, 198/224 data-points were available for analysis. Shapiro-Wilk W test showed that none of the eight groups except Control Recheck 2 was normally distributed. 1/CV of phase shift varied substantially amongst the cohort ranging from 1.4 to 43.7, but the medians, 2.1 – 2.3, were similar across all groups.

\[^{bb}\] JMP 11.0.0 (64-bit), 2013, SAS Institute Inc.
Following log transformation to mitigate the highly skewed distribution, statistical analysis showed 1/CV of phase shift was unaffected by group and chronicity; thus neither chondroitinase ABC administration nor injury chronicity had a significant effect on pelvic-thoracic limb coordination (Table 3.3). Baseline had a significant effect with $P = 0.00$, i.e. dogs tend to maintain the same level of pelvic-thoracic limb coordination measured by 1/CV over the duration of the study.

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Table 3.2 1/CV of Pelvic-thoracic Limb Phase Shift.

<table>
<thead>
<tr>
<th>Group</th>
<th>Q25</th>
<th>Median</th>
<th>Q75</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control Baseline</td>
<td>2.0</td>
<td>2.2</td>
<td>2.5</td>
</tr>
<tr>
<td>Control Recheck 1</td>
<td>2.0</td>
<td>2.2</td>
<td>2.6</td>
</tr>
<tr>
<td>Control Recheck 2</td>
<td>2.0</td>
<td>2.1</td>
<td>2.4</td>
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<tr>
<td>Control Recheck 3</td>
<td>1.9</td>
<td>2.2</td>
<td>2.7</td>
</tr>
<tr>
<td>Intervention Baseline</td>
<td>2.1</td>
<td>2.3</td>
<td>2.6</td>
</tr>
<tr>
<td>Intervention Recheck 1</td>
<td>2.0</td>
<td>2.2</td>
<td>2.5</td>
</tr>
<tr>
<td>Intervention Recheck 2</td>
<td>1.8</td>
<td>2.1</td>
<td>2.5</td>
</tr>
<tr>
<td>Intervention Recheck 3</td>
<td>2.0</td>
<td>2.1</td>
<td>3.0</td>
</tr>
</tbody>
</table>

Figure 3.3 1/CV of Pelvic-thoracic Limb Phase Shift.
Control = Groups 1, 3cc, 5 and 7; Intervention = Groups 2, 4, 6 and 8.

cc Data-point from Subgroup 3 with the value of 43.7 was excluded as an outlier.
Table 3.3 Statistical Summary of Log-transformed 1/CV of Coordination.

| Parameter           | Coef | Std Err | z    | P>|z| | 95% CI    |
|---------------------|------|---------|------|------|-----------|
| Study Group         | 0.05 | 0.04    | 1.08 | 0.28 | -0.04 – 0.13 |
| Injury Chronicity   | 0.01 | 0.02    | 0.48 | 0.64 | -0.03 – 0.04 |
| Baseline            | 0.78 | 0.07    | 10.73| 0.00 | 0.34 – 0.93  |

Discussion

Analysis of gait coordination, measured by 1/CV of pelvic-thoracic limb phase shift, revealed that chondroitinase ABC has no statistically detectable treatment effect on locomotor function. Failure to regain pelvic-limb gait coordination in the chondroitinase ABC-treated Intervention group does not support our original hypothesis that pelvic-thoracic limb gait coordination would improve, quantified by an overall increase in 1/CV of pelvic-thoracic limb phase shift.

Since quadrupedal gaits that require thoracic-pelvic limb coordination are executed by spinal cord central pattern generators under the direction of supraspinal centers via pyramidal and extrapyramidal tracts\textsuperscript{103,109,110}, our results suggests that the current drug formulation and/or delivery was unsuccessful in restoring the functional continuity of these supraspinal and spinal pathways.

Nevertheless, our current method for analysing and quantifying gait coordination provides a novel means for assessing inter-limb phase shift. Our method offers a more detailed, sensitive analytic compared with some of the more conventional metrics for assessing inter-limb coordination such as the pelvic-thoracic step ratio and the proportion of coordinated pelvic limb steps; it also provides a straight-forward alternative to the more advanced gait metrics such as the ‘mean diagonal coupling interval’ that measures the
temporal variability in pelvic limb placement and the lateral stability of paw placement\textsuperscript{80,91–94}.

One key limitation is the large number of data-points excluded for analysis. For pelvic-thoracic limb phase shift, 11.6\% of the available data-points were excluded because the number of pelvic limb steps were < 20\% of the corresponding thoracic steps, which had been recorded from study participants with lower levels of pelvic limb motor function. This method of selection could have created a certain degree of sampling bias, or more specifically exclusion bias, that would have favored individuals within the cohort that possessed superior motor function. On the other hand, inclusion of animals that had very few pelvic limb steps would have led to an inaccurate estimate of 1/CV of pelvic-thoracic limb phase shift based on a very small number of samples from each gait recording.
CHAPTER 4

ELECTROPHYSIOLOGIC EVALUATION

Introduction

Electrophysiologic techniques can help evaluate the functional integrity of neurologic pathways and in spinal cord injury research, electrophysiologic parameters for spinal cord conduction can be used as minimally invasive, inexpensive outcome measures for monitoring post-injury functional recovery and quantifying the treatment effects of potential interventions. Transcranial magnetic motor-evoked potentials or TMMEPs and somatosensory-evoked potentials or SSEPs are two of the most well-established electrophysiologic techniques in humans and animals for evaluating the functional integrity of motor and sensory spinal cord pathways, respectively.\textsuperscript{80,132 - 139}

Both techniques involve applying an external stimulus, magnetic or electrical, to elicit transmembrane ion movement or current flow that depolarizes the neuron and triggers action potentials to travel along the spinal cord; the characteristics of action potential propagation can be quantified using parameters such as ‘onset latency’ or the time it takes for the nerve impulses to reach a certain location along the neurologic pathway, and ‘waveform amplitude’ or magnitude of the response elicited.\textsuperscript{140}

Somatosensory-evoked potentials, elicited by tibial nerve electrical stimulation and recorded from dorsal lamina of the vertebral body, can be used to evaluate ascending sensory pathways in dogs with acute spinal cord injury secondary to intervertebral disc extrusion.\textsuperscript{139} One study shows that the potentials recorded from injury-adjacent T10 – T11 segments differ
considerably from the controls; further, waveform can serve as a reliable prognostic indicator for neurologic recovery, defined as regaining ambulation and urinary continence\textsuperscript{139}.

Similarly, scalp recording of somatosensory-evoked potentials from the contralateral sensory cortex can be used for monitoring change in spinal cord conduction following therapeutic intervention in spinal-injured pet dogs in a clinical trial setting\textsuperscript{80}.

Tibial nerve stimulation-evoked potentials recorded from the spinal cord are thought to be the ascending potentials generated by the ipsilateral proximal dorsal root, dorsal funiculus and afferent collaterals to the dorsal horn; this occurs when the ascending action potentials arrive following synaptic modulation within the lumbosacral intumescence in L4 – S3 segments in the dog\textsuperscript{11,141,142}.

Selective spinal cord transection studies suggest that while the ascending potentials propagate in all cross-sectional areas of the spinal cord, the ipsilateral dorsal quadrant, especially dorsolateral fasciculus and dorsal column, has the greatest contribution\textsuperscript{143}. While onset latency is strongly correlated with body length in dogs, the correlation between amplitude and body length is only moderate\textsuperscript{144}.

Transcranial magnetic motor-evoked potentials, the counterpart of somatosensory-evoked potentials, examine the functional integrity of spinal cord motor pathways. The potentials are elicited, in accordance with Faraday’s law of electromagnetic induction, when the magnetic coil-induced transitory magnetic field generates electrical impulses in the motor cortex, which is propagated along the spinal cord motor pathways to reach the periphery, such as cranialis tibialis and extensor carpi radialis muscles in the contralateral limbs, from which compound muscle action potentials are typically recorded\textsuperscript{138,140}.

In humans, the magnetic field-induced action potentials descend primarily in corticospinal pathways\textsuperscript{145}, while in veterinary species such as rats and cats, extrapyramidal
tracts, including reticulospinal and vestibulospinal tracts, play larger roles in the propagation of transcranial magnetic motor-evoked potentials\textsuperscript{146,147}. Similar to somatosensory-evoked potentials, quantifiable parameters of motor-evoked potentials, such as onset latency and amplitude, can be extracted from pet dogs in clinical studies as objective outcome measures\textsuperscript{93,137}.

