The Long-Term Efficacy Study of Multiple Allogeneic Canine Adipose Tissue-Derived Mesenchymal Stem Cells Transplantations Combined With Surgery in Four Dogs With Lumbosacral Spinal Cord Injury

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Abstract
Severe lumbosacral pain, paraparesis or paraplegia, and urinary incontinence are common but frustrating problems in dogs with lumbosacral spinal cord injury (SCI). The surgical interventions including stabilization and decompression may not restore satisfying neurological functions in severe SCI. Adipose tissue-derived mesenchymal stem cells (Ad-MSCs) show benefits in immunomodulation, anti-inflammation, and promotion of axonal growth and remyelination, and also display efficacy in several diseases in veterinary medicine. In this report, four dogs presented with fracture of sacrum vertebrae or fracture of seventh lumbar and lumbosacral displacement after road traffic accidents. The clinical signs include lumbosacral pain (4/4), paraparesis (3/4), paraplegia (1/4), and urinary incontinence (4/4). All dogs were treated by surgical decompression with or without stabilization 1 to 7 weeks after trauma. Allogeneic canine Ad-MSCs (cAd-MSCs) were injected locally on nerve roots through the surgical region in all dogs. One dose of intravenous transplantation and 4 doses of local transplantation were also performed within 8 weeks after the surgery separately. All dogs showed significant neurological improvements with normal ambulatory ability (4/4) and urinary control (3/4) 3 months after the surgery and the first cAd-MSCs transplantation. No side effect was related to multiple cAd-MSCs transplantations during 6 months monitoring in all dogs. In conclusion, multiple cAd-MSCs transplantations could be a recommended treatment combined with surgery in dogs with lumbosacral SCI.

Keywords
lumbosacral spinal cord injury, stem cells, decompression surgery, urinary incontinence, dogs

Introduction
Spinal cord injury (SCI) is a complex pathophysiological cascade resulted from mechanical injury to the spinal cord, and could be a serious public health issue in human and veterinary medicine.¹ ². The worldwide incidence of SCI is 40–80 per one million people per year.³ However, the incidence of SCI in dogs is unknown. SCI pathophysiology consists of primary and secondary injury mechanisms. In dogs, the most common causes of primary injury to the spinal cord are intervertebral disk herniation (IVDD) and vertebral injury from motor vehicle accidents, it could lead to the result of compression, concussion, shearing, laceration, distraction, and contusion to the spinal cord parenchyma. Pathophysiological changes of resulting from the mechanical force of the primary injury include hemorrhage, disruption of cell membrane integrity of axons, and ion and neurotransmitter imbalance that immediately compromises neural function.

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Secondary injury results from (1) vascular damage and loss of autoregulation, (2) excessive release of the excitatory neurotransmitters aspartate and glutamate, (3) intracellular neuronal calcium accumulation, (4) cellular damage from the production of reactive oxygen species, and (5) the inflammatory response to acute SCI \(^4,5\). Furthermore, the evolution of the lesion after SCI involves both necrosis and apoptosis. The direct damage to myelin and the death of oligodendrocytes in white matter tracts continues for many weeks after injury, both contribute to demyelination\(^6\).

Lumbosacral SCI in dogs can result in paraparesis or paraplegia with lumbosacral pain, decreased anal and tail tone, urinary incontinence, and even loss of deep pain perception (DPP) in severe cases. The neurological deficits vary depending on the degree of SCI \(^7,8\). The standard therapeutic strategies for lumbosacral SCI are stabilization and decompression of lesion of SCI. A poor or guarded prognosis should be anticipated if there is absence of DPP of pelvic limb, perineal sensation, or bladder tone\(^9,10\). Only half of lumbosacral SCI dogs without anal reflex and perineal sensation regain urinary and fecal continence\(^1\). Since a limited recovery rate in such patients, it is important to design a novel therapy combined with standard surgical treatment to promote the regeneration of the spinal cord.

Stem cells are cells with specific functions, including the ability of self-renewal, differentiate into multilinesages, and there are different effects\(^10\). Due to the increasing interest in mesenchymal stem cells (MSCs) and their multipotential potential has led to an expansion in research involving the regeneration of tissue\(^11\). MSCs are multipotent stem cells, and can be derived from adipose tissue, bone marrow, umbilical cord, placenta, or other resources\(^12\). According to the powerful ability to renew themselves while maintaining their versatility and the ability to differentiate into adipocyte, chondrocyte, and osteoblast in vitro, MSCs are considered a suitable candidate for cell therapy\(^13\).

