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Therapeutic efficacy of microtube-embedded chondroitinase ABC in a canine clinical model of spinal cord injury

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Abstract

Many hundreds of thousands of people around the world are living with the long-term consequences of spinal cord injury and they need effective new therapies. Laboratory research in experimental animals has identified a large number of potentially translatable interventions but transition to the clinic is not straightforward. Further evidence of efficacy in more clinically-relevant lesions is required to gain sufficient confidence to commence human clinical trials. Of the many therapeutic candidates currently available, intraspinally applied chondroitinase ABC has particularly well-documented efficacy in experimental animals. In this study we measured the effects of this intervention in a double-blinded randomized controlled trial in a cohort of dogs with naturally-occurring severe chronic spinal cord injuries that model the condition in humans. First, we collected baseline data on a series of outcomes: forelimb-hindlimb coordination (the pre-specified primary outcome measure), skin sensitivity along the back, somatosensory evoked and transcranial magnetic motor evoked potentials and cystometry in 60 dogs with thoracolumbar lesions. Dogs were then randomized 1:1 to receive intraspinal injections of heat-stabilized, lipid microtube-embedded chondroitinase ABC or sham injections consisting of needle puncture of the skin. Outcome data were measured at 1, 3 and 6 months after intervention; skin sensitivity was also measured 24 hours after injection (or sham). Fore-hind coordination was affected by neither time nor chondroitinase treatment alone but there was a significant interaction between these variables such that coordination between forelimb and hindlimb stepping improved during the 6-month follow-up period in the chondroitinase-treated animals by a mean of 23%, but did not change in controls. Three dogs (10%) in the chondroitinase group also recovered the ability to ambulate without assistance. Sensitivity of the dorsal skin increased at 24 hours after intervention in both groups but subsequently decreased to normal levels. Cystometry identified a non-significant improvement of bladder compliance at 1 month in the chondroitinase-injected dogs but this did not persist. There were no overall differences between groups in detection of sensory evoked potentials. Our results strongly support a beneficial effect of intraspinal injection of chondroitinase ABC on spinal cord function in this highly clinically-relevant model of chronic severe spinal cord injury. There was no evidence of long-term adverse effects associated with this intervention. We therefore conclude that this study provides strong evidence in support of initiation of clinical trials of chondroitinase ABC in people with chronic spinal cord injury.

Keywords: glial scar, chondroitin sulfate proteoglycan, translation

Introduction

During the past two decades many interventions have successfully improved functional and histological outcome measures in animals with experimental spinal cord injury (Kwon *et al*., 2011; Tetzlaff *et al*., 2011). In contrast, this research has not yet delivered an indisputably effective treatment for human patients. Achievement of this underlying objective is impeded, in part at least, because the many differences between clinical spinal cord injury in humans and traditional experimental animal models mean that statistical improvement in a laboratory model does not imply that there will also be similarly meaningful benefit in clinical injuries (Kwon *et al*., 2015).

Most critically, laboratory rats commonly used in spinal cord injury studies are young, genetically near-identical and their experimental injuries are homogenous in character and severity. Such homogeneity is desirable in the laboratory because it enables the signal of the investigated intervention to be discerned amongst the noise of other variables that might influence outcome. In contrast, human spinal cord injury patients and their injuries are highly heterogeneous - even within clinical sub-categories there is a great deal of variation in demographic features, co-morbidities and outcome (Fawcett *et al*., 2007) - which means that the functional benefit that might be associated with a therapeutic intervention is less easily recognized. On the other hand, unless an intervention is sufficiently effective to make a substantial change in the lives of individual patients, for instance by altering their dependency on others for care, then it will not become adopted as a worthwhile clinical intervention.

Pet dogs frequently suffer acute spinal cord injury (Moore *et al*., 2017) and these dogs undergo similar diagnostic, surgical and rehabilitation procedures to their human counterparts. Also similar to human patients, some will fail to recover with conventional therapy alone. This leaves a large population of chronically-injured dogs for which there is no available effective therapy and that can serve as a spontaneous model for testing therapies thought to have promise for translation from laboratory to clinic. Lesions in these dogs (Griffiths, 1972; Smith & Jeffery, 2006; Levine *et al.*,

2011) closely model many features of chronic spinal cord injury in humans. Such a translational model is difficult to replicate in laboratory animals.