In this study, we used (1) the cranial-most vertebral body level at which somatosensory-evoked potentials could be recorded and (2) the presence of transcranial magnetic motor-evoked potentials in pelvic limbs as outcome measures for assessing the therapeutic effect of chondroitinase ABC on restoring the electrophysiological integrity of sensory and motor pathways in severely spinal-injured dogs. Pre-intervention data were collected on Day 0 from Intervention and Control groups, followed by three rechecks at Months 1, 3 and 6.

We hypothesized that Intervention group would have a smaller distance, measured in the number of spinal cord segments, between the caudal level of injury and the cranial-most level of recordable somatosensory-evoked potentials (Figure 4.1). The rationale of using this distance as an outcome measure is that chondroitinase ABC, a drug with the putative effect of promoting axonal regeneration, should theoretically promote spinal cord conduction across the lesion, such that the distance would be expected to decrease over time.

![Figure 4.1 Distance Measured in Number of Spinal Cord Segments.](image-url)
We also hypothesized that Intervention would have a larger number of animals with recordable transcranial motor-evoked potentials in the pelvic limbs than Control at Months 1, 3 and 6. The proportion of animals with positive pelvic limb transcranial magnetic motor-evoked potentials was expected to increase in the Intervention group over time since chondroitinase ABC is thought to promote axonal regeneration and thus, theoretically, should restore functional continuity of the injured spinal cord.

Materials and Methods

Study approval, written consent, patient selection and chondroitinase ABC administration are outlined in Chapter 2. Following evaluation of vital parameters including heart rate, respiratory rate, mucous membrane color, capillary refill time and rectal temperature, each dog was sedated with 0.005 mg/kg dexmedetomidine and 0.2 mg/kg butorphanol intravenously. During the procedure, the dog was placed in sternal recumbency and monitored using the same vital parameters every 5 minutes until the end, when 0.05 mg/kg atipamezole was given intramuscularly to reverse the effect of dexmedetomidine and the dog was ambulatory.

Somatosensory-evoked potentials were recorded on a conventional electrodiagnostic unit from the dorsal lamina immediately cranial to dorsal spinous process using a bipolar recording electrode (Figure 4.2). Reference bipolar electrode was placed in the epaxial muscle ~ 1 cm lateral to the recording electrode ipsilateral to the side of stimulation.

---

dd Nihon Kohden Neuropack M1 MEB-9200 EMG/EP/IOM System, discontinued
e Recording electrode: Ambu Neurol ine Concentric 38 x 0.45 mm, Ref. 74038-45/25
ff Reference electrode: Ambu Neurol ine Concentric 38 x 0.45 mm, Ref. 74038-45/25
Subdermal ground electrode\textsuperscript{gg} was placed subcutaneously over cranial aspect of the stifle joint. To minimize noise, pelvic limbs were minimally stimulated with subdermal needles\textsuperscript{hh} placed near the tibial nerve at the hock; stimulation was confirmed by observation of slight digital twitching.

![Figure 4.2 SSEP and TMMEP Recording](image)

Figure 4.2 SSEP and TMMEP Recording. Somatosensory-evoked potentials (left) recorded from L5 – L3 dorsal laminae; note amplitude attenuation at each recording site. Transcranial magnetic-evoked potentials (right) recorded from contralateral thoracic and pelvic limbs. Arrows indicate onset latency.

Somatosensory-evoked potentials were considered present if the same waveform with a peak-to-peak amplitude of > 0.15 µV could be repeated at least once; amplitude and latency were recorded after signal averaging of > 200 sweeps. On each side, the recording started at L6 and progressed cranially one vertebra at a time until potentials could no longer be recorded; location, amplitude and latency of the potentials at the cranial-most vertebra were recorded for analysis. The vertebra immediately cranial to the level of spinal cord injury was tested to confirm loss of conduction across the lesion epicenter.

\textsuperscript{gg} Ground electrode: Ambu Neuroline Subdermal 12 x 0.40 mm, Ref. 74512-100/24

\textsuperscript{hh} Stimulating electrodes: Ambu Neuroline Subdermal 12 x 0.40 mm, Ref. 74512-100/24
Transcranial magnetic motor-evoked potentials were tested by stimulating the motor cortex at 70 % and 80 % stimulation intensities with a magnetic coil\textsuperscript{ii} held 1 – 2 cm from the skull and recording from the contralateral cranialis tibialis muscle with a bipolar recording electrode\textsuperscript{ii} (Figure 4.2). Extensor carpi radialis muscle potentials recorded from the right thoracic limb served as control to ensure that a response could be elicited in an unaffected limb. Subdermal ground electrode\textsuperscript{kk} was subcutaneously placed over the cranial aspect of the stifle. Response was considered present if three consecutive potentials with an amplitude of > 0.15 mV were obtained from three separate stimulations at either intensity. Due to time constraint, only amplitude and latency of the last waveform were recorded.

For both somatosensory-evoked and transcranial magnetic motor-evoked potentials, onset latency was defined as the time difference between Time 0 and onset of deflection from the baseline in either positive or negative direction; peak-to-peak amplitude was defined as the difference between two largest peaks of adverse polarity following the initial deflection from baseline. Statistical analysis\textsuperscript{ll} for the presence of pelvic limb motor-evoked potentials was done to detect any treatment effect associated with chondroitinase ABC by using time series logistic regression with the inclusion of time (Day 0, Month 1, 3 and 6) and baseline values as covariates; inclusion of the latter was to allow for differences when the participants were enrolled in the study.

\textsuperscript{ii} Magstim 200\textsuperscript{2}, The MAGSTIM Company Limited, Whitland, Wales, UK.
\textsuperscript{jj} Recording electrode: Ambu Neurolife Concentric 38 x 0.45 mm, Ref. 74038-45/25
\textsuperscript{kk} Ground electrode: Ambu Neurolife Subdermal 12 x 0.40 mm, Ref. 74512-100/24
\textsuperscript{ll} Statistical analysis was performed by Dr. Nick Jeffery using Stata 11 Data Analysis & Statistical Software, StataCorp LP, College Station, TX.
Results

Sixty dogs were recruited but results were not available from all of them at all time-points because of patient dropout, missed rechecks, and inability to perform procedure due to animal’s behavioural issues or technical difficulties (Table 4.1). Pre-intervention baseline data were collected at Day 0 from 60/60 patients between Intervention and Control; at Rechecks 1, 2 and 3, data were available from 58/60, 55/60 and 52/60. General summary data of the two groups are reported in Chapter 2.

<table>
<thead>
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<th></th>
<th>Sensory-evoked</th>
<th></th>
<th>Motor-evoked</th>
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<tr>
<td></td>
<td>Intervention</td>
<td>Control</td>
<td>Intervention</td>
<td>Control</td>
</tr>
<tr>
<td>Baseline</td>
<td>27</td>
<td>30</td>
<td>30</td>
<td>30</td>
</tr>
<tr>
<td>Recheck 1</td>
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<td>29</td>
<td>29</td>
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</tr>
<tr>
<td>Recheck 2</td>
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<tr>
<td>Recheck 3</td>
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</table>
For somatosensory-evoked potentials, the distance, measured in the number of spinal cord segments, between the caudal level of spinal cord injury and the cranial-most level of recordable potentials, was calculated and compared between Intervention and Control at the four time-points, resulting in eight groups (i.e. Control Baseline, Recheck 1, Recheck 2 and Recheck 3; Intervention Baseline, Recheck 1, Recheck 2 and Recheck 3). The median distance was similar across all groups and remained the same for both groups during the study period; the four Control groups all had a median of 2 spinal cord segments and the four Intervention groups all had a median of 3 (Table 4.2). For the entire cohort, the distance ranged between -5 and 6 segments. Where the potentials were not recordable, mostly due to excessive background noise levels, the number 15 was assigned to graphically reflect these data points (Figure 4.3).

Table 4.2 Distance of Somatosensory-evoked Potentials (No. of Cord Segments).

