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RESEARCH ARTICLE

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Transplantation of encapsulated autologous olfactory ensheathing cell populations expressing chondroitinase for spinal cord injury: A safety and feasibility study in companion dogs

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Abstract

Spinal cord injury (SCI) can cause irreversible paralysis, with no regenerative treatment clinically available. Dogs with natural SCI present an established model and can facilitate translation of experimental findings in rodents to people. We conducted a prospective, single arm clinical safety study in companion dogs with chronic SCI to characterize the feasibility of intraspinal transplantation of hydrogel-encapsulated autologous mucosal olfactory ensheathing cell (mOEC) populations expressing chondroitinase ABC (chABC). mOECs and chABC are both promising therapies for SCI, and mOECs expressing chABC drive greater voluntary motor recovery than mOECs alone after SCI in rats. Canine mOECs encapsulated in collagen hydrogel can be matched in stiffness to canine SCI. Four dogs with complete and chronic loss of function caudal to a thoraco-lumbar lesion were recruited. After baseline measures, olfactory mucosal biopsy was performed and autologous mOECs cultured and transduced to express chABC, then hydrogel-encapsulated and percutaneously injected into the spinal cord. Dogs were monitored for 6 months with repeat clinical examinations, spinal MRI, kinematic gait and von Frey assessment. No adverse effects or significant changes on neurological examination were detected. MRI revealed large and variable lesions, with no spinal cord compression or ischemia visible after hydrogel transplantation. Owners reported increased pelvic-limb reflexes with one dog able to take 2–3 unsupported steps, but gait-scoring and kinematic analysis showed no significant improvements. This novel combination approach to regeneration after SCI is therefore feasible and safe in paraplegic dogs in a clinical setting. A randomised-controlled trial in this translational model is proposed to test efficacy.

KEYWORDS
canine translational model, cell therapy / transplantation, chondroitinase ABC, hydrogel encapsulation, neurology, neuroscience, olfactory ensheathing cells, spinal cord injury, spontaneous animal model

1 | INTRODUCTION

Paralysis and incontinence caused by spinal cord injury (SCI) have clear and substantial effects on the health of affected individuals, and there is currently no curative treatment. SCI is also common in dogs, often resulting from natural intervertebral disc herniation (Hansen, 1951; Jeffery et al., 2013). Affected dogs sustain heterogenous contusive-compressive injuries similar to those observed in humans (Griffiths, 1978; Norenberg et al., 2004; Smith & Jeffery, 2006), and with a size closer to human SCI than experimental rodent models. Pet dogs with chronic SCI also provide a more heterogenous group of animals than laboratory cohorts, with co-morbidities, clinical interventions and logistical constraints that are more reflective of the human population. These dogs therefore provide a translational model of human SCI which is valuable for screening promising experimental rodent therapies for "real-world" effect (Moore et al., 2017).

Mucosal olfactory ensheathing cells (mOECs) have undergone a successful randomized controlled trial in pet dogs with chronic SCI, demonstrating increased fore-hindlimb coordination compared to controls (Granger et al., 2012). However, the improvement was unpredictable and variable between dogs, and there was no evidence of long tract regeneration, implying further development and/or combination therapies will be required. There is considerable evidence intraspinal transplantation of OECs improves walking recovery after SCI in experimental rodents (Nakhjavan-Shahraki et al., 2018; Watzlawick et al., 2016), and transplantation is safe in people (Li et al., 2015), but controlled clinical trials are lacking.

Delivery of chondroitinase (chABC) into the spinal cord improves walking and skilled voluntary movement reliably across multiple rodent models (Muir et al., 2019), as well as in experimental cats (Tester & Howland, 2008) and non-human primates (Rosenzweig et al., 2019). In a clinical trial in pet dogs, microtubule-delivered chABC showed similar improvements in fore-hindlimb coordination to mOEC transplantation (Hu et al., 2018). However, rapid degradation of chABC at body temperature presents a barrier to its clinical use.

We have developed canine mOECs modified by a 3rd generation lentivirus to express chABC (mOEC-chABC) (Carwardine et al., 2016), providing a clinically applicable combination therapy. We have further demonstrated proof-of-principle that modified canine mOECs provide better recovery of voluntary movements than mOECs alone in a rat forepaw reaching model of SCI (Prager et al., 2021).

A key barrier to cell transplantation strategies in regenerative medicine is a lack of cell survival in the hostile environment of injured tissue. Hydrogel encapsulation of cells increases cell survival, and hydrogels modulate the injury environment, stimulate axon regeneration and improve outcomes after experimental SCI (Führmann et al., 2017; Tam et al., 2014). Consideration of the biomechanical properties of transplanted hydrogels is also important to ensure integration of the transplanted construct with the host, reduce inflammatory reactions, and prevent iatrogenic damage. Canine mOECs encapsulated in collagen hydrogel can match the stiffness of the injured canine spinal cord determined using a clinically applicable ultrasound elastography method (Prager et al., 2020). Collagen is highly biocompatible and minimally immunogenic (Aurand et al., 2012; Straley et al., 2010), has been shown to be safe in experimental canine transplants (Han et al., 2015), and implanted in human SCI (Xiao et al., 2016), giving confidence in safe delivery of this collagen hydrogel in canine clinical cases.

We therefore tested the feasibility and safety of delivering hydrogel-encapsulated mOECs transduced to express chABC in chronic and severe SCI, in a clinical environment, using the canine translational model.