Adipose tissue-derived mesenchymal stem cells (Ad-MSCs) can be easily obtained in healthy individuals, and are abundant in adipose tissue. Many studies have proposed data to prove that Ad-MSCs are very safe, which makes the use of autologous or allogeneic Ad-MSCs a suitable research tool and cell therapy\(^14-16\). The therapeutic effects of Ad-MSCs transplantation have been demonstrated in several animal models of SCI and are considered to show beneficial effects include immunomodulation, anti-inflammation, and promotion of axonal growth and remyelination\(^17-19\). Several clinical studies concluded that transplantation of canine Ad-MSCs (cAd-MSCs) or MSCs from other tissue sources including bone marrow and deciduous teeth were beneficial, and could improve neurological function in dogs with IVDD or traumatic SCI \(^20-28\). However, there still lacks study about the indication of cAd-MSCs in lumbosacral SCI and urinary incontinence in dogs.

Our study aimed to understand the long-term efficacy of multiple allogeneic cAd-MSCs transplantations combined with surgery in lumbosacral SCI dogs suffering from urinary incontinence and neurological deficits of the pelvic limb. The efficacy, safety, and feasibility of multiple allogeneic cAd-MSCs transplantations were evaluated for at least 6 months.

### Materials and Methods

#### Patient Selection

Four client-owned dogs diagnosed with lumbosacral SCI, with a history of hit by car for 1 to 7 weeks, were selected in the study. All the owners signed informed consent for participation in the study. Clinical signs of dogs included lumbosacral pain, paraparesis or paraplegia, and urinary incontinence. The preoperative examinations included complete cell count, biochemical profile, coagulation test (prothrombin time and activated partial thromboplastin time), and thoracic/abdominal radiography. The infectious diseases included canine heartworm disease, Lyme disease, canine ehrlichiosis, canine anaplasmosis, canine distemper virus, canine adenovirus, canine influenza virus, canine parvovirus, canine corona virus, and giardia were tested and presented negative before the cAd-MSCs transplantation. Any life-threatening issues were treated preoperatively in the study.

The detailed information of all dogs lists in Table 1. Dog 1 has a history of surgical repair of the left sacroiliac (SI) joint at the previous clinic, and the dog was presented with pelvic limb ataxia before the treatment. Dog 2 was presented with non-ambulatory paraparesis and improved to ambulatory paraparesis before the treatment. Dog 1 was presented with large distended urinary bladder, turgid bladder wall, and difficulty of manual expression for urinary bladder, and the condition is referred as upper motor neuron bladder. Detrusor sphincter dyssynergia is a possible condition in Dog 1 when UMN bladder was presented in sacral lesion\(^29\). Dog 2, Dog 3, and Dog 4 was presented with occasional leakage of urine and the bladder was easily evacuated manually, and the condition is referred as lower motor neuron bladder.

#### Neurological Examination and Radiography

Full neurological examination included testing of postural reaction, spinal nerve reflexes, muscle tone, anal reflex, perineal sensation, and DPP was performed by one surgeon preoperatively in all dogs. All dogs were presented with normal DPP. The clinical presentation of neurological examinations is described in Table 2. The severity of neurological dysfunction is divided into five grades based on Scott H.W. and McKee W.M. (Table 3)\(^30\). Fracture of seventh lumbar (L7) and 100% lumbosacral displacement was confirmed by obtaining lateral radiographic projection of hip region in three dogs except for Dog 1 (Fig. 1). Radiographs of the thorax, abdomen, and spine were performed in all dogs to detect any presence of internal bleeding, lung trauma, long bone...
fracture, or organ damage. The radiography of the hip region in Dog 1 showed a comminuted fracture and malalignment of the sacral vertebrae (S1), one screw fixation in the left SI joint, and comminuted fracture of bilateral ilium and ischium. Dog 1 was arranged for computed tomography (CT) to assess the integrity of the sacrum and spinal canal before the cAd-MSCs transplantation. CT revealed a comminuted fracture of the sacral vertebra and narrowed spinal canal with several bone fragments in the L7-S1 segment in Dog 1 (Fig. 2).

**Resource, Isolation, and Culture of Canine Ad-MSCs**

The adipose tissue was collected from the abdomen cavity of a 4-year-old male neuter Miniature Poodle dog. The dog has received annual rabies vaccine with the non-detected antibody of *Brucella* spp. before sampling. This study was carried out in strict accordance with the recommendations of the Institutional Animal Care and Use Committee (IACUC) of Industrial Technology Research Institute (ITRI), Taiwan, under approval number ITRI-IACUC-2015-046.