There are many interventions that could be suitable for testing in this canine model of chronic spinal cord injury – specifically, those that have undergone repeated successful testing in experimental animals in multiple laboratories throughout the world. In this study we selected chondroitinase ABC, which has been demonstrated to improve outcome in numerous experiments on spinal cord-injured rodents (Bradbury *et al*., 2002; Bartus *et al*., 2014), cats (Jefferson *et al*., 2011) and non-human primates (Bowes *et al*., 2012). Chondroitinase ABC is a bacterial enzyme that can digest the chondroitin sulfate proteoglycans that constitute a major part of the scar that forms in spinal cord lesions and blocks axonal regeneration (Bradbury and Carter, 2011). Current obstacles to translation of this agent into human spinal cord injury patients are: i) the need for a formulation with stability at mammalian body temperature so as to provide persistent activity without the need for repeated administration (see Bradbury and Carter, 2011); and, ii) the need to demonstrate efficacy and safety in realistic translational models. The first obstacle can be overcome by buffering in trehalose and embedding in lipid microtubes which, together, render chondoitinase ABC heat-stable and long-acting (Lee *et al*., 2010). Here we addressed the second obstacle by conducting a randomized controlled clinical trial to measure the effects of lipid microtube-embedded chondroitinase ABC in dogs with severe chronic clinical spinal cord injury; this could be considered a final prelude to commencement of formal regulatory approval processes for translation into similarly-injured humans.

Materials and methods

Methods

The study design, primary and secondary outcomes measures and analytical methods were all pre-specified and carried out in accordance with the submitted funding proposal (held by the sponsor, the International Spinal Research Trust). The pre-specified primary outcome measure was a measure of temporal coordination between forelimb and hindlimb motion that we have previously described (Hamilton *et al.*, 2007); further details of the methods are available below and in

Supplementary Material. All procedures and the trial design were approved by the Institutional Animal Care and Use Committee at Iowa State University (Log number: 3-13-7526-K).

Animals

We aimed to recruit a sample size of 60 (see sample size calculations below) dogs weighing less than 20 kg and with chronic severe spinal cord injury confined between T3 and L3 vertebrae; dogs have 13 thoracic and 7 lumbar vertebrae. For inclusion, dogs had to have persistent loss of urinary continence and voluntary motor function in the hindlimbs following an acute spinal cord injury occurring at least 3 months before recruitment. Most of these dogs had suffered acute intervertebral disc herniation, which is common in small dogs (Moore *et al*., 2017). Typical cases had no voluntary motor function in the hindlimbs, no discernible sensory function to any part of the hindquarters (including the tail) and were both urinary and fecally incontinent. Dogs were excluded from the study if they had lesions affecting the lumbosacral intumescence (L4 to S3 spinal cord segments), had concurrent orthopedic disorders that would preclude recovery of walking, or had any condition from which they were expected to die within 1 year. Dogs that were too aggressive or anxious to be controlled when they walked on a treadmill were excluded.

Materials

Preparation of chondroitinase ABC

Chondroitinase ABC was obtained from a commercial supplier (AMSBIO) as a lyophilized powder in a sterile ampoule (see Supplementary Material). The powder was reconstituted in filter-sterilized 38% trehalose solution (10 Units per 1600 μL trehalose solution), which was divided into 400 μL aliquots that were kept frozen at -80 ^{o}C until mixed with the lipid microtubes. The lipid microtubes were prepared according to the previously published protocol (Lee *et al*., 2010; see Supplementary Material). On the day before intraspinal injection, a stock 400 μL aliquot of reconstituted chondroitinase ABC in 38% trehalose was thawed and mixed with one batch of microtubes until it formed a homogenous milky suspension; this was then stored overnight at 4 ^{o}C to allow adsorption of the chondroitinase ABC solution onto the microtubes. Each dog received 200 μL of the trehalose/microtube suspension re-diluted in a further 200 μL of 38% trehalose solution immediately before it was injected into the spinal cord. The total 400 μL suspension (containing 1.25 Units of chondroitinase) was divided into an injection of 200 μL (625 mU) at

each injected site; each of these injections was administered in two aliquots, with the needle bevel facing caudally and cranially respectively (see below). The dose was selected based on 'scaling up' calculations from rodent experiments as described in Supplementary Material.

Procedures

Pre-study

Each dog underwent neurological examination to confirm the site and severity of the lesion. This included routine examination of the level of the injury through assessment of the *cutaneous trunci* muscle reflex (Gutierrez-Quintana *et al.*, 2012). After the neurologic examination and obtaining written informed consent from the owners, dogs were formally admitted to the trial.

Each dog then underwent a series of baseline functional tests, including analysis of coordination of gait during treadmill walking, von Frey filament testing of skin sensitivity, cystometry and electrophysiological recordings. On the fourth day of hospitalization each dog was randomized to receive either a percutaneous intraspinal injection of chondroitinase ABC or to undergo needle puncture of the dorsal skin (so as to blind the observer and owner regarding treatment allocation). Allocation was equal between groups and determined by opening the next in a numbered series of sealed opaque envelopes each containing a slip of paper labeled 'ChAse' or 'Control'. These were prepared in batches of 20; the batching method was not known to the observer who recorded the functional outcomes.