2 | METHODS

2.1 | Case recruitment

This study was approved by the Royal Veterinary College Animal Welfare and Ethical Review Board and licensed (P302A3B70) under the UK Home Office Animal Scientific Procedures Act (1996). Four dogs were recruited sequentially after advertising, with inclusion criteria as per a previous trial (Granger et al., 2012) (see Supplementary Information). These dogs have complete motor and sensory loss with no improvement a minimum of 3 months post-injury, considered equivalent to thoracic ASIA "A" grade in human patients (Fawcett et al., 2007; Granger et al., 2012; Olby et al., 2003).

A specialist veterinary neurologist (Joe Fenn or Nicolas Granger) performed an initial clinical and neurological examination to confirm eligibility. Owners provided written informed consent and committed to baseline kinematic and von Frey measures prior to intervention then 4 follow-up visits at 2 weeks, 1, 3 and 6 months after transplantation (study outline in Supplementary Figure 1).

2.2 | Gait analysis

Kinematic gait analysis was performed on a treadmill with the dog supported by a sling under the abdomen (Granger et al., 2012; Hamilton et al., 2007) (see Supplementary Information). Fore-hindlimb coordination was summarized by calculating the mean cumulative lag between the hindlimb and forelimb over a minimum of

30 forelimb steps. Lateral stability was calculated from the coefficient of variation of the distance between fore and hind limb paws, expressed as the ratio of hind-fore limb variation.

Gait analysis was performed at each visit. Videos of each treadmill session were scored by an observer blinded to time-point (Leticia Escauriaza) using the Texas Spinal Cord Injury Score (TSCIS) (Levine et al., 2009) and a modified Open Field Gait Score (OFS) (Olby et al., 2016).

2.3 | Pressure testing with von Frey aesthesiometer

An electronic von Frey aesthesiometer (World Precision Instruments; Hertfordshire, UK; 1000 g probe, rigid tips) was used to quantify response to pressure (Moore et al., 2013). Included dogs have no sensation caudal to the level of their lesion, so thresholds were recorded for three parameters bilaterally: (i) initiation of hind limb withdrawal reflex in response to pressure between dorsal metatarsal IV and V (assessment of sciatic function), (ii) initiation of *cutaneous trunci* reflex (at the most cranial point it was found using a pinch), (iii) sensation (conscious reaction) at the skin overlying the lesion site. Each location was repeated 5 times on each side and the mean of the 10 values for each site calculated. All tests were performed by the same observer.

2.4 | MRI

Animals were anesthetized (see Supplementary Information) for MRI, mucosal biopsy and transplantation. MR imaging (1.5 T Intera CX, Philips Medical Systems, Eindhoven, the Netherlands) was performed at baseline prior to olfactory mucosa biopsy, repeated at the next visit 30 min after transplantation and at 2 months after transplant. Sequences included: T1W transverse and sagittal, T2W transverse and sagittal, BAL TGRAD transverse and STIR sagittal.

MRI characterization included (Lewis et al., 2018): lesion length from T2 hyperintensity on BAL TGRAD; maximal spinal cord compromise (MSCC), the percentage of spinal cord cross sectional area with abnormal signal intensity at the worst point; length of MSCC; volume of the cystic lesion (based on T2W BAL TGRAD images) calculated from sequential regions of interest marked by hand using a commercial DICOM viewer (OsiriX MD version 11.0.2, OsiriX Foundation, Geneva, Switzerland) by a specialist veterinary radiologist blinded to case and time-point (Mark Plested).

2.5 | Olfactory mucosal biopsy and cell culture

Blind olfactory mucosal samples were taken via the nostril (Ito et al., 2019) (see Supplementary Information). Biopsies were processed and cultured as previously described (Granger et al., 2012; Ito et al., 2008, 2019). To select for mOECs, blood vessels, nerves and cartilage were removed from biopsies to leave only olfactory mucosa as identified by its brownish color. During culture mOECs were separated from contaminant fibroblasts by differential trypsinization (see Supplementary Methods).

Once approximately a quarter of the cells for transplant were obtained (target 1.25 million), cells were transduced to express chABC with a 3rd generation lentivirus at multiplicity of infection 10. Synthesis of lentivirus and transduction protocol is described elsewhere (Carwardine et al., 2016; Prager et al., 2021). 50 μl samples of media from 3 wells were taken at 1 week after transduction to confirm presence of active chABC by Morgan Elson reaction (Carwardine et al., 2016). Enzyme activity values were calibrated to a standardization curve from commercial chABC (Merck C2905) of concentrations between 0.01 and 0.5 U to allow comparison between Morgan Elson runs.

2.6 | Cell encapsulation and transplantation

Once sufficient modified cells were cultured (target 5 million per dog), they were trypsinized, divided into 3 and resuspended in a volume of collagen hydrogel based on MRI measurement at time of biopsy (if cavities were present at a given level on MRI) or 100 μl media (if no cavity was seen).

Transplant media was Dulbecco's Modified Eagle Media with added neuregulin and forskolin (culture media without serum or antibiotics, see Supplementary Information). Type 1 rat tail collagen hydrogel was synthesized as previously described by dissolving in acetic acid to 4.1 mg/ml and neutralizing with sodium hydroxide (Prager et al., 2020). Transplant suspensions were kept on ice to prevent gelation and transplanted within 30 min of encapsulation. A sample of media from each transplant population was tested for bacterial and mycoplasma contamination. A sample of cells were fixed in paraformaldehyde and characterized by immunofluorescence for fibronectin and p75 (Prager et al., 2021).