Approximately 3.5 g of canine adipose tissue was cut into small pieces and washed in phosphate buffered saline (PBS) twice. Then the pieces were submitted to enzymatic digestion for 60 min at 37°C, in a solution of SF1 hMSC Medium (serum-free medium, Unimed Healthcare Inc., Taipei, Taiwan) and 1 mg/ml of collagenase NB 4 Standard Grade (SERVA Electrophoresis GmbH). The digested material was centrifuged at 1500 rpm for 10 min, and the pellet was resuspended in SF1 hMSC Medium and then seeded in tissue culture dishes (CORNING, USA). The cultures were incubated

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**Table 1. Characteristics of Four Dogs.**

| Dog   | Breed     | Age (year) | Body weight (kg) | Sex   | Duration between surgery and SCI | Diagnosis                                      |
|-------|-----------|------------|------------------|-------|----------------------------------|------------------------------------------------|
| Dog 1 | Shiba-Inu | 3          | 8.7              | Female| 7 Weeks                          | Comminuted fracture of the sacral vertebra      |
| Dog 2 | Mix       | 1          | 9.2              | Female| 2 Weeks                          | Fracture of L7 and 100% lumbosacral displacement|
| Dog 3 | Mix       | 3          | 25.7             | Male  | 3 Weeks                          | Fracture of L7 and 100% lumbosacral displacement|
| Dog 4 | Mix       | 4          | 16.4             | Male  | 1 Week                           | Fracture of L7 and 100% lumbosacral displacement|

SCI: spinal cord injury.

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**Table 2. Results of Neurological Examination and Grade of Neurological Dysfunction of Four Dogs.**

| Dog   | Proprioception | DPP | Sacral pain reflex | Patella reflex | Flexor reflex | Anal reflex/perineal sensation | Motor function | Type of urinary incontinence | Grade of neurological dysfunction (Table 3) | Lesion of spinal cord segments |
|-------|----------------|-----|--------------------|---------------|--------------|------------------------------|----------------|-------------------------------|----------------------------------------|-------------------------------|
| Dog 1 | 1              | 2   | 2                 | 2             | 2            | 0/1                          | 2              | UMN                           | Grade 2                                | S1-S3                          |
| Dog 2 | 1              | 2   | 2                 | 2             | 2            | 0/0                          | 2              | LMN                           | Grade 2                                | S1-S3                          |
| Dog 3 | 0              | 2   | 3                 | 1             | 1            | 0/0                          | 0–1            | LMN                           | Grade 4                                | L4-S3                          |
| Dog 4 | 0              | 2   | 3                 | 2             | 2            | 0/0                          | 2              | LMN                           | Grade 3                                | S1-S3                          |

DPP: deep pain perception; LMN: lower motor neuron; 1: weak; 3: strong; 2: normal; UMN: upper motor neuron; 0: absent.

**Table 3. Grade of Neurological Dysfunction**

| Grade | Description                                                                 |
|-------|-----------------------------------------------------------------------------|
| Grade 1 | Thoracolumbar pain with no neurological deficits                               |
| Grade 2 | Ambulatory paraparesis                                                       |
| Grade 3 | Non-ambulatory paraparesis                                                   |
| Grade 4 | Paraplegia with or without urinary retention and overflow                     |
| Grade 5 | Paraplegia with loss of both bladder control and deep pain perception (DPP) |

Figure 1. Lateral radiographic projection of hip region in Dog 4. There are fractures of L7 (white arrow), lumbosacral displacement, and a large bladder from lower motor neuron deficit.
at 37°C with 5% CO₂, and the medium was changed every 72 h. When 80%–90% confluence was attained, the cultures were treated with 0.05% trypsin-enzyme-linked immunosorbent assay (EDTA; Gibco, USA) and subcultured. For cell passage, the cells were washed with PBS then treated with 0.05% trypsin-EDTA. Trypsin was inactivated by the addition of SF1 hMSC Medium and the cells were centrifuged at 1500 rpm for 10 min. Cell number and viability were evaluated using the Automated Cell Counter (ADAM MC, NanoEntek, Korea).

**Immunophenotyping of Canine Ad-MSCs**

The cAd-MSCs phenotypes were evaluated by performing flow cytometry. The cells were pelleted at 1500 rpm for 5 min, washed with MACS buffer (Miltenyi Biotec), and then distributed into groups according to requirement. Here we had four markers including CD34 (R-Phycoerythrin conjugated, clone 1H6; BD), CD44 (ALEXA FLUOR® 488-conjugated, clone YKIX337.8.7; Bio-Rad), CD45 (R-Phycoerythrin conjugated clone YKIX337.8.7; Bio-Rad), and CD90 (clone YKIX337.8.7; Bio-Rad). Apart from CD90, other groups were immunostained for 30 min at 4°C in the dark. CD90 requires another 30 min of incubation for secondary antibody (R-Phycoerythrin conjugated, Rabbit anti-rat, Bio-Rad). Furthermore, there were three relative isotype control antibodies that need to be prepared, including Rat IgG2a (ALEXA FLUOR® 488 isotype control, Bio-Rad), Rat IgG2b (R-Phycoerythrin isotype control, Bio-Rad), and Mouse IgG1 (R-Phycoerythrin isotype control, Beckman Coulter), which were also incubated 30 min at 4°C in the dark. After incubation, the cells were washed and resuspended with MACS buffer. The acquisition was performed by using a FACScan (BECTON DICKINSON) with 5,000 events per sample. Lastly, data were analyzed by using FlowJo software (Tree Star).