Study procedures

Treadmill gait recordings were made similarly to previous reports (Hamilton *et al*., 2007; Granger *et al*., 2012). Briefly, each dog was walked at constant speed on a treadmill while held on a leash. The hindquarters were supported by a sling placed under the abdomen to maintain the vertebral column in a normal walking position parallel to the treadmill belt. Reflective markers were placed on the lateral aspect of each paw and both elbows and their motion was recorded by the Vicon infra-red motion analysis system. The primary outcome measure was temporal coordination between each fore paw and the contralateral hind paw strike (*i.e.* diagonal coupling). The mean value for coupling of both right and left forelimbs with their diagonal pairs was used for the final statistical analysis. More details are provided in Supplementary Material.

Von Frey filaments assessed skin sensitivity before and after chondroitinase ABC injection or sham treatment. At each time point the von Frey filaments were applied to the skin on both sides of the dorsal aspect of each dog starting at the level of L6 vertebra and progressing cranially in steps corresponding to the length of one vertebra up to the scapulae (the region of T6 vertebra). A positive response was defined as a behavioral response suggestive of cranial perception of the stimulus (whether noxious or non-noxious). The sum total number of positive responses at each time point was used for analysis.

Cystometry was used similarly to a previous report (Granger *et al*., 2012) to determine the compliance of the bladder during filling with room-temperature sterile 0.9% saline solution as is routine in human patients (Biering- Sørensen *et al*., 2008). Briefly, the bladder was catheterized and then filled at a rate of 10 mL/minute for dogs <10 kg and 20 mL/min for dogs >10 kg, while measuring the bladder pressure. The end-point was detrusor contraction and (partial) bladder voiding or an intravesicular pressure of 40 cmH_2O (because pressures higher than this can risk damage to the ureters and kidneys).

Transcranial magnetic evoked motor potentials were obtained with dogs under sedation with butorphanol (0.2 mg/kg) and dexmedetomidine (5 μg/kg), as described previously (Sylvestre *et al*., 1993; da Costa *et al*., 2006; Granger *et al*., 2012). Briefly, a 90 mm single coil powered by a current generator (Magstim 200, Wales, UK) was positioned tangentially over the skull (lateral and rostral to the vertex and 2 cm from midline) and discharged at 80% maximum power (~2 T on the skull surface, see Nollet *et al.*, 2003) while recording the latencies of the evoked compound muscle action potentials in the *cranial tibialis* and *extensor carpi radialis* muscles using concentric recording needles. [The *extensor carpi radialis* was used as a control for the sedation level because excessive sedation can eliminate this motor potential in normal limbs.] The test was repeated three times for each hindlimb (*i.e*. stimulation was directed at each side of the brain in turn) after we had obtained a positive response from the forelimb. We recorded the latency and amplitude of the last wave of the series; only waves of amplitude greater than 0.15 mV were considered a positive response.

Sensory evoked potentials were recorded using a monopolar needle electrode placed percutaneously to lie on the laminae of the thoracolumbar vertebrae or the interarcuate ligament, during stimulation of the tibial nerve just proximal to the hock (ankle) joint, with the subcutaneous reference electrode placed ~2 cm laterally, as previously described (Poncelet *et al*., 1993). The stimulus intensity was set to be just sufficient to evoke an observable response in the distal musculature. We recorded the latency and amplitude of this wave at each vertebral level from L5 moving cranially until a response could not be detected. Sensory evoked potentials were designated as 'intact' if the same waveform, with a peak-to-peak amplitude of >0.15 μV could be repeated at least once during signal averaging of at least 200 sweeps. Each tibial nerve were stimulated and recorded individually and the site of the most cranial intact response was used for subsequent analysis.

Intraspinal injection

Under general anesthesia each dog was positioned for fluoroscopy in right lateral recumbency so that one 22 Gauge, 1.5 or 2.5 inch, spinal needle could be placed into the lesion epicenter and another spinal needle placed into the spinal cord at the L3/4 vertebral interspace (the cranial margin of the spinal cord segments containing the lower motor neurons of the central pattern generator for the hindlimbs). If the primary lesion was at L3/4 then the L4/5 site was also injected. Each needle was initially placed midline into the subarachnoid space so that cerebrospinal fluid flowed from the hub and then repositioned so that the bevel would lie within the spinal cord parenchyma. Injections were made using a 1 mL Luer lock syringe with the needle bevel in the center of the spinal cord parenchyma - a depth of approximately 3 mm from the dorsal dura. A total of 200 μL chondroitinase preparation, divided into two aliquots, was injected at each site; one aliquot was injected with the bevel facing cranially and one with it facing caudally. Each 100 μLaliquot injection was timed to be completed within at least 120 seconds. The volume of injection is discussed in Supplementary Material.