Each dog was re-anesthetized and positioned in right lateral recumbency for transplantation. Lumbar cerebrospinal fluid (CSF) and jugular venous blood samples were obtained prior to transplant for baseline cytology and protein content, and hematology and biochemistry respectively.

Spinal needles (22G, 1.5–2.5 inches) were placed percutaneously through the intervertebral space in the midline into the center of the spinal cord under fluoroscopic guidance (Axiom Iconos R200, Siemens AG, Erlangen, Germany) (Granger et al., 2012). Needles were placed at three levels; at the center of the lesion area and adjacent cranial and caudal spaces. The transplant was injected over 1 min, with half the cells at the center, and the remaining half split equally between cranial and caudal injections. Needles were flushed with 0.1 ml of hydrogel or media to match transplant vehicle.

After anesthesia dogs were monitored for pain overnight, and received follow up behavioral measures the next day before discharge.

2.7 | Post-transplantation follow-up

Follow-up visits were planned for 2 weeks, 1, 3 and 6 months after transplantation based on previous interventional trials in canine SCI (Granger et al., 2012; Hu et al., 2018). Due to the UK COVID-19 lockdown, repeat MRI, CSF and blood samples were taken at 2-month instead of 1-month, when only phone update was able to be obtained. At all visits dogs received repeat clinical and neurological examination by Joe Fenn or Nicolas Granger and repeat gait analysis and von Frey test. Owners were also asked to report changes using a standardized questionnaire and score their dog's quality of life, walking ability and ease of bladder expression on a visual-analogue scale (1–10).

2.8 | Statistical analysis

As this study was primarily aimed at determining safety and feasibility statistical analysis was exploratory and not powered, and we did not pre-specify primary or secondary outcome measures. Data was assessed for normality using QQ plots and Shapiro-Wilk test. Non-parametric data is shown with median and range, parametric data as mean ± SD. Analysis of tests with measures repeated at serial time points (MRI, gait analysis, von Frey) used a two-way ANOVA or mixed effects model if there were missing values (stated in results). $p < 0.05$ defined statistical significance. Statistical analysis was performed in Prism (Graphpad, v9.1) and RStudio (RStudio, 2012).

3 | RESULTS

3.1 | Case recruitment

Case details are summarized in Table 1. All injuries were caused by intervertebral disc extrusion and surgically decompressed at the time of paralysis. At transplantation, median age was 5 years (range 4–6 years), median weight was 11 kg (5.3–15.3 kg), and median time since injury was 9 months (5–29 months). Injury sites ranged between T12 and L4.

3.2 | Cell culture

No adverse effects were noted from nasal biopsy apart from mild epistaxis lasting less than 30 min. All dogs were returned home to their owners the same day.

Cells were cultured, transduced to express chABC and maintained sterile in all cases. The median number of cells transplanted was 3.95 million (range 1.8–6.5 million), and contained a median of 46% mOECs (range 4.5%–99%) (Figure 1a and b). There was no significant difference in activity of chABC secreted by cell cultures from each case (Kruskall-Wallis $p = 0.73$) (Figure 1c).

3.3 | Clinical and owner reported outcomes

There were no adverse effects reported after transplantation. The neurological examination did not change during the trial, except in case 3 where the *cutaneous trunci* reflex cut-off progressively moved cranial from the level of the L2/L3 intervertebral disc space (left/right respectively) to T13/L1.

Hematology and biochemistry were unremarkable pre and post-transplant in all cases. In 3 of 4 cases CSF could be collected and there was no significant change in protein level or cytology pre and post-transplant. The 2 cases with more recent injury (5 and 7 months) both showed mildly elevated protein (0.35–0.5 g/L), mild mononuclear pleocytosis (3–12 total nucleated cell count/ml), aggregates of myelin, and evidence of prior hemorrhage in 1 case. This profile did not change over time. CSF from the case 29 months after injury was cytologically unremarkable.

Owners (who were not blinded) reported an increase in reflexive hind limb movement in 3 cases, a decrease in frequency of urinary incontinent episodes in 2 cases, and the ability to take 2–3 steps unsupported in 1 dog from 5 months after transplantation. There were no reports of behavior suggestive of pain at any timepoint.

Visual analoge scale reporting of ease of bladder expression and quality of life did not change over the course of the study. Bladder expression consistently rated very easy (median score 0, range 0–4) and quality of life consistently rated very good (median 10, range 6–10). Walking ability rating increased from 0 (no walking ability) to 5 (out of 10) in 1 case and did not change in all others.

TABLE 1 Clinical details of cases

Case	Sex	Age	Breed	Injury location	Hind limb muscle tone/reflex activity	Time between injury and transplant (months)	Weight (kg)
1	MN	5years 2 m	Miniature Dachshund	T13-L2 Left-sided	Rigid/present	5	7.0
2	MN	4years 8 m	Cross breed	T13-L1	Flaccid/absent	11	14.5
3	ME	5years 2 m	French Bulldog	L2-L4 Right-sided	Rigid/present	7	15.3
4	FN	6years 9 m	Miniature Dachshund	T12-13 Right-sided	Rigid/present	29	5.3

Abbreviations: E, Entire; F, Female; M, Male; N, Neutered.