<table>
<thead>
<tr>
<th>Group</th>
<th>Q25</th>
<th>Median</th>
<th>Q75</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control Baseline</td>
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</tr>
<tr>
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<tr>
<td>Control Recheck 3</td>
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<td>2</td>
<td>4</td>
</tr>
<tr>
<td>Intervention Baseline</td>
<td>1</td>
<td>3</td>
<td>5</td>
</tr>
<tr>
<td>Intervention Recheck 1</td>
<td>1</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>Intervention Recheck 2</td>
<td>2</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>Intervention Recheck 3</td>
<td>2</td>
<td>3</td>
<td>4</td>
</tr>
</tbody>
</table>
Figure 4.3 Comparison of Distance for Somatosensory-evoked Potentials. Animals from which potentials could not be recorded were assigned 15.
Control = Groups 1, 3, 5 and 7; Intervention = Groups 2, 4, 6 and 8.

Transcranial magnetic motor-evoked potentials were evaluated at each recheck and compared with Baseline at Day 0, where 3/30 of Control and 3/30 Intervention participants had recordable pelvic limb potentials; at Recheck 1, 5/29 Control and 6/29 Intervention did; by Recheck 2, 5/28 Control and 3/27 Intervention did; and at Recheck 3, the numbers were 3/25 and 4/27 for Control and Intervention, respectively (Figure 4.4). Statistical analysis\textsuperscript{nm} showed that neither group (Intervention or Control) nor time (Day 0, Month 1, 3 and 6) had a significant effect on the presence of pelvic limbs motor-evoked potentials; albeit baseline appeared to have a significant effect on the outcome with $P = 0.003$, i.e. dogs that had recordable potentials at the start of the study were more likely to still have them at the end (Table 4.3).

\textsuperscript{nm} Stata 11 Data Analysis & Statistical Software, StataCorp LP, College Station, TX.
Figure 4.4 Proportion of Positive Motor-evoked Potentials.

Table 4.3. Statistical Summary of Pelvic Limb Motor-evoked Potentials.

| Parameter  | Coef | Std Err | z   | P>|z| | 95% CI  |
|------------|------|---------|-----|-----|--------|
| Study Group | 0.32 | 0.88    | 0.36| 0.72| -1.41 – 2.05 |
| Time       | -0.40| 0.37    | -1.09| 0.28| -1.12 – 0.32  |
| Baseline   | 4.61 | 1.55    | 2.97| 0.00| 1.57 – 7.65   |

Discussion

The distance, measured in the number of spinal cord segments, between the caudal level of spinal cord injury and the cranial-most level of recordable sensory-evoked potentials remained the same during the six-month study participation period for both Intervention and Control. Chondroitinase ABC also had no treatment effect on the number of participants from which motor-evoked potentials were recordable from the pelvic limb.

One potential source of bias would be the variable effect of sedation agents used, namely, butophanol and dexmedetomidine. While poorly investigated in veterinary medicine, human
studies report variable effects of these pharmacological agents on the recorded evoked potentials. For example, opioids, such as fentanyl, sufentanil and alfentanil, are shown, in various degrees, to decrease the amplitude and increase the latency of evoked potentials\textsuperscript{148–150}. By contrast, dexmedetomidine, an alpha-2 receptor agnostic, appears to promote good conditions conducive to evoke-potential monitoring and has a negligible effect on the waveform amplitude\textsuperscript{151–152}.

Thus, it is possible that the effect of the sedation agents confounded the results collected due to differences in, for instance, the pharmacodynamics of individual participants. On the other hand, this confounding factor might have been mitigated in the current study as we only compared the presence and absence of evoked potentials as a gross assessment for detecting any potential treatment effect of chondroitinase ABC, and did not further carry out detailed comparison of the amplitude and latency of individual waveforms.

Further, after confirming reproducibility, only one reading of the amplitude and latency of the last recorded waveform were measured for analysis due to time constraint, since we had to perform sensory-evoked and motor-evoked potential recording then manually measure the amplitude and latency on the conventional electrodiagnostic unit\textsuperscript{nm} within the 30 minutes of reliable, adequate sedation. Measurement of multiple waveforms recorded from the same individual may be more time-effectively obtained on newer electrodiagnostic units.

\textsuperscript{nm} Nihon Kohden Neuropack M1 MEB-9200 EMG/EP/IOM System, discontinued
CHAPTER 5
CYSTOMETRIC EVALUATION

Introduction

Persistent lower urinary tract dysfunction can be debilitating in spinal-injured human and veterinary patients. Both bladder filling and voiding are affected in T3 – L3 spinal-injured pet dogs: during filling, spontaneous detrusor contraction, or ‘detrusor overactivity’, periodically elevates intravesical pressure and causes urine leakage. During voiding, uncoordinated detrusor and sphincter contraction, or ‘detrusor-sphincter dyssynergia’ causes ineffective bladder emptying.\(^{153}\)

Normal bladder filling and voiding are coordinated by both segmental spinal cord reflexes and supraspinal centers. During filling, gradual bladder distention stimulates the bladder wall mechanosensitive A-delta and C-fibers that in turn stimulate: (1) sympathetic innervation via hypogastric nerves to simultaneously contract the bladder outlet and inhibit the detrusor, and (2) somatic innervation of the external urethral sphincter via pudendal nerves; these mechanisms facilitates urine storage and prevents leakage\(^{11,103,153 – 156}\) (Figure 5.1(a)).

Although the pontine storage center may facilitate involuntary sphincter control, bladder filling is largely an involuntary process regulated by sympathetic thoracolumbar and somatic sacral segmental spinal cord reflexes in normal individuals\(^{103,156 – 159}\). In contrast, micturition is an active, voluntary process coordinated by supraspinal centers that ensure reciprocal detrusor contraction and outlet relaxation occur simultaneously when socially appropriate (Figure 5.1(b)).
Once a certain intravesical pressure or volume threshold, 12 – 32 cmH₂O in conscious humans\textsuperscript{155,160,161} and 18 mL/kg or 50 cmH₂O in anaesthetized dogs\textsuperscript{162}, is reached, action potentials are triggered in the bladder wall afferent to convey this information to the pontine
micturition center and cerebral cortex\textsuperscript{157 – 159,162}. Activation of the former simultaneously: (1) inhibits sympathetic and somatic innervation to trigger outlet relaxation, and (2) stimulates parasympathetic innervation to initiate and sustain detrusor contraction until urine voiding is complete.

Lower urinary tract dysfunction as the result of traumatic spinal cord injury, affecting urine storage and/or voiding, can result from damage anywhere within the micturition pathway between the sacral spinal cord and the brain. Following suprasacral spinal cord injury, bladder wall C-fiber afferents, which are usually functionally silent in spinal-intact animals, can become activated or over-sensitized and trigger spontaneous detrusor contractions during bladder filling\textsuperscript{155,159,161,162}.

Our recent unpublished study suggest that a large proportion of chronically paralyzed pet dogs, secondary to severe, traumatic thoracolumbar spinal cord injury, exhibit C-fiber-mediated detrusor contraction during bladder filling, or ‘detrusor overactivity’, that can lead to urinary incontinence. Further, along with other pathologic spinal cord reflexes such as involuntary pelvic limb stepping, ‘reflex voiding’ or involuntary voiding that can be elicited by segmental afferent stimulation of the perineum, perigenital area, tail and pelvic limbs, is also common in severe or chronic thoracolumbar spinal-injured dogs.

Ironically, these animals often require regular manual bladder expression to prevent urine retention and potential bladder rupture as they are not able to initiate urination, which requires simultaneous detrusor contraction and sphincter relaxation mediated by supraspinal centers. In spinal-injured humans and dogs, pathologic spinal cord micturition reflex, mediated by hypersensitive C-fibers, can trigger concurrent detrusor and sphincter contractions, or ‘detrusor-sphincter dyssynergia’, that can lead to inefficient emptying, high residual volume, bladder over-distension and detrusor hypertrophy from chronic, persistent intravesical pressure elevation\textsuperscript{157 – 159,162,163}. 
C-fiber mediated detrusor-sphincter dyssynergia during voiding and detrusor overactivity during filling can be diagnosed using cystometry, an urodynamic test that assesses urinary bladder function. Although currently underutilized in veterinary medicine, cystometry is commonly indicated for diagnostic and monitoring purposes in people with urinary incontinence, including that caused by neurogenic lower urinary tract dysfunction such as traumatic spinal cord injury\textsuperscript{163–165}. In both species, cystometric outcome measures, such as bladder compliance, intravesical pressure and bladder storage capacity, can be obtained for detecting common abnormalities such as detrusor overactivity; when combined with sphincter electromyogram, detrusor-sphincter dyssynergia can also be diagnosed\textsuperscript{163–165}.