Follow-up protocol

The functional tests were repeated by an observer blinded to treatment allocation at 1, 3 and 6 months after injection of chondroitinase (or skin puncture control); von Frey filament testing was also repeated at 24 hours after intervention. At each re-visit, each owner was interviewed with a

specific set of questions about changes in their dog's general health, behavior, locomotor and bladder function and then each dog stayed in the clinic for 5 days. During this period each dog underwent the functional tests described above and also received 30-60 minutes daily physical therapy tailored to their individual needs by a certified canine rehabilitation technician who was blinded to their treatment category. Briefly, exercises consisted of swimming, underwater treadmill walking, sit-to-stand repetition, weight shifting, balancing exercises and encouragement to walk with hindquarter support using slings and carts. Owners were instructed to continue appropriate physical therapy at home and encourage dogs to ambulate in their home environment. Urination in these incontinent dogs was managed at home and in the clinic by manual compression of the caudal abdomen to trigger reflex urination. Owners were instructed to express urine as fully as possible at least three times daily.

Owners remained blinded to treatment allocation group of their dogs until after collection of all follow-up outcome measurements and completion of their final interview.

Statistics

Sample size calculation

We estimated the need for 24 dogs in each group to detect a 25% difference in the primary outcome measure at 6 months after intervention with power of 80% and α of 0.05. Because dropout was expected in this type of trial (death from complications of paraplegia, owners unable to travel, *etc*) we aimed to recruit 30 dogs in each group.

Codes were broken after completion of all data collection, including owner interviews, and checking to ensure data completeness (bearing in mind missed data points through bad weather, owner withdrawal *etc*) and after processing to provide data on primary outcome temporal relationships. Raw primary outcome data was analyzed by an investigator who had no knowledge of treatment group and supplied processed data that summarized diagonal coupling relationships to another investigator who carried out the statistical analysis.

Data analysis: For all outcomes, data were assessed for Gaussian distribution and then transformed, if necessary, using Box-Cox analysis and the 'gladder' command to determine

suitable transformation using Stata 11 for Windows (StataCorp, College Station, TX, USA). The primary outcome data were analyzed with a multilevel linear regression model ('xtmixed') using Stata 11, using random effects for subjects and fixed effects for the intervention and including a term for interaction between chondroitinase and time, whilst adjusting for baseline measurements by their inclusion as a covariate. Standard *post hoc* commands ('contrast') in Stata were used to determine main effects and to further explore interactions. Similar analyses were applied to the bladder compliance data. The remaining outcomes data were plotted to check distributions and pre- and post-intervention values compared using paired Student's t tests or equivalent non-parametric analyses. $P < 0.05$ was taken to indicate a significant relationship between dependent and independent variables or differences between the control and active intervention (chondroitinase ABC) groups.

Sensitivity of the primary outcome analysis to data lost to follow-up was assessed through two secondary analyses: i) inclusion of only those animals for which there was a complete dataset; and, ii) derivation of a more complete dataset by multiple imputation in Stata (see Supplementary Material).

Results

A total of 60 dogs was recruited and randomly allocated between the intervention and control groups in a 1:1 ratio as planned (Fig.1; Table 1; Table 2). As explained in Supplementary Material, dogs with severe chronic spinal cord injury sometimes develop a pattern of so-called 'spinal walking' (Gallucci *et al.*, 2017) and this was noted at enrolment in 5/30 dogs allocated to the chondroitinase group and 4/30 dogs in the control group.

Primary outcome measure

Treadmill locomotion

Plots summarizing the 'before' and 'after' values for the control and intervention groups suggested improvement associated with intraspinal chondroitinase injection (Fig. 2). Corresponding summary statistics reveal a 23% improvement (*i.e.* a reduction in numerical score) in mean coordination score from baseline to the 6-month follow-up in the chondroitinase group (from 2.16

to 1.67; paired Student's t test $P = 0.008$) whereas in the control group there was a 2% deterioration in mean score (1.99 to 2.03; paired Student's t test $P = 0.677$). Graphs of coordination against time at a group level revealed this change to be a gradual restoration, in the chondroitinase-injected group only, of a more normal temporal association of forelimb and hindlimb stepping as time elapsed (Fig. 2). At the start of the trial, control and chondroitinase groups exhibited similar dysfunction but, with increasing length of follow-up, the chondroitinase group regained progressively better function, whereas the control group did not, commensurate with significant interaction between chondroitinase and time. Multilevel modeling and *post hoc* analysis revealed that there was no overall effect of either time ($\chi^2 = 4.91$; $P = 0.178$) or chondroitinase injection ($\chi^2 = 1.69$; $P = 0.194$) alone following the intervention but a significant interaction between these two variables ($\chi^2 = 9.17$; $P = 0.027$). Specifically, coordination in chondroitinase-injected dogs at 6 months was significantly improved compared to their baseline ($\beta = -0.555$; 95%CI: -0.956/-0.155; $P = 0.007$) and compared to coordination in control animals at 6 months ($\beta = -0.484$; 95%CI: -0.790/-0.178; $P = 0.002$) (Supplementary Material, Tables 1 and 2 show complete model results).