**Canine Ad-MSCs Differentiation Assays**

The cAd-MSCs were seeded in duplicate at 8 × 10³ cells/well in Costar 12-well plates (CORNING), and transferred to differentiation media one day later. The cells were maintained for 30 days with a media change every 3 to 4 days. The Mesenchymal Adipogenesis Kit (CHEMICON) was used for Adipogenic differentiation. Briefly, 0.5 mM isobutylmethylxanthine, 100 μM indomethacin, 1 μM dexamethasone, and 10 μg/mL insulin were added to the fetal bovine serum (FBS) containing media (MEM alpha with 10% FBS). The cells were fixed with 4% paraformaldehyde (Sigma-Aldrich) for 40 min, and Oil Red O (CHEMICON) was used to stain the lipid drops. For osteogenic differentiation, 0.1 μM dexamethasone (Sigma-Aldrich), 10 mM β-glycerophosphate (Sigma-Aldrich), and 200 μM ascorbic acid (Sigma-Aldrich) were added to the FBS containing media. Then, they were fixed and incubated with 0.2% AgNO₃ solution (Sigma-Aldrich). For chondrogenic differentiation, 173 μM ascorbic acid (Sigma-Aldrich), 10 ng/mL transforming growth factor-β (PEPROTECH), and 10 mM ITS (Gibco) were added to the FBS containing media. Then, the cells were fixed, cut into slices section, and stained with Alcian Blue (Sigma-Aldrich).

**Cell Quality Verification After Cell Shipment and Storage**

United Specialists Animal Hospital is located 280 kilometers away from ITRI. The cell quality was verified before this study to eliminate the influence of shipment. Three conditions of cells with low to high cell concentration were loaded in the syringe, and transported to United Specialists Animal Hospital in the refrigerated storage (2°C–8°C) for about 19–24 h, and then transported back to the laboratory in the same refrigerated storage for about 19–24 h. Count the cell concentration and cell viability with an automated cell counter before and after 2 times shipping. Calculate the cell concentration and viability again after storing in the refrigerator for 4 days. The counted cell concentration divided by the original cell concentration in the syringe is the cell recovery rate.

**Anesthesia and Surgical Procedures**

Four dogs received IV pre-med of fentanyl (3 mcg/kg), midazolam (0.3 mg/kg), cefazolin (22 mg/kg), and meloxicam (0.1 mg/kg). The dogs were inducted with propofol (4 mg/kg) and maintained with isoflurane (1%–2%) and a constant rate infusion of fentanyl (2–4 mcg/kg/h IV) after intubation.

All surgeries were performed by the same surgeons. The dogs were placed in sternal recumbency after the aseptic
A towel was supported under the abdomen of dogs to maintain elevation of the spines and pelvis from the table. An IobanTM2 (3M Health Care, Germany) drape was attached on the skin of the surgical region. A dorsal skin incision was made from the level of sixth lumbar to sacral vertebrae. The subcutaneous tissue was separated to expose the superficial sacral fascia. The superficial and deep sacral fascia was incised. All muscular attachments were elevated from the dorsal spinous processes, articular facets, and vertebral lamina by periosteal elevators and bipolar electrocautery. Dog 1, Dog 2, and Dog 3 with a history of more than 2 weeks were difficult to restore the integrity of the sacrum or alignment of L7-S1. Dorsal laminectomy was performed in these three dogs. A high-speed pneumatic drill was used to remove the outer cortical bone and medullary bone. The inner cortical bone was removed by Kerrison rongeur. Some bone fragments and fibrotic tissues which adhered to the nerves were removed in Dog 1. In Dog 4, the alignment of L7-S1 was restored by Hohmann retractors, and the stabilization was performed using two 2.7 mm string of pearls (SOP) locking plates bilaterally. Both SOP locking plates were contoured and twisted to fit the shape of the dorsolateral aspect of the L7 and the dorsomedial aspect of the SI articulation. Four screws were placed in the L7 vertebral body and SI articulation bilaterally. Dorsal laminectomy was performed in the space between both SOP locking plates. After the first local transplantation of cAd-MSCs, the muscles, fascia, subcutaneous layer, and skin were closed routinely in all dogs.