Bladder compliance is a measure of the bladder wall’s response to stretching during filling and it is defined as the amount of change in intravesical pressure associated with a specific change in intravesical volume or $\Delta V/\Delta Pa$. Although 12.5 – 30 mL/cmH$_2$O has been used as the lower end of normal in humans, bladder compliance can vary greatly amongst both spinal-intact and spinal-injured individuals\textsuperscript{163–167}. While the generally accepted safe intravesical pressure threshold is in the vicinity of 40 – 50 cmH$_2$O for humans, the physiologic bladder storage capacity is yet to be determined, although the range of 300 – 600 mL/patient has been used in many human studies and 18 mL/kg has been reported in dogs\textsuperscript{162–167}.

In spinal cord injury translational research, cystometric outcome measures can be utilized to assess the treatment effect of potential interventions in pre-human clinical models such as spinal-injured pet dogs. In this study, we used bladder compliance as the outcome measure for assessing the therapeutic effect of chondroitinase ABC on autonomic or lower urinary tract function. Pre-intervention cystometry data were collected on Day 0 from Intervention and Control groups, followed by three rechecks at Months 1, 3 and 6. We hypothesized that Intervention would have significantly higher compliance than Control.
Materials and Methods

Study approval, written consent, patient selection and chondroitintase ABC administration are outlined in Chapter 2. Cystometry was performed on a conventional urodynamic unit\(^{oo}\) by a blinded investigator\(^{pp}\) on fully conscious dogs with minimal manual restraint (Figure 5.2(a)). A urinary catheter\(^{qq}\) was aseptically placed in the urinary bladder and 0.9% sterile saline solution was infused via a rate-adjustable pump at 10 mL/min for dogs < 10 Kg or 20 mL/min for dogs 10 – 20 Kg.

![Image](image1.png)

Figure 5.2 Cystometry Recording. (a) Recording with minimal manual restraint; (b) real-time recording of intravesical pressure (red) and total volume infused (blue).

Total volume infused and intravesical pressure were recorded in real-time (Figure 5.2(b)) and infusion was stopped when: (1) leaking was apparent at the external urethral orifice; or (2) physiologic bladder capacity of 20 mL/Kg was reached; or (3) intravesical pressure threshold of 50 cmH\(_2\)O was reached. Compliance was determined using the formula \(\Delta V/\Delta P_a\), or change in bladder volume per unit change in intravesical pressure in mL/cmH\(_2\)O.

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\(^{oo}\) Mercury Urodynamic Module with in-built UROLAB software, Life-Tech Inc. Stafford, TX, USA  
\(^{pp}\) Hilary Hu BSc BVSc  
\(^{qq}\) Dual lumen 6.0 and 7.0 French urinary catheter, Life-Tech Inc. Stafford, TX, USA
Two standard points of intravesical pressure and volume were used for calculation of compliance: (1) at the start of infusion when both were zero; and (2) when infusion was stopped immediately before the start of any detrusor contraction that resulted in urine leakage at the external urethral orifice, or when the pressure or volume thresholds were reached. Statistical analysis of bladder compliance was done by using cross-sectional time series regression with the inclusion of injury chronicity and time (i.e. baseline at Day 0, Month 1, 3 and 6) as covariates to allow for potential differences between the two groups at the beginning of the study.

Results

Sixty dogs were recruited but cystometry data were not available from all at all time-points due to patient dropout, missed rechecks, and inability to place urinary catheter (Table 5.1). Pre-intervention baseline data were available from 57/60 between Intervention and Control; at Month 1, data were available from 55/60; at Month 3 and Month 6, data were available from 52/60. General summary data comparing Intervention and Control are reported in Chapter 2.

<table>
<thead>
<tr>
<th>Time/Group</th>
<th>Intervention</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline</td>
<td>28</td>
<td>29</td>
</tr>
<tr>
<td>Recheck 1</td>
<td>27</td>
<td>28</td>
</tr>
<tr>
<td>Recheck 2</td>
<td>25</td>
<td>27</td>
</tr>
<tr>
<td>Recheck 3</td>
<td>27</td>
<td>24</td>
</tr>
</tbody>
</table>

Statistical analysis was performed by Dr. Nick Jeffery using Stata 11 Data Analysis & Statistical Software, StataCorp LP, College Station, TX.
Bladder compliance was measured at all four time-points and compared between Intervention and Control, resulting in eight groups (Table 5.2, Figure 5.3). Shapiro-Wilk W test showed that none of the eight groups was normally distributed. While compliance was highly variable amongst the cohort ranging from 0.4 to 339 ml/cmH$_2$O, the medians were within a narrow range of 3.2 – 6.7 ml/cmH$_2$O.

Table 5.2 Summary of Bladder Compliance (ml/cmH$_2$O).

<table>
<thead>
<tr>
<th>Group</th>
<th>Q25</th>
<th>Median</th>
<th>Q75</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control Baseline</td>
<td>2.0</td>
<td>4.3</td>
<td>16.0</td>
</tr>
<tr>
<td>Control Recheck 1</td>
<td>2.0</td>
<td>4.2</td>
<td>10.2</td>
</tr>
<tr>
<td>Control Recheck 2</td>
<td>1.9</td>
<td>3.2</td>
<td>6.0</td>
</tr>
<tr>
<td>Control Recheck 3</td>
<td>1.7</td>
<td>3.9</td>
<td>9.0</td>
</tr>
<tr>
<td>Intervention Baseline</td>
<td>2.8</td>
<td>4.8</td>
<td>11.4</td>
</tr>
<tr>
<td>Intervention Recheck 1</td>
<td>2.0</td>
<td>6.7</td>
<td>43.3</td>
</tr>
<tr>
<td>Intervention Recheck 2</td>
<td>2.1</td>
<td>4.1</td>
<td>13.2</td>
</tr>
<tr>
<td>Intervention Recheck 3</td>
<td>2.6</td>
<td>4.7</td>
<td>13.3</td>
</tr>
</tbody>
</table>

Figure 5.3 Comparison of Bladder Compliance.
Control = Groups 1, 3, 5 and 7; Intervention = Groups 2, 4, 6 and 8.

88 JMP 11.0.0 (64-bit), 2013, SAS Institute Inc.
Following log transformation to mitigate the highly skewed data distribution, statistical analysis showed that neither group (Intervention versus Control) nor injury chronicity had a significant effect on compliance (Table 5.3). Baseline appeared to have a significant effect on bladder compliance, meaning that the dog’s bladder compliance at the beginning of the study significantly affected its value at subsequent rechecks. A mild study group effect may be present as $P = 0.095$; if it were, it would largely be explained by the unusually high or improved median compliance in Intervention Recheck 1 group, which was 6.7 ml/cmH$_2$O, while medians of the other seven groups ranged between 3.2 and 4.8 ml/cmH$_2$O (Table 5.2).

### Table 5.3 Statistical Summary of Log-transformed Chronicity-adjusted Compliance.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Coef</th>
<th>Std Err</th>
<th>z</th>
<th>$P&gt;[z]$</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Study Group</td>
<td>0.46</td>
<td>0.28</td>
<td>1.67</td>
<td>0.10</td>
<td>-0.08 – 1.00</td>
</tr>
<tr>
<td>Injury chronicity</td>
<td>0.01</td>
<td>0.01</td>
<td>1.13</td>
<td>0.26</td>
<td>-0.01 – 0.02</td>
</tr>
<tr>
<td>Baseline</td>
<td>0.44</td>
<td>0.10</td>
<td>4.25</td>
<td>0.00</td>
<td>0.24 – 0.65</td>
</tr>
</tbody>
</table>
Intravesical pressure (P\text{ves}) and bladder storage volume at urine leaking were measured for calculating compliance (i.e. compliance = ΔV/ΔPa). Shapiro-Wilk W\text{II} test showed that P\text{ves} data were not normally distributed in 3/8 groups: Control Baseline, Intervention Baseline and Intervention Recheck 1. The results were highly variable amongst all groups and their medians were 15.0 – 25.0 cmH\text{2}O; the range for the cohort was 1 – 50 cmH\text{2}O (Table 5.4, Figure 5.4).