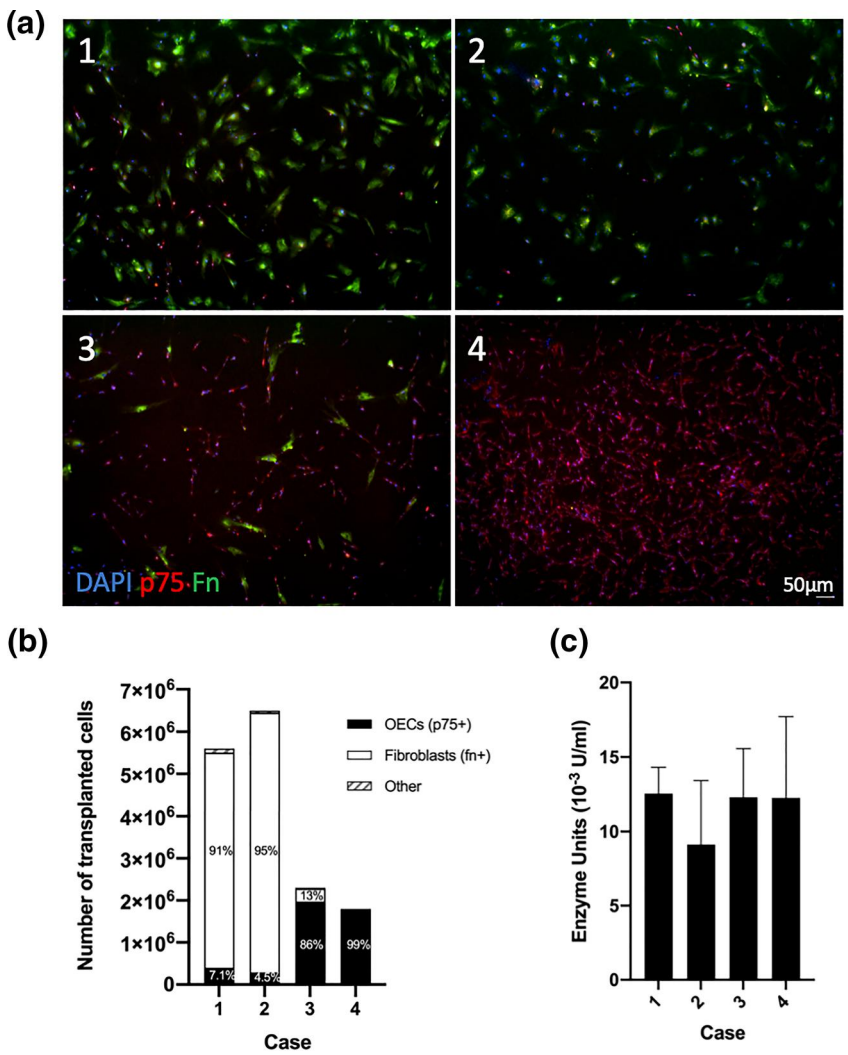

FIGURE 1 Characterization of transplanted cell population. Cell population was characterized by immunofluorescence. Example images showing p75+ (mOECs, red) and fibronectin+ (fibroblasts, green) cells, with all nuclei (DAPI, blue), for each case are shown (a). Total number of cells transplanted and the proportion of each cell type (% shown in black text) is displayed in (b). Transplanted cells were transduced to express chABC. chABC activity in vitro was determined by Morgan Elson Assay (c) with no significant difference in chABC activity seen between cases [Colour figure can be viewed at wileyonlinelibrary.com]

3.4 | MR imaging

At baseline MRI, all cases showed abnormalities in the spinal cord cranial and caudal to the previously reported site of surgery with severe spinal cord parenchymal architecture loss. This consisted of large cystic cavities surrounded by a rim of spinal cord tissue and/or cord atrophy surrounded by dural adhesions. The intramedullary hyperintense signal on T2 weighted images extended over 3 to 8 vertebral bodies, consistent with extensive cavitation and glial scarring (Figure 2a). There was no remaining compression other than in case 3 where circumferential cord constriction from adhesions was suspected. In case 4 a T2W hypointense signal and T1W mild hypointense signal was seen caudal to the lesion, interpreted as intramedullary hemorrhage. Lesions were highly heterogeneous between dogs and the cystic cavities within the spinal cord had complex morphology, as shown on 3D render images (Figure 2b).

Immediately after transplantation, small areas of T2W hypointensity were visible in case 2, 3, 4 in the central and dorsal spinal cord at the site of the lesion. This was interpreted as air introduced from injection and was reduced (case 2) or absent (case 3, 4) by post-transplant MRI 2–3 months later. No signs of spinal cord compression, ischemia or hemorrhage were seen after hydrogel transplantation.

Lesion volume at baseline was used to guide volume of hydrogel transplanted. Lesions had a mean (SD) volume of 581 (374) mm^3. All dogs had a maximum spinal cord compromise of 100%. Lesion volume, lesion length and length of MSCC were all significantly different between dogs [$F(3,6) = 16.0$, $p = 0.0029$; $F(3,6) = 14.3$, $p = 0.0039$; $F(3,6) = 19.8$, $p = 0.0016$ respectively] but did not change significantly over time, that is, between pre-transplant, immediately after transplant, and post-transplant [$F(2,6) = 3.9$, $p = 0.082$; $F(2,6) = 0.64$, $p = 0.56$; $F(2,6) = 4.9$, $p = 0.055$] (Figure 2c–e). Note the post-transplant in cases 1, 3, and 4 is 2 months, while in case 3 it is 3 months, a delay caused by the UK COVID-19 lockdown.