**Post-Operation Care**

All dogs were hospitalized and confined in the cage for 3–9 days after the surgery. The fentanyl patch (25 mcg/h, Durogesic D-trans Transdermal Patch, Janssen Pharmaceutica N.V., Belgium) was used for analgesia according to the body-weight of each dog for 5 days. Prophylactic antibiotic therapy (cephalexin, 22 mg/kg) and meloxicam (0.1 mg/kg) were administrated orally for 5 days. The physical and neurological examinations were evaluated every day. The urinary bladder was checked or expressed four times a day. During hospitalization, all dogs received acupuncture every other day post-operation, and also received daily manual physical therapy of pelvic limb included passive range of motion exercises and massage therapy. Daily manual physical therapy was performed by owners after discharge in all dogs. All dogs received acupuncture every two weeks after discharge. After the dog was returned to the status of ambulatory paraparesis, the rear support harness was used for rehabilitation and leash walking every day.

A subcutaneous seroma at the surgical region was noted in Dog 3 five days after surgery. Non-infectious inflammation was confirmed according to the negative result of the bacterial culture. The seroma was treated by aspiration through needle every other day for 2 weeks until the seroma vanished.

**Transplantation of Canine Ad-MSCs**

The cAd-MSCs were prepared and put into the room temperature during the dorsal laminectomy. The cAd-MSCs were mixed adequately to ensure there was no sediment in the syringe. The first dose of cAd-MSCs (5 X 10⁶, 0.5 ml) was transplanted on the nerve roots at the level of the SCI on the region of dorsal laminectomy before the wound closure. The second dose of cAd-MSCs (4 X 10⁶/kg) was diluted into 20 ml normal saline and administrated intravenously for 30 minutes immediately after the surgery. Other four doses of cAd-MSCs were transplanted epidurally every 2 weeks after the surgery and the first cAd-MSCs transplantation (Fig. 3).
After the aseptic scrub, 5 X 10⁶ cAd-MSCs were injected into the surgical decompression region by 22G X 40 mm spinal needle. In Dog 4 with double SOP locking plates, 5 X 10⁶ cAd-MSCs were injected into the decompression region between both SOP locking plates (Fig. 4). In Dog 3, the local cAd-MSCs transplantation was stopped due to the subcutaneous seroma on the surgical region. This dog only received the first local cAd-MSCs transplantation during the surgery and following IV cAd-MSCs transplantation post-operation.

Evaluations of Post-Operation

All dogs were followed for at least 6 months by regular rechecks and telephone interviews (Fig. 3). Complete neurological examinations of pelvic limbs were performed during every treatment of cAd-MSCs transplantation until the dog showed normal postural reaction and walking. Urinary continence included voluntary urinary voiding and ability of urine retention, anal reflex, perineal sensation, and locomotion of tail were evaluated every 2 weeks until 8 weeks after the first cAd-MSCs transplantation, and 12 weeks after the first cAd-MSCs transplantation. There was no side effect related to cAd-MSCs transplantations shown during the 6 months study in all dogs.

Results

Characteristics of the Canine Ad-MSCs Isolated from the Canine Adipose Tissue

The cAd-MSCs had fibroblastic-like morphology, adhered to plastic, and formed homogenous monolayers (Fig. 5A). The cAd-MSCs attained 80%–90% confluence on the fifth day. The plastic adherent cAd-MSCs were multipotent and capable of differentiating into adipocytes, osteoblasts, and chondrocytes when they were cultured in adipogenic, osteogenic, and chondrogenic mediums, respectively. To demonstrate differentiation into these cell types, staining with oil-red O, Vonkossa staining, and Alcian Blue were performed. Oil-red O staining showed lipid droplets indicating induction of adipogenesis (Fig. 5B). Osteogenesis was confirmed by the presence of a mineralized matrix (Fig. 5C) while the presence of glycosaminoglycans verified by Alcian Blue staining demonstrated induction of chondrogenesis (Fig. 5D). To characterize the stem cells, flow cytometry was also used to determine the expression pattern of CD markers. The results showed that more than 95% of the cells expressed characteristic CD markers of mesenchymal stem cells, for example, CD90 (Thy1), CD44 (HA receptor), and 1%–4% of cells were positive for hematopoietic cell CD markers, for example, CD45 (leukocytes) and CD34 (hematopoietic stem cells) (Fig. 5E).

Cell Quality Verification after the Canine Ad-MSCs Shipment and Storage

5 x 10⁵ to 1 x 10⁷ per milliliter cells were loaded in the syringe for refrigerated transportation to the animal hospital and transported back to our laboratory. The data showed that the cell viability and recovery can still be maintained for more than 90% when the cells in the syringe were transported in a refrigerated environment for 2 days. After the cells were stored in a refrigerator for another 4 days, they can still maintain the survival and recovery rate for more than 90%. This means that the cells can be stored in a refrigerated environment for at least 6 days (Fig. 6).