<table>
<thead>
<tr>
<th>Group</th>
<th>Q25</th>
<th>Median</th>
<th>Q75</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control Baseline</td>
<td>7.0</td>
<td>18.0</td>
<td>28.5</td>
</tr>
<tr>
<td>Control Recheck 1</td>
<td>10.3</td>
<td>19.0</td>
<td>31.0</td>
</tr>
<tr>
<td>Control Recheck 2</td>
<td>12.0</td>
<td>21.0</td>
<td>28.0</td>
</tr>
<tr>
<td>Control Recheck 3</td>
<td>14.0</td>
<td>23.5</td>
<td>30.8</td>
</tr>
<tr>
<td>Intervention Baseline</td>
<td>13.3</td>
<td>17.0</td>
<td>29.5</td>
</tr>
<tr>
<td>Intervention Recheck 1</td>
<td>7.0</td>
<td>15.0</td>
<td>26.0</td>
</tr>
<tr>
<td>Intervention Recheck 2</td>
<td>11.5</td>
<td>19.0</td>
<td>34.5</td>
</tr>
<tr>
<td>Intervention Recheck 3</td>
<td>10.0</td>
<td>25.0</td>
<td>34.0</td>
</tr>
</tbody>
</table>

Table 5.4 Summary of Intravesical Pressure (cmH\text{2}O).

Figure 5.4 Comparison of Intravesical Pressure. Control = Groups 1, 3, 5 and 7; Intervention = Groups 2, 4, 6 and 8.

\text{II} JMP 11.0.0 (64-bit), 2013, SAS Institute Inc.
Storage capacity expressed as a percentage of the physiologic bladder capacity was compared across the eight groups. Shapiro-Wilk $W^{uu}$ test determined that all groups but Control Baseline, Recheck 1 and Recheck 2 were not normally distributed (Table 5.5, Figure 5.5). The group medians ranged from 46.3% to 71.3% and the storage capacity for the cohort ranged from 8.8% to 100%.

**Table 5.5 Summary of Bladder Storage Capacity (%).**

<table>
<thead>
<tr>
<th>Group</th>
<th>Q25</th>
<th>Median</th>
<th>Q75</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control Baseline</td>
<td>39.0</td>
<td>62.3</td>
<td>87.2</td>
</tr>
<tr>
<td>Control Recheck 1</td>
<td>40.8</td>
<td>65.1</td>
<td>93.5</td>
</tr>
<tr>
<td>Control Recheck 2</td>
<td>30.9</td>
<td>46.3</td>
<td>82.5</td>
</tr>
<tr>
<td>Control Recheck 3</td>
<td>24.1</td>
<td>55.5</td>
<td>80.9</td>
</tr>
<tr>
<td>Intervention Baseline</td>
<td>45.9</td>
<td>71.3</td>
<td>100.0</td>
</tr>
<tr>
<td>Intervention Recheck 1</td>
<td>31.2</td>
<td>63.3</td>
<td>100.0</td>
</tr>
<tr>
<td>Intervention Recheck 2</td>
<td>22.3</td>
<td>56.4</td>
<td>83.5</td>
</tr>
<tr>
<td>Intervention Recheck 3</td>
<td>38.2</td>
<td>61.6</td>
<td>92.7</td>
</tr>
</tbody>
</table>

**Figure 5.5 Comparison of Bladder Storage Capacity.**
Control = Groups 1, 3, 5 and 7; Intervention = Groups 2, 4, 6 and 8.

$^{uu}$ JMP 11.0.0 (64-bit), 2013, SAS Institute Inc.
Discussion

Urinary bladder compliance, the predefined outcome measure for autonomic function in this study, was unaffected by the administration of chondroitinase ABC and remained largely unchanged during the six month study participation period for the cohort. Similar to compliance, the two explanatory variables from which compliance was directly or indirectly derived, intravesical pressure and bladder storage capacity, were highly variable within the 60-dog cohort.

While compliance ranged from 0.4 to 339 ml/cmH$_2$O, the medians for all groups encompassing both experimental groups and all four time-points were within a narrow range between 3.2 and 6.7 ml/cmH$_2$O, which was consistent with our recent unpublished study on 84 severely thoracolumbar spinal-injured pet dogs. While data on normal bladder compliance in dogs is currently unavailable, in humans the range of 12.5 – 30 mL/cmH$_2$O has been used as the lower end of normal, with high variability amongst both spinal-intact and spinal-injured individuals$^{163–167}$.

Our data thus suggest that spinal-injured dogs tend to have reduced bladder compliance, likely caused by C-fiber-mediated detrusor contraction during bladder filling, or ‘detrusor overactivity’, that can lead decreased bladder storage capacity and increased intravesical pressure, the latter could at least in part be the result of detrusor-sphincter dyssynergia as the bladder is triggered to contract against closed sphincters during the bladder filling stage$^{155,159,161,163}$.

Indeed, when compared with conscious humans (since veterinary studies are currently unavailable) that have a intravesical pressure threshold of 12 – 32 cmH$_2$O before urination is triggered$^{155,160,161}$, the Q75 of all of our eight groups (Intervention and Control at the four time-points) were around 30 cmH$_2$O, or 26.0 – 34.5 cmH$_2$O, suggesting that ~ 25% of the 60-
dog cohort had intravesical pressure that exceeded what would be considered normal for humans. Further, during cytometry recording, the saline infusion was manually stopped in a small subset of animals in all eight groups because the intravesical pressure had reached 50 cmH$_2$O, or the established upper safety limit for intravesical pressure in humans and dogs$^{162–167}$.

Further, bladder storage capacity was consistently lower, with group means ranging between 46.3% and 71.3% of the expected storage capacity based on body weight in this cohort, further contributing to the overall reduced bladder compliance. One likely explanation would be the spontaneous detrusor contractions during bladder filling resulting in urine leakage or incontinence. Another possible explanation would be the presence of concurrent urinary tract infection, which spinal-injured humans and dogs are at high risks for developing, resulting in pollakiuria during the participant’s clinical trial visits when cystometric evaluation was performed.

Thus, the overall reduced bladder compliance, or a decreased capacity to accommodate volume expansion, in this 60-dog cohort in both Intervention and Control at all four time-points could be explained by the pathologic increase in intravesical pressure and decrease in bladder storage capacity as compliance measures the bladder’s response or resistance to volume expansion during filling, which is mathematically expressed as $\Delta V/\Delta Pa$. Further, in a small subset of animals, the bladder compliance could have been artificially elevated as the result of unresponsive, ‘over-stretched’ bladders (e.g. due to infrequent manual bladder expression) such that the intravesical pressure would remain low despite significant bladder expansion.

Additionally, the highly scattered distribution of data-points at all time-points in both Intervention and Control, characterized by large ranges and large inter-quartile ranges, might have rendered the detection of potential treatment effect more challenging due to the higher
probability of random errors. Although not statistically significant, study group (Intervention vs. Control) did appear to have a mild effect on compliance as $P = 0.095$, which may be more detectable in a larger and/or more homogenous population.

Our ability to detect chondroitinase ABC’s therapeutic effect, if any, in future investigations may hence be enhanced by selecting a more homogenous group of subjects based on, for instance, similar spinal cord injury severity, comparable injury chronicity or baseline compliance that falls within a narrower range. Alternatively, larger sample sizes could be used in future studies to identify potential subgroup(s) that may be more responsive to the therapeutic effect of chondroitinase ABC.

Further, Intervention Recheck 1 at one month post-injection had an unusually high or improved median compliance compared to Control, but returned to baseline level at Recheck 2 and remained low at Recheck 3. The current chondroitinase ABC formulation has a reported *in vivo* efficacy for up to six weeks. Our finding suggests that improvement in bladder compliance could possibly be sustained or even enhanced by repeat dosing after one month, or by extending the therapeutic life of a single delivery beyond one to three months by, for instance, using gene therapy and a lentiviral vector to transduce the host cells to express chondroitinase ABC.
CHAPTER 6
CLINICAL EVALUATIONS

Introduction

Objective outcome measures, as presented in Chapters 3 – 5, were used in the current 60-dog Phase II clinical trial for detecting change in neurologic function over six months primarily because of their higher degrees of reproducibility. Since Phase II trials are usually preliminary efficacy studies for detecting therapeutic and adverse effects of a novel intervention, less readily quantifiable clinical outcome measures can help further our understanding of chondroitinase ABC by providing detailed, individualized and clinically relevant data regarding its efficacy and safety.