Together with the effect of chondroitinase to improve coordination in the recipient group overall, three dogs (of 27 available for follow-up at 6 months) in the chondroitinase group regained the ability to walk unaided, but this occurred in none (of 25 available at 6 months) of the controls (Fisher's exact test $P = 0.236$). All three of these individuals recovered the ability to walk by 1 month and this persisted throughout the remainder of the study; between baseline and 6 months one showed better coordination and two worse coordination (pre-post scores were: 2.10-1.06, 2.64-2.85, 1.96-2.59 respectively). In Figure 2b two dogs show 6-month post-chondroitinase coordination scores approaching zero (*i.e.* near-normal). One of these dogs was able to weakly ambulate at the commencement of the study (one of the five in the group showing 'spinal walking', with a coordination score of 2.08) but improved following the intervention; the other dog (with an entry score of 2.39) did not recover the ability to ambulate independently during the 6-month study period but limb movements were described as 'stronger' by the owner.

All dogs included in this study received the treatment to which they were randomly allocated meaning that intention-to-treat and *per protocol* analyses would not differ. However, we wished to determine whether the results may have been influenced by missing data points and so we

carried out two further analyses: first, including only dogs for which we had complete datasets and the second on data for which missing values had been imputed (see Material and Methods and Supplementary Material). The results of both these analyses were similar to those of the original analysis (see above), confirming the combinatorial effect of chondroitinase injection and time in improving coordination at 6 months (with respect to baseline) in the chondroitinase group (compete datasets: β = -0.594; 95%CI: -0.1.04/-0.151; P = 0.009; multiple imputation dataset: β = -0.518; 95%CI: -0.917/-0.118; P = 0.011) and also providing confidence that this outcome was not biased by missing data.

Secondary outcome measures

Von Frey filament testing of skin sensitivity

Responses were highly variable between individuals but the median score was zero responses in both groups at all time points. At 24 hours after intervention, both control and chondroitinase-injected dogs showed similar increase in responses but this rapidly regressed and remained comparable between groups at both 3 and 6 months after intervention (Fig. 3). Statistical analysis revealed no evidence for a differentially detrimental effect of injection of the chondroitinase ABC preparation compared with the sham-treated animals (Mann-Whitney test pre-post scores: P = 0.671) and no owners in either group reported evidence for pain behavior at any follow-up interview. More detailed assessment of individual animal data is provided in Supplementary Material.

Bladder compliance

At baseline, compliance was highly variable in both groups, ranging from 0.7-180 mL/cmH_2O in the chondroitinase group and 0.96-150 mL/cmH_2O in the control group, with medians of 4.8 and 4.6 respectively (the reference interval is not well-established in dogs but thought likely to be similar to that in humans, *i.e.* ~12-40mL/cmH_2O [Toppercer and Tetreault, 1979; Combrisson & Cotard, 1989; Harris *et al.*, 1996]). Overall, there appeared to be a differing pattern of change after the interventions, with a tendency for compliance to increase in the chondroitinase group and decrease in the control group at the 1 month re-examination (Fig. 4). Examination of individual animal responses suggested that compliance increased in more dogs in the chondroitinase group than in the control group, with substantial increases occurring in a small minority of dogs in both

groups (Fig. 4). In dogs with abnormally low compliance (<12.5 mL/cmH_2O) there was an increase to within reference interval in 1/28 of control cases at the 1-month follow-up; a similar change occurred in 2/27 chondroitinase-injected dogs. The apparent difference between groups at 1 month did not persist and by 6 months after injection compliance in both groups had returned to values that were similar to those at baseline. Statistical analysis indicated that, even when controlled for the possible confounding effect of the duration of paralysis in each dog, neither the overall effect of injection of the chondroitinase ABC preparation ($\beta = 0.028$; 95%CI: -0.643/0.691; $P = 0.933$), nor its specific effect at 1 month follow-up ($\beta = 0.600$; 95%CI: -0.120/1.32; $P = 0.103$) had a significant association with compliance.

Transcranial magnetic motor evoked potentials

At entry to the trial, 3/30 control dogs and 3/30 dogs allocated to receive chondroitinase exhibited recordable transcranial magnetic motor evoked potentials in at least one *cranialis tibialis* muscle. One of the control dogs also exhibited so-called spinal walking but the other five (two controls and three chondroitinase-treated dogs) did not. During follow-up, transcranial magnetic motor evoked potentials continued to be recorded from similar numbers of dogs in both control and chondroitinase groups concluding with positive responses elicited from 3/25 controls and 4/27 chondroitinase dogs at 6 months.

transcranial magnetic motor evoked potentials were recorded *de novo* during the trial in two dogs in the chondroitinase group that also recovered independent ambulation (see above) and were also recorded during the trial in a third chondroitinase-injected dog, in which the fore-hind coordination score returned almost to normal values at 6 months. Positive transcranial magnetic motor evoked potential recordings at some, but not all, follow-up examinations were also noted in three dogs in the control group, two of which showed spinal walking at entry to the trial.