3.5 | Von Frey aesthesiometer results

Threshold pressure required to trigger hindlimb withdrawal (Figure 3a) was significantly different between dogs [$F(2,161) = 181$, $p < 0.0001$, mixed effects model] and over time [$F(3,920) = 5.2$, $p = 0.0007$]. Case 2 did not show hind limb reflexes at any point

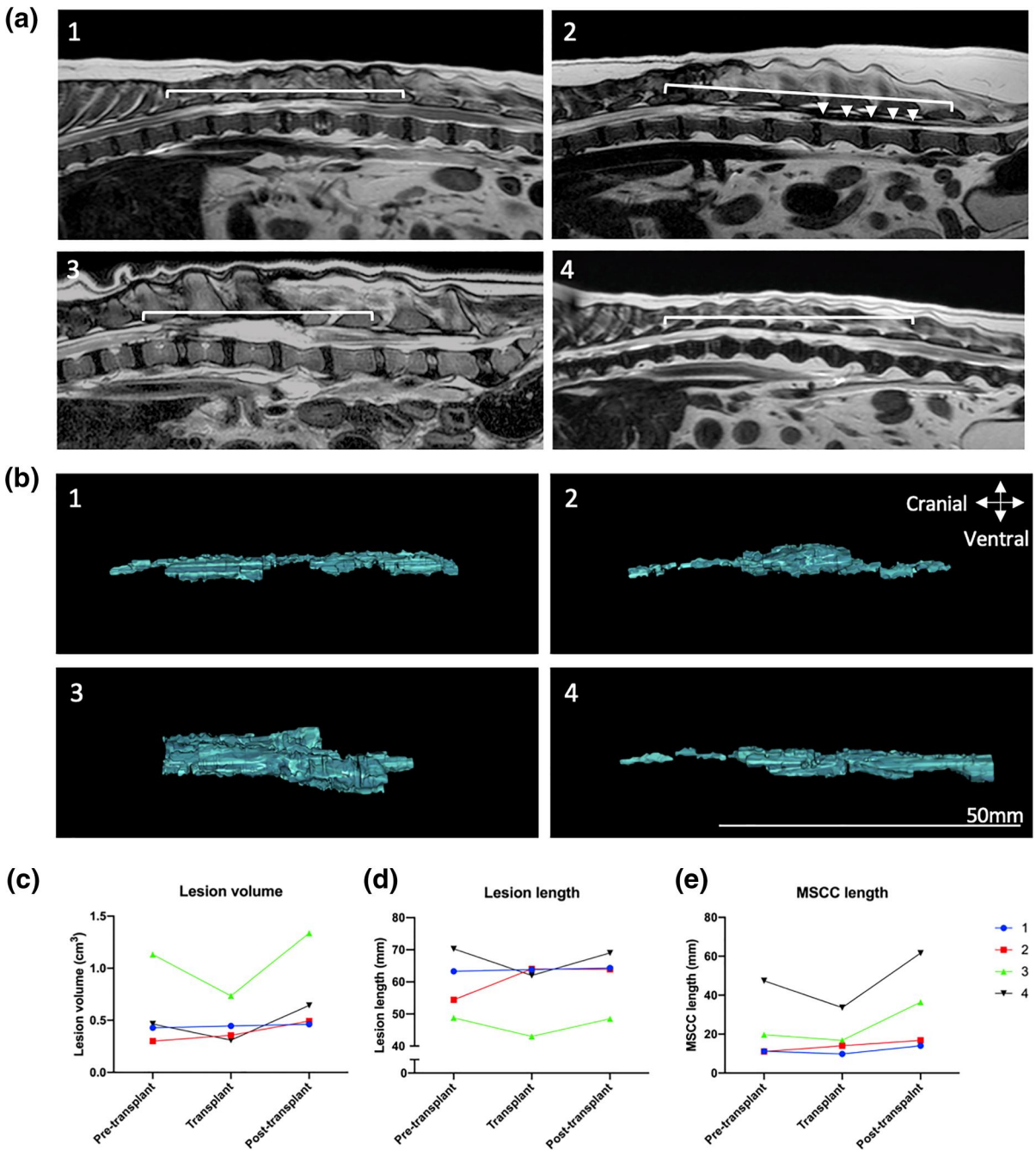

FIGURE 2 MR imaging. Example T2W sagittal images from each case are shown ((a); numbers indicate case). White bracket highlights extent and variability of T2 hyperintensity and abnormal spinal cord parenchyma. White arrow heads in case 2 mark area of spinal cord hemorrhage. Lesion volume was calculated by ROI volume measures on transverse T2W BAL TGRAD images and resulting volume renders of lesion morphology in each case are presented here viewed from left lateral (b). For each case, lesions were quantified pre-transplant, immediately after transplant, and 2–3 months after transplant and measurements of lesion volume (c), lesion length (d) and lenght of MSCC (e) are graphed. MSCC: maximal spinal cord compromise [Colour figure can be viewed at wileyonlinelibrary.com]

and was excluded from this analysis. There was no significant change in any animal between baseline and final time-point measures. There was significant variation at interim time-points: a decreased threshold in case 4 at 6 months after transplantation compared to all other time-points except baseline ($p < 0.05$ post hoc Tukey), with an increased threshold compared to baseline at 2 weeks ($p < 0.05$); a decreased threshold at 3 months compared to baseline and 24 h after transplant in case 3 ($p < 0.05$); and an increased threshold at 2 months compared to baseline and 2 weeks in case 1 ($p < 0.05$).