Clinical evaluation made by the investigator and pet owners can be highly valuable in translational spinal cord injury research. Firstly, the neurologic function of a spinal-injured pet dog, such as ambulation, can vary substantially from day to day even in the chronic phase\(^{\text{vv}}\) of injury. Owners’ observations of functional changes at home would thus supplement in-hospital evaluations at a given time.

Secondly, some study-associated complications, such as urinary tract infections secondary to urinary catheterization for cystometric evaluation, may not be apparent during hospitalization. Thus, investigator would need to rely on the owners’ observations for estimating the true incidences of study-related events in the cohort.

Moreover, certain clinical parameters are highly relevant for spinal-injured pet dogs because: (1) some, such as deep pain, are widely accepted standards for assessing and

\(^{\text{vv}}\) Chronic phase typically begins ~ 3 months post-injury when the dog’s neurologic status plateaus. Spontaneous neurologic recovery is unlikely to occur at this point.
communicating the degree of neurologic impairment and treatment-related recovery amongst clinicians and investigators; (2) some, such as urinary continence, are functional parameters that owners highly value and (3) failure to regain these functions can lead to clinically-significant decisions such as euthanasia.

Additionally, potential adverse drug effects that are specific to intrathecal chondroitinase ABC administration, such as injection-site neuropathic pain as evidenced by hyperalgesia and allodynia that might occur secondary to glial scar digestion and neuroplasticity-induced aberrant axonal sprouting, can be detected by simple means including clinical examination and von Frey sensory testing.

We herein present the clinical evaluations made by the investigator and owners regarding the potential therapeutic and adverse effects of chondroitinase ABC, as well as the study-related complications. Subjectivity of these outcome measures were mitigated by providing clear definitions of specific outcomes, with the goal of enhancing inter-observer agreeability and experimental reproducibility.

Materials and Methods

Study approval, written consent, patient selection and chondroitintase ABC administration are outlined in Chapter 2. Assessments performed at pre-intervention Day 0 and Months 1, 3 and 6 included: (1) von Frey filament sensory testing as a mechanical nociceptive threshold test to detect abnormal sensation; (2) owner questioning regarding potential treatment effect, adverse reaction and study-related complications; (3) presence of deep pain in pelvic limbs; and (4) ability to ambulate.
Von Frey sensory test was performed on fully conscious dogs with minimal manual restraint in a familiar, quiet room with little distraction by a blinded investigator. The test was performed the day before injection to obtain a baseline, the morning after injection, and at three rechecks. Each of the 17 filaments was applied one at a time perpendicular to the dog’s dorsum, one side at a time ~ 2 cm lateral to the dorsal midline, until the filament slightly deformed.

The filaments were applied starting from the level of L5–L6 dorsal spinous processes and were moved cranially one vertebra at a time up to the level of T6–T7, which was > 2 vertebrae cranial to the site of injury or injection. A response was considered ‘abnormal’ if the dog showed any reaction suggestive of discomfort or pain, including vocalization, exaggerated movement or aggression in response to the stimulus. A response was considered ‘present’ if the dog turned around to look or exhibited panniculus reflex.

The number of positive response on both sides were added to obtain the total for each animal.

Owner questioning was done during their pet’s clinical trial participation and after completion. At Month 1, 3 and 6 rechecks, each blinded owner was asked by the blinded investigator ‘Have you noticed any problems since the last visit?’ and ‘Have you noticed any changes since the last visit?’. At the last visit after all relevant data had been collected, the blinded owner was asked ‘Do you think your pet received the injection six months ago?’. Statistical analysis was done by first scoring if the owners had correctly guessed the group assignment, then comparing the score with random probability, or guessing correctly 50% of the time, using Fisher’s exact test.

Post-study follow-up for the first 42 participants was done six months after their last visits via email with owners and, due to thesis deadline, up to five months after their last

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ww Hilary Hu BSc BVSc
xx Panniculus reflex is observed as a brief paraspinal skin twitch mediated by cutaneous trunci muscles in response to a sensory stimulus.
yy Stata 11 Data Analysis & Statistical Software, StataCorp LP, College Station, TX.
visits for the last 18 participants. Each owner was asked ‘*Have you noticed any problems since completion of the clinical trial?’* and ‘*Have you noticed any changes since completion of the clinical trial?’*.

Pelvic limb deep pain perception was tested on fully conscious dogs with minimal manual restraint in a familiar, quiet room with minimal distraction by firmly crushing the nail bed and digit with forceps in dogs < 10 kg or pliers in dogs 10 – 20 kg\(^{87-89}\). Deep pain was considered ‘present’ if the dog repeatedly vocalized and/or turned his/her head towards or attempted to bite the source of stimulus.

Ambulation was assessed by observing the dog’s ability to walk on non-slippery surfaces such as grass or carpet. A dog was considered ‘ambulatory’ if he/she was able to walk ten consecutive steps unaided without falling or the lateral aspect of the foot or metatarsus touching the ground.

**Results**

Sixty dogs were recruited but results were not available from all at all time-points due to patient dropout and missed rechecks (Table 6.1). Pre-intervention baseline data were collected at Day 0 from 60/60 between Intervention and Control; at Month 1, data were available from 58/60; at Month 3, from 55/60; and at Month 6, from 52/60. General summary of Intervention and Control are reported in Chapter 2.

<table>
<thead>
<tr>
<th>Time/Group</th>
<th>Intervention</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline</td>
<td>30</td>
<td>30</td>
</tr>
<tr>
<td>Recheck 1</td>
<td>29</td>
<td>29</td>
</tr>
<tr>
<td>Recheck 2</td>
<td>27</td>
<td>28</td>
</tr>
<tr>
<td>Recheck 3</td>
<td>27</td>
<td>25</td>
</tr>
</tbody>
</table>

Table 6.1 Data Availability for Clinical Evaluation.
No abnormal response suggestive of neuropathic pain was detected with Von Frey in the cohort. The total number of response was the highest in both Control and Intervention shortly (12 – 24 hours) following injection, but decreased to sub-baseline levels by Recheck 1 and remained low until study completion (Table 6.2, Figure 6.1). Shapiro-Wilk W\textsuperscript{zz} test showed that none of the groups was normally distributed. Total response were 0 – 32 for the cohort; while medians for all groups were 0.

Table 6.2 Von Frey Sensory Test Summary.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Q25</th>
<th>Median</th>
<th>Q75</th>
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</thead>
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<tr>
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<td>0</td>
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<td>3</td>
</tr>
<tr>
<td>Control B2</td>
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<td>0</td>
<td>6</td>
</tr>
<tr>
<td>Control R1</td>
<td>0</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>Control R2</td>
<td>0</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>Control R3</td>
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<td>0</td>
<td>6</td>
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<tr>
<td>Intervention B1</td>
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<td>0</td>
<td>4</td>
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<tr>
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<td>0</td>
<td>0</td>
<td>8</td>
</tr>
<tr>
<td>Intervention R1</td>
<td>0</td>
<td>0</td>
<td>1</td>
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<tr>
<td>Intervention R2</td>
<td>0</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Intervention R3</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Figure 6.1 Comparison of Number of Von Frey Response.
Control = Groups 1, 3, 5, 7 and 9; Intervention = Groups 2, 4, 6, 8 and 10.

\textsuperscript{zz} JMP 11.0.0 (64-bit), 2013, SAS Institute Inc.

\textsuperscript{aaa} B1 = baseline before injection; B2 = baseline after injection; R = recheck.
Ambulation was evaluated at rechecks and compared with Baseline, where 4/30 of Control and 5/30 Intervention were ambulatory (Figure 6.2). At Recheck 1, 4/30 Control and 8/29 Intervention were ambulatory. By Recheck 2, 4/28 Control and 8/27 Intervention were ambulatory. At Recheck 3, the numbers were 4/25 and 8/27 for Control and Intervention, respectively. In total, three participants regained ambulation during the study, all from Intervention.