Sensory evoked potentials

In the control group there were 21 animals with pre- and post-intervention percutaneous sensory evoked potential recordings and 22 in the chondroitinase group but there appeared to be no difference between groups in change in cranial-caudal level of response (Fig. 5; Mann Whitney test: $P = 0.926$).

Assessment of adverse events

Over the entire follow-up period owners reported a total of 19 adverse events that they associated with the periods when dogs stayed in the hospital during which interventions, tests of outcome and physical therapy were given: 11 occurred in the chondrotinase group and 8 in the controls. These reported adverse events were all transient, lasting for up to 3 days; the majority were periods of diarrhea, evidence of urinary tract inflammation or infection or, on three occasions, reduced activity for 1-3 days.

Adverse events noted by the owners after the first visit - during which baseline functional data were collected and the intervention applied - included eight events in chondroitinase dogs and one in a control animal. Three dogs that had received chondroitinase showed reduction in mobility that lasted for up to 3 days (two of these dogs subsequently recovered independent ambulation) and one additional dog appeared painful for the first 12 hours after injection. Two chondroitinase-injected dogs showed evidence of urinary tract infections and one had a generalized seizure immediately upon recovery from anesthesia. One chondroitinase-injected and one control dog developed diarrhea during hospitalization.

At the 1-month re-examination, five adverse events were noted: two episodes of diarrhea (one in each group), two dogs had skin lesions overlying bony prominences of the pelvis (one in each group) and one dog (chondroitinase-injected) showed periods of spasmodic limb muscle activity for a week following this visit. Five further adverse events (four of lower urinary signs and one of diarrhea) in control animals were recorded during the following two re-visits. None of the owners reported evidence of abnormal sensitivity on their dogs (*e.g.* flinching, crying, whining or biting when being touched) at any stage throughout the study.

Discussion

The results of this study confirm that intraspinal injection of heat-stabilized chondrotinase improves locomotor function in this chronic, severe, naturally-occurring model that mimics clinical spinal cord injury in humans. Importantly, the effect became increasingly prominent with

increasing time after injection, during which dogs received tailored physical therapy, supporting a previous intimation that chondroitinase and directed physical activity are synergistic in restoration of spinal cord function (Garcia-Alias et al., 2009). In addition, while at home the dogs were encouraged to move around their home environment, which may have played a role similar to that of an enriched environment for spinal cord-injured rats (Lankhorst *et al*., 2001). Such formal and voluntary physical therapy might contribute to the strong response in this outcome in the chondroitinase-injected dogs.

Interestingly, it appears that we detected two types of recovery of locomotor activity associated with intraspinal chondroitinase injection. First, there was widespread improvement in fore-hind coordination throughout the group as a whole, with two dogs recovering near-normal values (see Fig. 2). In addition three dogs developed independent locomotion that was not associated with improved fore-hind coordination. We consider that each type of response could be associated with activity of the chondroitinase at either one or both injection sites. First, improvement of coordination implies transmission of impulses across the lesion site (so that fore and hind limbs become temporally coordinated) and can be explained either by regeneration of axons across the lesion site (Bradbury *et al.*, 2002; Yick *et al.*, 2003; Barritt *et al.*, 2006) or restoration of functionality to pre-existing fibers through chondrotinase-mediated effects on the damaged tissue (for instance *via* release of matrix-bound factors [Crespo *et al.*, 2007]). Such effects may or may not also require reorganization of targets in the destination tissue that may have been facilitated by the more caudal chondroitinase injection. We propose that this mechanism of action explains the increased coordination noted within the group as a whole, and especially in the two dogs whose coordination scores improved to near-normal values (and one of which also showed recovery of recordable transcranial magnetic motor evoked potentials). In contrast, in some animals - perhaps those in which axon regeneration or restoration of functionality across the lesion site was not feasible because of its character or severity - chondroitinase effects at the more caudal injection site may have been sufficient to allow reorganization of synaptic contacts, *via* disruption of perineuronal nets (Massey *et al.*, 2006; Cafferty *et al.*, 2008; Garcia-Alias *et al.*, 2009), thus facilitating development of 'spinal walking'. We propose that this mechanism may underlie the recovery of independent locomotion in the three dogs that did not exhibit improved coordination; however, there is a need for further examination of this possible effect since few chondroitinase-

injected dogs recovered in this way and there was not a significant difference in its incidence between control and chondroitinase groups.

The absence of evidence to suggest that intraspinal chondroitinase injections caused problematic adverse effects is of critical importance. A particularly worrisome aspect of any intervention that involves intraspinal administration of an agent that might induce plastic change in the nervous system is that it might also engender abnormal pain sensation, especially in the dermatomes of the injected region. The data we collected here on responses to von Frey filament stimulation over the dorsum are consistent with development of hypersensitivity in the immediate post-intervention period. However, there was a similar incidence of increased sensitivity in both chondroitinase and control dogs, providing strong evidence that the chondroitinase injection was not the cause. Instead, heightened sensitivity is better attributed to the combination of hair clipping and needle damage to the skin, which were factors common to both groups. During the remainder of the follow-up period skin sensitivity gradually decreased in both groups to that observed at enrolment.