Improvement of Neurological Functions in Dogs

After the surgery and first cAd-MSCs transplantation, all dogs showed improvement of neurological functions within 2 weeks, and improved progressively within 12 weeks (Table 4). Lumbosacral pain was absent within 1 week in all dogs. Dog 3 with paraplegia and Dog 4 with non-ambulatory paraparesis both restored ambulatory paraparesis within 2 weeks after the first cAd-MSCs transplantation, and regained normal proprioception 4–6 weeks after the first Ad-MSCs transplantation. Dog 1 and Dog 2 with different degrees of paraparesis or ataxia restored normal proprioception within 4 weeks after...
the first cAd-MSCs transplantation. The ability of urine storage and periodic voiding regained normal 8 weeks after the first cAd-MSCs transplantation in Dog 2 and Dog 4. Dog 1 with a longer history regained urinary continence 12 weeks after the first cAd-MSCs transplantation. Weak anal reflex and normal perineal sensation were presented in these three dogs within 12 weeks, which were absent before the cAd-MSCs transplantations. Dog 3 showed absent anal reflex, perineal sensation, and locomotion of the tail till the end of the study, and the retention of bladder returned to normal with intermittent urinary incontinence.

Discussion

Lumbosacral SCI is a devastating condition that could lead to severe and permanent neurological deficits in human and veterinary medicine. In humans, an early surgical decompression is suggested in preventing secondary damage within 24 h after traumatic SCI. Unlike in human medicine, a large percentage of SCI dogs were euthanized due to delayed treatment or irreversible neurological dysfunction. In spite of the dogs with lumbosacral SCI may regain ambulatory status after surgical treatment, dogs and their owners may still suffer from the condition of urinary incontinence. Management of lower urinary tract dysfunction is frequently a long-term problem in dogs that suffered from severe chronic SCI. Caring for patients with SCI is an important and necessary topic for human and veterinary medicine as well as all those families involved, especially when the quality of life in people is impacted. The impacts of SCI go beyond the level of socially and financially of the patient and its entire family, which extends further to the community and society. The reverse of urinary incontinence and neurological dysfunction is extremely important to improve the quality of life in patients and their families.

All SCI dogs with lost anal reflex, absent perineal sensation, and urinary incontinence were selected in this study. In general, the prognosis is varied depending on the severity of SCI, duration of trauma, and treatment plans. However, lost anal reflex, absent perineal sensation, and urinary incontinence also represented a poor prognostic factor in lumbosacral SCI dogs before treatment. Dog 1, Dog 2, and Dog 4 regained weak anal reflex and normal sensation of the tail base at the end of the study. Dog 3 remained absent anal reflex.
reflex, perineal sensation, and locomotion of tail. The neural control of urinary bladder, urethral sphincter, anal reflex, and perineal sensation is origin from the lumbosacral (L4-S3) spinal cord\textsuperscript{32,38}. This may indicate the presence of anal reflex and perineal sensation after the surgical treatment and the cAd-MSCs transplantation are highly correlated with the good outcome of urinary incontinence. Furthermore, the presence of anal reflex and perineal sensation after the treatment may

**Figure 5.** Cell morphology, differentiation, and fluorescence-activated cell sorting (FACS) analysis of cAd-MSCs. (A) cAd-MSCs had fibroblastic-like morphology and formed homogenous monolayers (40X). (B) There were lipid droplets (red) in cells shown by Oil-red O staining after adipogenesis induction (100X). (C) Under osteogenic culture conditions, presence of a mineralized matrix in cAd-MSCs cells was confirmed by Vonkossa staining (100X). (D) Alcian Blue staining revealed that cAd-MSCs cells synthesized chondroitin sulfate after the induction of chondrogenesis (100X). (E) cAd-MSCs were characterized by flow cytometry using cluster of differentiation (CD) markers; and were positive to CD90 and CD44, and negative to CD45 and CD34. cAd-MSCs: canine adipose tissue-derived mesenchymal stem cells.
be helpful for surgeons to evaluate the outcome in lumbosacral SCI dogs. A study showed fewer lumbosacral SCI dogs regained urinary continence than thoracolumbar SCI dogs, and only 55% large (>15 kg), noncondrodystrophic lumbosacral SCI dogs with retained DPP regained both ambulatory function and urinary continence after decompression surgery. SCI dogs with LMN incontinence have less chance to regain urinary continence. About one-third of the dogs with intervertebral disk herniation that recovered motor function had intermittent urinary incontinence, this makes bacteriuria a common concurrent problem in dogs with chronic SCI and urinary incontinence. However, the treatment in SCI-induced urinary incontinence is limited, and only few papers mentioned the improvement of urinary incontinence in dogs with SCI after stem cells transplantation. This is the first study that demonstrated chronic urinary incontinences in dogs with SCI could be reversed after multiple administrations of allogeneic cAd-MSCs combined with surgical treatment. In this study, all lumbosacral SCI dogs with retained DPP were presented with urinary incontinence and different level of pelvic limb neurological deficits for 1 to 7 weeks. After the decompression surgery with or without stabilization and multiple cAd-MSCs transplantsations, all dogs regained normal ambulatory ability within 8 weeks. Dog 1 with UMN bladder and possible detrusor sphincter dyssynergia, and Dog 2 and Dog 4 with LMN bladder fully recovered from urinary incontinence within 12 weeks. Dog 3 with LMN bladder restored the ability of urinary retention and returned to intermittent urinary incontinence within 12 weeks. According to the limited information for the effect of stem cells therapy on SCI-caused urinary incontinence, this study may provide a promising treatment protocol for further research in SCI patients with urinary incontinence.