Deep pain perception was evaluated at each recheck and compared with the baseline, where 2/30 of Control and 4/30 Intervention had deep pain (Figure 6.3). At Recheck 1, 5/30 Control and 4/29 Intervention participants were deep pain-positive. By Recheck 2, 5/28 Control and 4/27 Intervention were deep-pain positive. At Recheck 3, the numbers were 6/25 and 5/27 for Control and Intervention. In total, six participants regained deep pain during the study, of which, four were from Control.
Owners were questioned regarding observable changes and/or problems since the previous visit (Figure 6.4). For Intervention, owners reported changes in 19/29, 12/27 and 12/27 participants at Rechecks 1, 2 and 3, respectively. Similarly, 21/29, 15/28 and 10/25 owners whose pets had been randomized to Control reported positive findings at the three rechecks. Main changes reported included: increased pelvic limb movement when on the floor and/or in their carts, improved ability to ambulate and stand and remain standing on their pelvic limbs, increased tail movement and more responsive to touch in the caudal half of the body including the pelvic limbs.
Owners were also asked about their observation of any problems associated with or since the previous visit (Figure 6.5). For Intervention, owners reported problems in 8/30, 3/29, 2/27 and 0/27 of the participants at Baseline and Rechecks 1, 2 and 3, respectively; while 1/30, 2/29, 5/28 and 3/25 owners whose pets had been randomized to Control reported problems at these four time-points. Problems mainly included diarrhea, urinary tract infection, reduced mobility for 1 – 3 days, skin ulceration and sensitivity to back palpation.

![Figure 6.5 Owner Questioning Regarding Problems.](image)

Forty-two of the 60 owners were asked at the end of the study following data collection but before unblinding to speculate which experimental group their dog had been assigned six months prior. For Control, 6/22 owners believed that their pets had been assigned to Intervention, while 11/20 of Intervention owners did (Figure 6.6). Owners correctly guessed group assignment in 27/42 or 64.3% of the cases and were incorrect in
15/42 or 35.7%; statistical analysis\textsuperscript{bbb} using Fisher’s exact test showed that, with $P = 0.34$, this was not significantly better than random probability of 50%.

![Figure 6.6 Owner Speculation of Group Assignment.](image)

Lastly, of the 52 participants that had completed the study, post-study follow-up data were available from 48, 24 from either group. None of the owners observed any problems since study completion and 10/24 or 42% owners of Intervention reported continued changes, which mainly included increased attempts and duration of weight-bearing and ambulation on their pelvic limbs and improved urinary continence; while 14/24 or 56% owners of Control that received chondroitinase ABC after study completion reported changes, which included increased attempts and duration of weight-bearing and ambulation on pelvic limbs.

\textsuperscript{bbb} Statistical analysis was performed by Dr. Nick Jeffery using Stata 11 Data Analysis & Statistical Software, StataCorp LP, College Station, TX.
Discussion

Clinical evaluation made by the investigator and owners suggested that while chondroitinase ABC had no clinically detectable adverse effects, it might have some detectable therapeutic effect in a subgroup of study subjects. As evidenced, neither observational evaluation nor von Frey sensory test identified evidence indicative of pain or discomfort associated with intrathecal chondroitinase ABC administration.

However, despite the lack of evidence for neuropathic pain, positive von Frey response peaked in Control and Intervention 12 – 24 hours post-injection, but declined to sub-baseline levels by Month 1 and remained low. One possible explanation for this surge of positive von Frey response, which coincided with an increased number of owner-reported problems following chondroitinase ABC injection at Day 0, would be that the intrathecal drug delivery could have caused some minor damage to the soft tissues at/near the injection sites.

Another potential cause of increased sensitivity to mechanical stimulation delivered by the von Frey filaments would be the surgical preparation protocol for the intrathecal injection, which involved processes that might have irritated the skin, such as hair clipping and surgical scrubbing.

Evidence supportive of the detectable therapeutic effect of chondroitinase ABC, on the other hand, was equivocal as while the owners were unable to correctly surmise which experimental group their pet had been assigned six months prior, twice as many Intervention owners thought their pet had received the injection compared with Control. Further, three dogs in Intervention regained ambulation during the six-month participation period, while none of the Control did.
Statistical analysis was not performed in this case because of the small number of participants that qualified as ‘ambulatory’. However, upon closer examination of these three dogs (Dogs 23, 28 and 34) that regained ambulation during the study, it appears that they possessed overall superior neurologic function than the rest of the cohort at the time of study enrolment.

Dogs 28 and 34 had only been paralyzed for four to five months at the time of enrolment and had intact deep pain and some motor function in their pelvic limbs, implying functionally incomplete or partially recovered spinal cord injuries. Dog 23, albeit remained deep pain negative during the study and had been non-ambulatory for 24 months at the time of enrolment, had remarkable pelvic limb stepping reflexes and was already able to weight-bear and walk several steps on her pelvic limbs.

Therefore, it is possible that chondroitinase ABC may benefit a certain subgroup, namely, individuals that have incomplete or partially recovered spinal cord injuries and/or a certain degree of pelvic limb motor function, either mediated by supra-spinal input (i.e. Dogs 28 and 34 that had intact deep pain) or segmental spinal cord reflex (i.e. Dog 23 that was deep pain negative but had strong reflex stepping).

Further, all three dogs were small breed that weighted 4.2 – 6.0 kg; their small body size could have made it easier for them to support most of their body weight with their thoracic limbs and thus rendered them more likely to ambulate and receive afferent stimulation via their pelvic limbs to facilitate reflex-mediated stepping through mechanisms similar to those reported in experimental dogs and cats that are deprived of normal supraspinal locomotor control, but can be trained and/or stimulated to repetitively step and ambulate \(^{111-113,119-125}\).

Additionally, all three Intervention dogs regained ambulation one month post-injection, which coincided with the temporary improvement in bladder compliance. Thus, it
can be deduced that the possible therapeutic effect would occur Day 0 – Month 1, and improvement in neurologic function could be sustained or even enhanced by repeat dosing after one month, or by extending the therapeutic life of a single delivery beyond one to three months by, for instance, using gene therapy and a lentiviral vector to transduce the host cells to express chondroitinase ABC76.

Interestingly, while three Intervention participants regained ambulation during the six-month study participation period, two Intervention and four Control participants regained deep pain perception over the same period. Since all participants were enrolled > 3 months post-injury, or during the chronic injury phase when spontaneous neurologic recovery is unlikely to occur, our results suggest that continued neurologic recovery is possible beyond the first three months of injury in a subset of patients, with or without chondroitinase ABC.

One possible explanation for the clinical improvement during the chronic phase would be the benefit of rehabilitation therapy, which the study participants underwent during each of their weeklong hospital visit. In experimentally spinal-injured rats, specific rehabilitation regimens, either administered alone or in conjunction with chondroitinase ABC, can improve motor outcomes68. While its benefit in chronically spinal-injured dogs is unknown, rehabilitation therapy can provide sensory stimulation that promotes segmental central pattern generator-mediated reflexes that trigger stepping and ambulation111 – 114,119 – 125.

Lastly, while statistical analysis indicated that the accuracy of owners’ guesses were no better than random probability, twice as many Intervention owners speculated that their pets had received the chondroitinase ABC than Control owners, or 55% versus 27%. The relatively small sample size of 48 could partially account for the lack of detection of statistical significance. It is also possible that there was a small, more treatment-responsive
subgroup in Intervention, in which case a larger study may be warranted to detect chondroitinase ABC’s treatment effect, if any, in a subpopulation.
The current Phase II double-blinded randomized controlled chondroitinase ABC clinical trial in 60 severely thoracolumbar spinal-injured pet dogs failed to demonstrate a statistically significant group treatment effect, pre-defined as 25% dogs in Intervention and 0% dogs in Control recovering thoracic-pelvic limb gait coordination measured by phase shift by Month 6. Secondary parameters, including motor-evoked and sensory-evoked potentials and bladder compliance, also detected no significant treatment effect of this putative drug for spinal cord injury.

The lack of detectable therapeutic effect of chondroitinase ABC in the current study could be multi-factorial. Firstly, since this was the first study in a large animal model, the dose of chondroitinase ABC had been unknown; therefore, it is possible that the dose administered was sub-therapeutic. The lack of report of potential side effects and adverse reactions in Intervention would indirectly support this speculation.

Secondly, intrathecal chondroitinase ABC administration was very challenging at times, especially in the study participants that had, presumably, injury- or surgery-induced soft tissue fibrosis and osseous proliferation at and/or near the injection sites, hindering the precision of spinal needle placement for full-dose drug delivery despite the use to fluoroscopy-guided spinal needle placement technique.

Due to the clinical nature of these cases, one significant limitation of this study was that labelling and tracing could not be practically implemented to confirm successful drug delivery; for instance, was the full dose actually administered into the spinal cord
parenchyma or was it only injected into the cystic cavity or syrinx, which would probably just ‘leak out’ as soon as the spinal needle was withdrawn?