Owners were encouraged, through specific interview questions, to report any adverse events following recruitment into the trial. Although many events were reported, these occurred at similar frequency in both control and chondroitinase groups. Furthermore, most of the adverse events were suggestive of non-specific effects of staying in our hospital or undergoing the investigative procedures. For instance, diarrhea is very common in dogs after periods of stress, and urinary tract irritation or infection can be associated with cystometry. One dog that had been injected with chondroitinase exhibited seizures upon recovery from anesthesia. Although this might appear rather alarming, it is unlikely that this was a consequence of the intraspinal injections. First, the volume of the injections was very small, meaning that it would be highly improbable for the injected material to reach the brain *via* the cerebrospinal fluid. Second, this dog may have been at inherent increased risk of seizures because the spinal cord injury was the result of a fracture-luxation at L1/2 vertebrae and head injury is common correlate of spinal fractures in dogs. This dog recovered uneventfully and showed no persistent abnormalities of brain function or repeat seizures during the follow-up period.

494 The compliance results recorded at the 1-month follow-up provide a slight suggestion that
495 chondroitinase injection might open an opportunity for improving bladder function. The group of
496 chondroitinase-injected dogs as a whole demonstrated much higher compliance (*i.e.* ability to
497 retain more urine) at the first follow-up assessment. However, this effect was not statistically
498 significant and faded by the time of later re-assessments. It is possible that the initial change in
499 function could have been an effect of chondroitinase that did not persist and it might be that more
500 effective or prolonged training of bladder function might make this improvement more permanent.
501 However, while the group effects look promising, analysis at an individual level (Fig. 4) suggests
502 that normal bladder compliance (estimated as 12-40 mL/cmH_2O) was restored in few dogs in either
503 group at this time point. Whilst it remains possible that chondroitinase may have a beneficial effect
504 on bladder function there was such a large degree of variability in compliance at enrolment that
505 detecting such an effect may be difficult unless trial participants are stratified for this variable.
506
507 At a group level, there were no readily attributable effects of chondroitinase ABC on the secondary
508 electrophysiological outcome measurements, which were designed to provide possible
509 explanations for any changes in overall function that we detected in the primary analysis. This lack
510 of change parallels the findings of our previous study on olfactory ensheathing cell transplantation
511 (Granger *et al*., 2012). There are two main possible explanations. First, the changes that occur in
512 the spinal cord to mediate improvement in limb girdle coordination do not necessarily rely on
513 changes in spinal cord long tract function. For instance, changes in propriospinal connections may
514 improve fore-hind coordination but will not be detected by the evoked potential recordings that
515 are dependent upon long tract integrity. The second possible explanation is that these measures of
516 long tract function are not sufficiently sensitive to detect changes that were elicited by the
517 chondroitinase injection. Evidence in support of this proposition is that chronic, histologically sub-
518 complete spinal cord injury in rats can abolish motor evoked potentials (Metz *et al*., 2000) and it
519 is known that, after acute spinal cord injury, transcranial magnetic motor evoked potentials can
520 even be abolished in dogs with purposeful movement (Sylvestre *et al*., 1993). Moreover, although
521 there is a general (inverse) correlation between latency of evoked potentials and white matter
522 preservation (Nashmi *et al*., 1997), the precise relationship between intact detected conduction and
523 the number of intact axons is unknown. Despite these limitations, in the five dogs that showed
524 recovery of independent ambulation (n = 3) or recovery of normal fore-hind coordination (n = 2),

transcranial magnetic motor evoked potentials could be recorded at some stage throughout the follow-up period.

There has long been vigorous debate about how much pre-clinical evidence is required before it is reasonable to translate a successful intervention from the laboratory to humans with spinal cord injury (Kwon *et al*, 2013; Kwon *et al*., 2015). The evidence we present here suggests that chondroitinase ABC is at this threshold: not only has its beneficial effect been demonstrated repeatedly in laboratory animals but, as we show here, it is sufficiently potent to ameliorate lost function following severe clinical injury in a large mammalian species and it is not associated with detectable detrimental effects, which should all augur well for clinical benefit in humans with spinal cord injury. The question then remains as to whether the effect size is of sufficient magnitude to be of benefit were the therapy to be translated into humans. The change we detected in the primary outcome measure was ~23%, which can be regarded as a large treatment effect, corresponding to our ability to detect this difference in a reasonably-powered (80%) study, even in such a relatively small sample population. It is also of similar magnitude to that reported in a meta-analysis of olfactory ensheathing cell transplantation in experimental animals that was recommended as supportive evidence to pursue human clinical trials (Watzlawick *et al.*, 2016). However, whether an intervention will translate from one species to another with the same magnitude of effect is almost impossible to predict, because the mechanisms of recovery may or may not translate between species. For instance, it is difficult to know whether the mechanisms underlying recovery of coordination in our dogs (or, similarly, recovery of open-field ambulation or forelimb reaching tasks in rats) will also lead to, for example, improved hand function in humans. The most plausible means to test the translatability is to trial the intervention in humans. Therefore the key value of our data is the detection of benefit in the face of real-life lesion heterogeneity and the absence of detectable adverse effects, because this combination provides a clear green light for the trials in humans that are necessary to categorically define the magnitude of effect in that species.