The threshold pressure required to stimulate the *cutaneous trunci* reflex at the most caudal level possible (Figure 3b) was significantly different between cases [$F(3,36) = 24.1$, $p < 0.0001$] but there was no statistically significant change over time [$F(3,250) = 118$, $p = 0.06$].

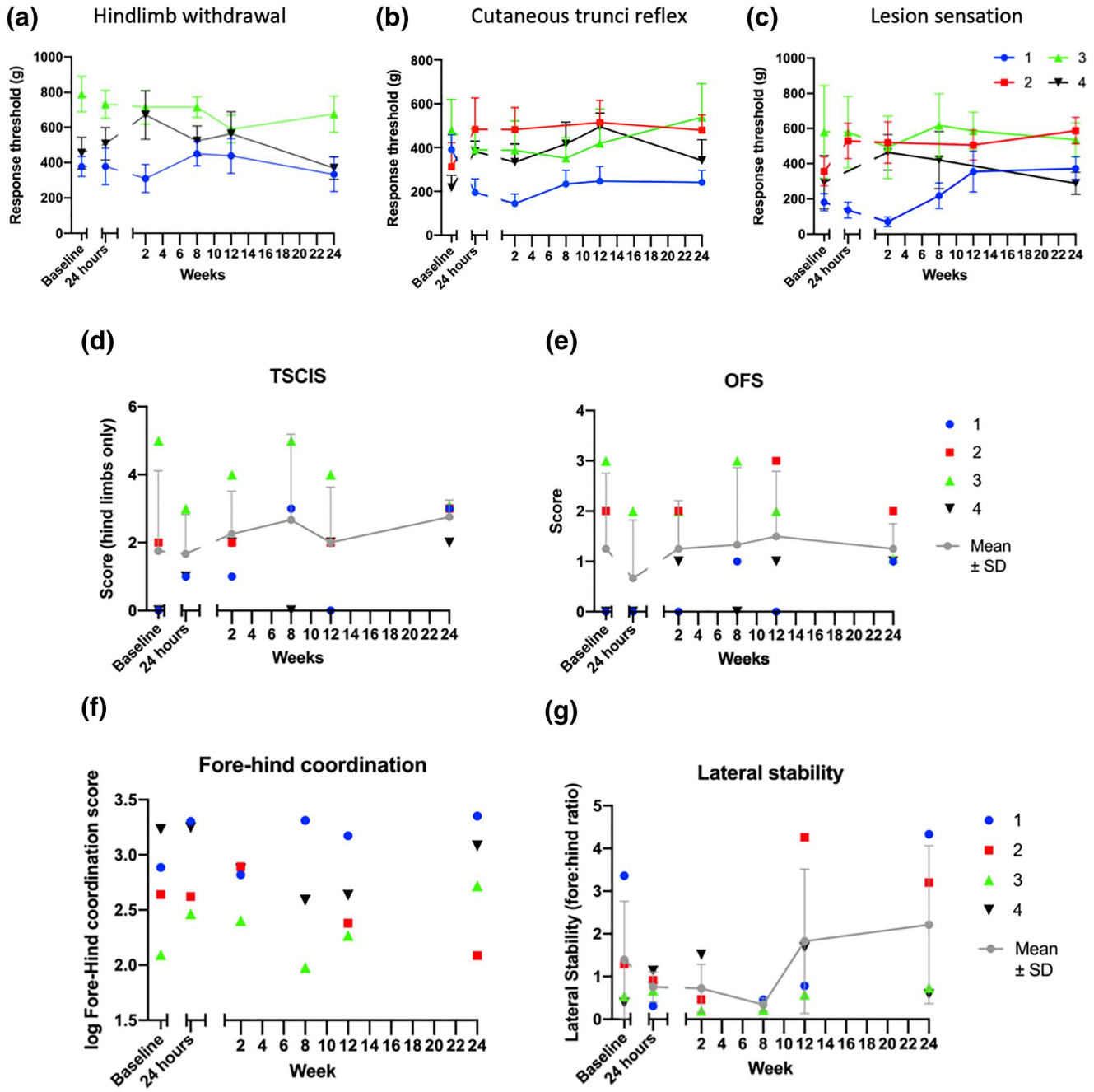

FIGURE 3 Functional testing: von Frey threshold responses and gait analysis. A von Frey aesthesiometer was used to determine pressure at which hindlimb withdrawal ((a). N.B. case 2 had flaccid paralysis and was therefore excluded from this analysis) or *cutaneous trunci* reflex (b) were triggered, and pressure at which the animal could feel and respond to stimulus at the skin overlying the lesion site (c). N.B. Case 3 was not able to return at 2 months due to the UK COVID-19 lockdown. There were significant differences between cases in all measures, and significant differences between time-points for hindlimb withdrawal and lesion sensation. There was no significant difference between baseline and final time-point for hindlimb withdrawal, but lesion sensation threshold increased significantly between baseline and 6 months in case 1 and 2 (c). Two video-based gait scores (d, e) showed no statistically significant change in gait over time. Kinematic gait analysis assessed fore-hind limb coordination (f) and lateral stability (g), with no significant change over time seen [Colour figure can be viewed at wileyonlinelibrary.com]