In this study, Dog 1 with the 7-week-history of urinary incontinence has a poor prognosis as described in a study: a poor outcome was 5.88 times higher for lumbosacral SCI dogs with urinary incontinence for history longer than 1 month. The dog has a limited improvement of urinary incontinence within 8 weeks after the surgery and the first sAd-MSCs transplantation. The sacral pain vanished, and the proprioception was returned to normal within 4 weeks after the first cAd-MSCs transplantation. After other 4 doses of local cAd-MSCs transplantation, the dog restored normal urinary function gradually 12 weeks after the surgery and the first sAd-MSCs transplantation. Dog 2, the youngest
dog in this study, had urinary incontinence and relatively mild pelvic limb neurological deficits before the surgery and the cAd-MSCs transplantations. The dog presented normal pelvic limb and urinary functions on the last local cAd-MSCs transplantation 8 weeks after the surgery and the first sAd-MSCs transplantation. Dog 3 and Dog 4 have a body weight of over 15 kg and were believed to have less chance of returning to normal neurological functions. Both cases lost ambulatory ability with urinary incontinence in the beginning. They returned to ambulatory status with ataxia within 1–2 weeks after the surgery and the first cAd-MSCs transplantation, and regained normal proprioception 1 month later. Dog 4 also presented normal pelvic limb and urinary functions on the last local cAd-MSCs transplantation. However, Dog 3 the biggest dog (25 kg) in this study developed subcutaneous seroma after surgery, and the following local cAd-MSCs transplantation was terminated. Although the dog regained the ability of urine storage, the owner still needs to do the manual expression of the bladder occasionally. In Dogs 3, multiple IV cAd-MSCs transplantations could be an alternative plan to achieve a better outcome when local cAd-MSCs transplantation was suspended.

The allogeneic cAd-MSCs were used in this study. As the SCI dogs may suffer from concurrent systemic problems after road traffic accidents, autologous cAd-MSCs may not available to obtain from the dogs with trauma. In addition, the isolation and culture of autologous cAd-MSCs spend more time than allogeneic cAd-MSCs. This may delay the treatment timing and prolong the recovery time of SCI dogs. Choosing allogeneic cAd-MSCs for time saving of cell culture, also bring convenience to clinics, surgeons, and owners.

The allogeneic cAd-MSCs used in this study were verified for cell quality before transplantation as the shipment takes about 1 day. The data showed the cell viability can be maintained for 6 days in a refrigerated environment. This time gap also makes the cAd-MSCs transplantation flexible to the owner and the surgeon to plan the surgical timing. In this study, every cAd-MSCs transplantation was performed the day after the cAd-MSCs arrival for the best efficacy of treatment in all dogs. The excellent outcome in these four dogs probably contributes to the excellent cell quality in this study.

There are different ways to deliver stem cells for SCI patients in human and veterinary medicine, included intravenous (IV), epidural application, and intrathecal (intraspinal). While IV transplantation may need more stem cells number to home the damaged spinal cord parenchyma, local transplantation could deliver stem cells directly to the damaged spinal cord. Intrathecal transplantation is proved to be safe in humans and dogs. In this study, the first cAd-MSCs transplantation was delivered on nerve root after the decompression surgery such as indicated in other studies. Another dose of cAd-MSCs was transplanted intravenously immediately after the operation. Other local cAd-MSCs transplantations were arranged for four times with an interval of two weeks after the first cAd-MSCs transplantation. The procedure of local cAd-MSCs transplantation was to inject the prepared cAd-MSCs suspension into the surgical region of nerve roots. The local injection site can be decided by recognition of the surgical anatomy by palpation or ultrasound-guided, which made the procedures simple and easy. Unlike other studies that SCI dogs need to receive general anesthesia for local cAd-MSCs transplantation. The advantage of the procedure in this study is that the dogs do not need to be sedated or anesthetized for multiple local cAd-MSCs transplantations.