A further question might be whether the drug preparation and delivery system we used here is the most clinically appropriate. Although there is evidence of persistence of effect of native chondroitinase ABC for at least 10 days after injection into the brain (Lin *et al.*, 2008), the

consensus of opinion, summarized by Bradbury and Carter (2008), is that a translatable long-acting form of the enzyme is likely to be required for therapy of spinal cord injury. The composite product used in this study stabilizes the chondroitinase ABC active ingredient, facilitates sustained delivery and is easily delivered, therefore fulfilling this requirement. For the next step of introduction into humans and getting approval for clinical trial from regulatory agencies, the preparation will need to be made under appropriately controlled aseptic conditions. There do not appear to be any obstacles to this process: the lipid backbone used in the manufacture of the microtubes can be made under Good Laboratory Practice conditions and has already been used in a human clinical trial (Wicki *et al.*, 2015). Percutaneous injection of chondroitinase was selected in this study because it readily permits blinding of study observers and owners of the participating dogs, but it may not be the optimal method of ensuring that the drug reaches its target. Open surgery would ensure delivery into precise locations within spinal cord parenchyma and could easily be applied in phase I trials in humans, but similar delivery in a phase II trial would necessitate sham surgery for controls, which can be ethically controversial (Albin, 2002; Frank *et al.*, 2008). However, because participants in such a clinical trial would have chronic lesions with static neurologic function, a crossover design, similar to that proposed for cell transplantation for multiple sclerosis (Freedman *et al.*, 2010), would be feasible. Although this would not avoid the need for sham surgery it would reduce the number of participants required and assure those recruited that, unless unforeseen safety issues arose, they would each receive the active intervention (chondroitinase).

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Figure legends

Figure 1: Flowchart showing how dogs were recruited to this trial. 60 dogs were randomized to chondroitinase ABC injection or control groups from a total pool of 196 possible candidate cases.

Figure 2: Trial primary outcome measure: coordination between fore and hind limb stepping in treadmill walking dogs before and after intervention. The score (on the y-axis) is a summary of accumulated time delays between forelimb and hindlimb steps, with lower scores indicating better coordination. **a:** individual records for control animals; **b:** individual records for chondroitinase-injected animals; **c:** group summary over time (symbols represent mean and bars are standard error of the mean). Controls are illustrated in red and chondroitinase animals in blue. In control animals, although there is some (expected) intra-animal variability there is no systematic change over the 6-month follow-up period (paired Student's t test, $P = 0.677$). In contrast, both individual records (**b**) and group summary (**c**) of chondroitinase-injected animals show systematic and progressive reduction in score corresponding to improved coordination. At 6 months chondroitinase-injected animals improved by a mean of 23% from baseline (paired Student's t test, P = 0.008) and was the result of a significant interaction between chondroitinase injection and time (see text); there was a significant difference between groups at 6 months (contrast = -0.484; 95%CI: -0.790 / -0.178; $P = 0.002$).

Figure 3: Summary of responses to von Frey filament stimulation, in which a higher score indicates greater sensitivity (symbols represent mean and bars are standard error of the mean). From a low baseline there is an increase in sensitivity on the day immediately after the intervention in both groups (chondroitinase injection or sham) that decreases over time. The lack of difference in scores between groups (Mann-Whitney test at 6 months P=0.107) and lower scores in the active treatment group indicate that there is no evidence for induction of neuropathic pain following chondroitinase injection.

Figure 4: Change in bladder compliance after intervention. **a:** changes in group means (bars indicate standard error of the mean) over the 6-month follow-up period. In chondroitinase-injected

dogs there is an apparent increase in compliance (improved ability to retain urine) at 1 month but this does not persist; statistical comparisons detect no difference between groups at any point. **b** and **c** are spaghetti plots illustrating change in compliance over the first month after intervention in (**b**) control and (**c**) chondroitinase-injected dogs. Although there was a tendency for a greater proportion of dogs in the control group to show decreased compliance and a greater proportion of dogs in the chondroitinase group to show increased compliance in the first month, accounting for the changes observed in **a**, few animals in either group improved from abnormally low values to achieve values within the reference interval (indicated by dashed lines) at 1 month.

Figure 5: Changes in cranial-most level of recording of spinal sensory-evoked potentials (SEP) in control and chondrotinase-injected dogs during the 6-month follow-up period. Although there was some individual variation in level at which the SEP could be recorded as the study progressed there was no indication of a systematic difference in this variable between treatment groups.

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