Sensory threshold at the skin overlying the lesion (Figure 3c) was statistically significantly different between cases [$F(3,201) = 67.5$, $p < 0.0001$] and over time [$F(4.3, 173) = 3.5$, $p = 0.008$]. In case 1, sensitivity threshold at 3 and 6 months was greater than at baseline, 24 h and 2 weeks ($p < 0.05$, post-hoc Tukey test). In case 2, baseline sensitivity was less than all other timepoints ($p < 0.05$) and in case 4 sensitivity threshold at 2 weeks was higher than at baseline and 6 months ($p < 0.05$). No significant changes were seen in case 3.

3.6 | Gait analysis

No dog could step with their pelvic limbs unsupported at the start of the trial. Case 1 showed evidence of spinal walking from before transplantation, making stepping movements when its tail was pinched.

Gait scores on the treadmill varied between follow-up time points (Figure 3d and e) but did not consistently exceed baseline scores (5 for TSCIS and 3 for OFS) and any variation was not statistically significant [$F(5,13) = 0.63$, $p = 0.74$ for TSCIS and $F(5,13) = 0.40$, $p = 0.84$ for OFS]. Average values remained consistent over the course of the trial.

Computerized kinematic gait analysis was used to assess fore-hind limb coordination and lateral stability (Figure 3f and g). There was variation in measures between time points but no significant change between baseline and final time-point. There was a significant difference between cases in fore-hind limb coordination [$F(3,14) = 10.7$, $p = 0.0006$] but no significant change over time [$F(5,14) = 0.76$, $p = 0.59$]. There was no significant difference between cases or over time in lateral stability [$F(3,13) = 1.6$, $p = 0.24$ and $F(5,13) = 1.1$, $p = 0.40$ respectively].

We did not see a relationship between the proportion of OECs in the transplant population and any measure of functional recovery in this pilot study.

4 | DISCUSSION

This study highlights several outcome measures that could be used to design a powered and randomized controlled trial investigating efficacy of intra-spinal hydrogel encapsulated autologous mOEC-chABC transplantation. We demonstrate this therapeutic approach is safe and feasible in companion dogs with chronic and severe SCI.

Autologous mOECs and microtubule-stabilized chABC have previously been delivered intra-spinally in paraplegic dogs with few adverse effects, mainly transient post-injection pain, gastro-intestinal disturbance or urinary tract infections (Granger et al., 2012; Hu et al., 2018). In humans, adverse events following OEC transplantation are reported at a similar low rate (7.7%) consisting most frequently of fever, mild anemia and syringomyelia (Li et al., 2015). There are no reports of chABC delivery into the spinal cord of humans.

We saw no adverse effects or clinical deterioration in this study. The *cutaneous trunci* reflex was found to advance two intervertebral spaces cranially in one dog but this dog's behavior under examination is likely to have limited the reliability of reflex cut-off identification. No other clinical pathology parameters or examination findings suggested a worsening injury in this or any of the other 3 dogs.

The mild mononuclear inflammation present in CSF in two dogs in this study suggests a microglial response in the chronic phase of injury, which has been reported at sub-acute time-points (Spitzbarth et al., 2011) but not been demonstrated in CSF samples or at chronic time-points. In dogs, greater than 3 months after injury is considered the chronic phase and equivalent to around 1 year in humans (Fawcett et al., 2007; Olby et al., 2003). It is interesting to note that an inflammatory profile is not seen at 29 months after injury (case 4), suggesting resolution of inflammation only at later time points as reported in people (Greenhalgh et al., 2020).

A theoretical concern after both OEC and chABC delivery is the development of increased and aberrant spinal cord plasticity with potential sequalae of hypersensitization and neuropathic pain. Corroborating a previous report (Hu et al., 2018) we do not see evidence of this, which is encouraging for future translation to humans.

We also utilized electronic von Frey as a novel method to quantify reflexive response to mechanical stimulation and so more objectively assess any change in lumbosacral spinal cord plasticity and strengthening of local reflex circuits. The use of electrophysiology, particularly F-waves and the H-reflex, have also been reported as a means of interrogating local circuitry (Lewis et al., 2017). It would be interesting to compare these electrophysiology findings to von Frey thresholds in the same animal in a future larger scale trial.

Calculating the volume of lesion from MRI prior to transplantation and delivery of this volume of stiffness-matched hydrogel appears a safe approach to deliver liquid ("pre-gelled") hydrogel encapsulating cells in clinical lesions. Small air artifacts after transplantation have been noted in previous spinal cord cell injections (McMahill et al., 2015) and provide confidence that percutaneous injection under fluoroscopy delivers therapy into spinal cord parenchyma.

Hydrogel delivered in this manner is expected to conform to lesion size and morphology. The sort of highly variable and complex lesion morphology we report here highlights the advantages of percutaneous liquid delivery (to gel in situ), where surgical implant of even a conforming pre-gelled hydrogel would be challenging and more invasive. These findings are of relevance to a range of biomaterials and support future trials of percutaneously deliverable formulations.