Further, albeit we used a thermo-stabilized, slow-release formulation of chondroitinase ABC that is shown in vivo to sustain its chondroitin sulfate proteoglycan-suppressive effect for up to 6 weeks post-treatment, the duration of drug effect in the current large animal model could not easily be confirmed by means such as in vivo labelling and harvesting spinal cord samples for examination.

On the other hand, clinical evaluations, that were focused on detecting clinically observable treatment effect and adverse effects, suggested that chondroitinase ABC is a very safe agent when delivered intrathecally with fluoroscopy guidance at the current dose. Conversely, one could argue that the dose used in the current study might have been sub-therapeutic, thus, at least in part, account for the lack of detectable treatment effect.

In addition to dose adjustment, challenges associated with the current method of drug administration should be addressed in future investigations to ensure more accurate delivery of the drug at its intended dose. One alternative would be to administer chondroitinase ABC during the acute phase of spinal cord injury, such as intra-operatively. Immediate drug delivery may also be more efficacious as most experimental animal models that report measurable therapeutic effect involve drug delivery during the acute phase of spinal cord injury.

While the power calculation was intended to detect a treatment effect of 25% improvement in thoracic-pelvic limb phase shift, it is possible that a smaller treatment effect could exist but would require a larger sample size for its statistical detection. A larger sample size may also assist with the identification of potential subgroups differentiated by clinical parameters such as injury severity and chronicity, which may be more responsive to the treatment effect of chondroitinase ABC.
Indeed, there was some evidence in the current study that would support the possible existence of more treatment-responsive subgroups as three Intervention dogs regained ambulation during the study participation period; all three possessed superior levels of motor function, suggestive of functionally incomplete or partially recovered and/or recovering spinal cord injuries, compared to the rest of the cohort when they entered the study.

Further, the permanent improvement in motor function temporally coincided with the temporary improvement in bladder compliance in Intervention one month post-treatment. It can thus be deduced that the possible therapeutic effect would likely occur Day 0 – Month 1, and that the neurologic improvement could be sustained or even enhanced by repeat dosing after one month, or by extending the therapeutic life of a single delivery by, for instance, utilizing novel means such as gene therapy.76.

In conclusion, while our study failed to detect a therapeutic effect in chronic, severe thoracolumbar spinal-injured dogs, it has established drug safety in a clinical large animal model for traumatic spinal cord injury. Thus, this study has provided a platform for future investigations in which the dose, route and timing of chondroitinase ABC administration, as well as patient selection, can be adjusted to maximize its potential therapeutic effect and benefit spinal-injured human and veterinary patients. Identification of more treatment-responsive subgroups in future studies would assist with the development of personalized treatment, rendering the successful clinical translation of putative spinal cord injury intervention more likely.
REFERENCES


APPENDIX A [CLINICAL TRIAL BROCHURE]

Clinical Trial Summary

- **Enrollment:** Now
- **Participation:** 6 Months
- **Duration:** 2014 - 2016
- **Species:** Canine
- **Condition:** Spinal cord injury
- **Treatment:** Chondroitinase
- **Sponsor:** International Spinal Research Trust (ISR T)
- **Cost:** $100 (administration)
- **Benefits:**
  - Free rehab
  - Free evaluation
  - Free hospitalization
  - Free cart adjustments

Background

Spinal cord injuries have devastating impacts on our veterinary patients and their families as they can lead to paralysis and loss of urinary control. This is largely because the spinal cord does not regenerate effectively.

Following injury, the nerves on two sides of the injured area are not able to communicate properly. This means that the brain is not able to tell the legs what to do and the legs are not able to tell the brain where they are and what they are doing. The same problem happens with the bladder and bowels, and this can result in incontinence.

The purpose of our clinical trial is to test the effectiveness of a potential drug, chondroitinase, in helping paralyzed dogs, and hopefully one day paralyzed people, regain spinal cord functions after severe injuries.

During our clinical trial, we will inject chondroitinase into the spinal cord and then perform various tests over the following six months to assess neurological functions - e.g. gait, bladder function, nerve conduction.

Fortunately, there have been no reports of damaging effects of this potential treatment; and we have not seen any side effects or adverse reactions in our patients since we started the clinical trial in 2014.
APPENDIX B [CHONDROITINASE ABC DOSING CALCULATIONS]

Prepping Microtubes

**General Concept:**

Dry microtubes can be loaded simply by resuspending in the chABC solution. The loaded concentration is equivalent to the concentration of the chABC solution with a loading volume of 7.5ul per 10mg of lipid (1 tube). After this, delivery of drug is thought to be entirely diffusion limited. It is possible to remove unloaded solution (centrifugation) or simply inject with loaded and unloaded drug (equivalent to including a bolus injection of free drug at the same time as delivering microtubes). This is beneficial when the injury is not new, and there may already be a build up for a scar (the current experiment).

**Protocol Design Considerations:**

- We want to initially resuspend in a high concentration of chABC solution to get maximal loading,
- Minimum resuspension volume is ~40ul/mg lipid; (this leads to a large amount of chABC wasted)
- Attempts to aliquot microtubes into smaller containers prior to lyophilization has (as of yet) proved unsuccessful so we are stuck at 10mg/tube.
- **Lipid microtubes begin releasing chABC immediately so should be prepped as closely as possible to the surgery date,** or kept in the high concentration solution until immediately before injection so there is no concentration gradient to drive diffusion.

**Detailed Protocol:**

**Supplies:**

- 10 mg Tube of Microtubes
- 1 5U Bottle of chABC
- Sterile 38% Trehalose Buffer (can be re-sterilized by syringe filter)

**Equipment:**

- Micropipettes + Tips
- Sterile Laminar Flood Hood (if possible)
- Needles/Syringes
- Sterile Eppendorf Tubes (0.5 ml preferred)
**Prepping ChABC:**

1. Thaw chABC (preferably in a dehumidying chamber) ~ 15 minutes prior to starting
2. In laminar flow hood, inject 800ul 38% Trehalose Buffer into chABC bottle using a sterile syringe.
3. Mix thoroughly by shaking/swishing chABC bottle until all white power is dissolved. (TIP: MAKE SURE TO TURN BOTTLE UPSIDE DOWN TO DISSOLVE chABC that stick to the lid).
4. Remove stopper and aliquot using sterile pipettes into 2 400ul aliquots.
5. Store these at -80°C or -20°C until needed.

**Resuspending Microtubes**  (Night before surgery --or possibly slightly before that).
1. Thaw 1 chABC aliquot.
2. In hood: add 400ul of chABC stock solution to 1 tube of 10mg lipid microtubes.
3. Mix tubes by gentle pipetting up and down with a P1000 micropipettes (TIP: Best done if the very tip of the pipette is cut off so the hole is bigger)
4. Store overnight at 4°C

**Aliquot Microtubes**  (aliquots at the high concentration will minimize loss of loaded drug).
1. In laminar hood, aliquot 4 X 100 ul of the microtubes/chABC solution.
2. These can be stored for a maximum of 2 weeks at 4°C for each dog.

**Day of Surgery: (2 options)**

*High Initial Release (Bolus Injection)*
1. Inject the 100ul as desired using the smallest gauge needle possible (18 or 20; largest internal diameter to minimize breaking the tubes).
   1b. can also increase the volume/reduce viscosity if desired using additional sterile trehalose solution

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
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<tbody>
<tr>
<td>Total chABC Delivered</td>
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<tr>
<td>Total Loaded chABC</td>
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</tr>
<tr>
<td>Total Free chABC</td>
<td>~613 mU</td>
</tr>
</tbody>
</table>

This can be altered by further dilution because will reduce viscosity.

*Lower Initial Release*
1. In hood, add extra trehalose buffer (300ul).
2. Centrifuge at 1500rpm for 10 min.
3. In hood, remove supernatant and add sterile trehalose solution up desired injection volume. (exact amount of free chABC will depend on how effectively the supernatant can be removed).

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</thead>
<tbody>
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</tr>
<tr>
<td>Total Free chABC</td>
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</table>

** assuming 100% removal of supernatant which is not physically reasonable

<table>
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<th>Total chABC Delivered**</th>
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<td>Loaded chABC</td>
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<tr>
<td>Free chABC</td>
<td>~153mU</td>
</tr>
</tbody>
</table>

** assuming 75% removal of supernatant