Ad-MSCs improve functional recovery of the pelvic limb in SCI rats by activation of angiogenesis from upregulation of cytokine-induced neutrophil chemoattractant (CINC)-1, and further activated extracellular signal-regulated kinase (ERK)1/2 phosphorylation and Akt phosphorylation, in turn promoting vascularization, cellular survival pathways, regeneration, and stimulating neurogenesis and axonal growth. In this study, we delivered Ad-MSCs directly into the injured neural tissue through the surgical window, which allowed Ad-MSCs to bring cytokines and anti-inflammatory action to the injured environment.

A phenomenon was observed in this study. In the third and the fourth local cAd-MSCs transplantation, the dogs could not really feel the pain during the injections. But in the fifth and the sixth local cAd-MSCs transplantation, all dogs showed aggressive reactions to the injections. This can presume the nerve root in the lumbosacral region regenerated sensitivity to the pressure from the cAd-MSCs suspension. Although dogs may try to escape the restraint during the local cAd-MSCs transplantation, the small volume of cAd-MSCs (0.5 ml) can be easily injected into the target area. The nerve roots are away from the spinal cord in the surgical decompression region of sacrum vertebrae, which made the procedure safe without the concern of damage to the spinal cord from needle penetration, especially in lumbosacral SCI dogs.

The frequency of MSCs transplantation is not determined yet in human and veterinary medicine. Multiple MSC transplantations were proposed to enhance neurological recovery in humans. In this study, a total of 6 doses cAd-MSCs transplantation, included one dose of IV transplantation and five doses of local transplantation, were arranged in each dog except for Dog 3. Dog 3 received only twice cAd-MSCs transplantations and showed less neurological improvement compared to other cases, and this may be due to the worse neurological deficits and less frequency of cAd-MSCs transplantations in Dog 3. Multiple MSCs transplantations or increased frequency of MSCs transplantation should be advocated in SCI dogs with severe neurological deficits. If local MSCs transplantation is not available, IV MSCs transplantation could be an alternative solution. Dog 2 and Dog 4 showed normal pelvic limb and urinary functions on the 6th cAd-MSCs transplantation, within 8 weeks after the first cAd-MSCs transplantation. In cases with less severity such as Dog 2, less frequency of cAd-MSCs transplantation may achieve the same outcome, compared to the total of 6 doses.
cAd-MSCs transplantation in this study. In severe cases such as Dog 1 with longer SCI history and Dog 3, a total of 6 doses cAd-MSCs transplantation may provide a good outcome.

This is the first study to demonstrated treatments of lumbosacral SCI dogs by surgery combined with local cAd-MSCs transplantation, and following multiple local cAd-MSCs transplantations. Other studies applied either only one dose of MSCs during the surgery, or one or multiple doses of MSCs after the surgical treatment. There is one study transplanted one dose of MSCs during the surgery and injected intraspinally one dose of MSCs percutaneously under anesthesia one week after the surgery. In the above studies, neurological and functional improvements were observed in SCI dogs without DPP. As the spinal cord in lumbosacral region controls the pain sensation of tail base and perineal area, we selected lumbosacral SCI dogs with clinical signs including urinary incontinence and absent perineal sensation. We conclude the safety, feasibility, and a good outcome by using 6 doses of allogeneic cAd-MSCs transplantations in lumbosacral SCI dogs. A study in horses indicated that increased numbers of circulating CD8(+) T cells after multiple IV injections of allogeneic Ad-MSCs, and no adverse reaction or systemic inflammatory response were recorded. However, any possible immune response related with multiple allogeneic MSC transplantations should be carefully monitored in future human or animal cases.

Nature SCI in dogs is rather common and this makes dogs an excellent model for the comparative study of human SCI. This study may bring benefits to the research of SCI in both human and veterinary medicine. All dogs were treated by using local and intravenous (IV) cAd-MSCs transplantation with a 6-months-monitoring without any complication, which can prove the safety and feasibility of multiple allogeneic cAd-MSCs transplantations.

Conclusion

In summary, the safety and feasibility of multiple allogeneic cAd-MSCs transplantations have been proved in this pilot study. The local cAd-MSCs transplantations could be applied simply in the lumbosacral region for dogs with SCI. Multiple cAd-MSCs transplantations combined with surgical treatment can positively contribute to urinary continence and neurological improvement, and can be a potential treatment protocol in lumbosacral SCI dogs. However, we need more clinical cases to standardize the multiple allogeneic cAd-MSCs transplantations in future research of dogs with SCI.

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Ethical Approval

We confirm that guidelines on animal rights and treatment have been met and any details of approval obtained are indicated within the text of the submitted manuscript.

Statement of Human and Animal Rights

All procedures in this study were conducted in accordance with the recommendations of the Institutional Animal Care and Use Committee (IACUC) of Industrial Technology Research Institute (ITRI), Taiwan, under approval number ITRI-IACUC-2015-046.

Statement of Informed Consent

Written informed consent was obtained from a legally authorized representative(s) for anonymized patient information to be published in this article.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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