Collagen hydrogel is hyperintense on T2 weighted images but was not clearly distinguishable within the lesion cavity on our MR images. Further investigation of hydrogel-lesion conformation and hydrogel degradation over time would be valuable and may be possible with (e.g., gadolinium-functionalized hydrogel [Bakker et al., 2018]). Encapsulation of OECs within collagen hydrogel could be readily extended to human patients by making use of neurosurgical grade collagen hydrogel based products such as dural sealants (e.g., DuraGen^{TM}), as has recently been demonstrated with neural stem cells (Finch et al., 2020).

Gait analysis results were variable across the 4 cases, with low TSCIS and OFS values reflecting the lack of weight supported stepping seen by investigators. Owners of case 1 reported rare weight-supported stepping from 5 months after transplantation, although it must be highlighted that the owners knew their dog received a transplant therapy. Further, the variation in locomotor outcome measures during the trial could have been caused by variation of the

dogs' motivation. It may be worth considering having owners present during gait analysis in future trials.

Two dogs did improve from no movement at baseline to limb protraction at 6 months based on blinded gait scoring (case 1 and 4), and reflected in increased hind limb reflexive movements reported by owners. This was not, however, associated with changes in objective kinematic gait assessment. The kinematic measures require a minimum level of stepping and are designed to assess long-tract regeneration, suggesting the changes seen might be due to plastic changes below the lesion.

Although our study was not powered to test for this association, we did not see a relationship between functional recovery and mOEC proportion in the transplant population, for example, case 1 had one of the lowest proportions of mOECs (4.5%) while case 4 had the highest (99%). This finding is consistent with existing literature on recovery after OEC transplantation: (i) mixed populations of OECs and fibroblasts varying from 50% down to 5% OECs have both been shown to induce similar recovery of forepaw reaching after SCI in rodents (Yamamoto et al., 2009), and (ii) a previous efficacy trial of OEC transplantation in pet dogs with SCI did not show a correlation between OEC proportion and functional recovery (Granger et al., 2012).

The wide variation in proportion of mOECs in the transplant population for each case is similar to that previously reported in canine autologous mOEC transplants (Granger et al., 2012; Ito et al., 2019), and to mOEC proportions after culture from human endoscopic biopsies (Choi & Gladwin, 2015). Purification of mOECs during culture has been an active area of research, with a number of potential methods tested including enzymatic and mechanical dissociation (as used in this study), alteration in culture media serum concentration, immunopanning, use of magnetic nanoparticles, and fluorescence activated cell sorting using a range of antibodies (for a comprehensive review see (Higginson & Barnett, 2011)). Using enzymatic and mechanical dissociation (differential trypsinization) provided the simplest approach for this pilot trial, but future work to optimize culture of OECs and obtain more consistent populations could be considered.

Despite variation in OEC proportion we do not see a significant difference in chABC expression between cases, chABC secretion therefore appears to be relatively stable between OEC and fibroblast cell types. In previous in vitro experiments we also saw minimal differences in chABC expression between transduced OECs, HEK and HeLa cell types (Carwardine et al., 2016).

Combination therapies are likely key to successful regenerative therapy, however, there has been a lack of translation of such therapies to clinics. Homogeneity of rodent models reduces variation and increases statistical power for experimental studies but is not reflective of the clinical situation and natural injuries. Companion dogs with chronic, naturally-occurring SCI may therefore provide a model to bridge this gap.

This pilot trial in a referral veterinary hospital demonstrates that percutaneous intra-spinal transplantation of hydrogel encapsulated, autologous mucosal OEC-chABC combination therapy is feasible in a clinical environment and is safe in paraplegic dogs equivalent to ASIA A grade people. Prior work in rodents has shown that mOEC-chABC mediates greater recovery of voluntary movement after SCI than mOECs alone (Prager et al., 2021), and while we do not show significant evidence of functional improvement after transplantation here in dogs, the study was not powered for this outcome. We therefore propose that a blinded and randomized controlled trial for efficacy, using the outcome measures highlighted in this report, is feasible and would be valuable in this canine clinical translational model.

AUTHOR CONTRIBUTIONS

Jon Prager: Conceptualization; Methodology; Software; Formal Analysis; Investigation; Data Curation; Writing – Original Draft; Visualization. **Joe Fenn:** Methodology; Investigation; Resources; Writing – Review and Editing; Supervision. **Mark Plested:** Investigation; Writing – Review and Editing. **Leticia Escauriaza:** Investigation; Writing – Review and Editing. **Tracy van der Merwe:** Methodology; Investigation; Resources; Writing – Editing and Reviewing; Project Administration. **Barbora King:** Investigation; Resources; Writing – Editing and Reviewing; Project Administration. **Divya Chari:** Resources; Writing – Editing and Reviewing; Supervision; Funding Acquisition. **Liang-Fong Wong:** Conceptualization; Methodology; Investigation; Writing – Reviewing and Editing; Supervision; Funding Acquisition. **Nicolas Granger:** Conceptualization; Methodology; Formal Analysis; Investigation; Writing – Reviewing and Editing; Supervision; Project Administration; Funding Acquisition.

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CONFLICT OF INTEREST

The authors declare no conflicts of interests.

DATA AVAILABILITY STATEMENT

Data will be made available on reasonable request.

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