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Case report: Amniotic fluid-derived mesenchymal stem cell treatment in a dog with a spinal cord injury

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ABSTRACT

Spinal Cord Injury (SCI) refers to complete or incomplete damage to the spinal cord, which comprises the central nervous system. SCI in dogs, like humans, is mostly caused by external trauma, and the degree of impact is dependent on the location of the injury in the spine. Stem cell therapy is a promising avenue for SCI research. In this report, we investigate the therapeutic potential of amniotic fluid-derived mesenchymal stem cells (AF-MSCs) in dogs with spinal cord injuries. A 2-year-old male beagle dog presented with sensory and motor incomplete symptoms resulting in an inability to control the legs, hips, and genitourinary system due to an injury in the lumbar region of the spinal cord. In addition to the administration of surgical decompression, AF-MSCs were directly injected into the damaged spinal tissue. Approximately 15–16 weeks after stem cell transplantation, the dog's hind limb movement improved, and spinal cord regeneration was confirmed through magnetic resonance imaging (MRI). Eventually, the dog was able to walk independently, although not perfectly. In conclusion, AF-MSC-based stem cell transplantation may be beneficial for SCIs.

Introduction

Spinal cord injuries (SCIs) damage the vertebral column, which affects sensory, motor, or autonomic functions below the level of the injured region (Hu et al., 2018). In the case of spinal injury, primary tissue damage ensues immediately after the injury has occurred; thereafter, cell death begins and biochemical and vascular damage progresses (Ghavami et al., 2014; Olby, 1999). Subsequent secondary damage involves an inflammatory response, ischemic cascade, and neurotransmitter imbalance, and can occur minutes or weeks after the injury (Yu & He, 2015).

Although recent advances in medical care services can alleviate progressive neurological deterioration, there are still limited therapies that allow the functional restoration of a completely damaged spinal cord (Toreih et al., 2018). Ongoing research is therefore needed to improve the treatment of degenerative neurological disorders, and clinical trials have recently been conducted in this regard (Kim et al., 2011; Prado et al., 2019).

One of the avenues being explored is that of stem cells; the therapeutic ability of stem cells renders them a promising treatment for humans as well as companion animals (Wei et al., 2013). Stem cells are undifferentiated cells that are able to differentiate into multiple specialized lineage cell lines and have the capacity for self-renewal. Stem cells are known to migrate to damaged regions in the body and are involved in the repair process (Kassem et al., 2004).

Mesenchymal stem cells (MSCs), a branch of adult stem cells, originate from the mesenchyme, which is connective tissue derived from the mesoderm of the embryonic germ layer (Alhadlaq & Mao, 2004). Amniotic fluid-derived mesenchymal stem cells (AF-MSCs) have recently been explored as a novel regenerative treatment for degenerative diseases (in 'tAnker et al., 2003). AF-MSCs have characteristics that make them attractive for this application: multipotent differentiation, easy isolation after birth, reduced donor damage, non-tumorigenesis, low immune response, and acceptable ethical considerations compared with other sources of MSCs (De Coppi et al., 2007; Galende et al., 2010). Thus, AF-MSCs are increasingly used in the treatment of several diseases as well as tissue damage, including spinal cord injuries (Kim et al., 2014a).

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Based on numerous studies, AF-MSCs are considered as a potential therapeutic stem cell source for the treatment of various neurodegenerative disorders (Gaggi et al., 2022). Furthermore, AF-MSCs are not considered ethically contestable stem cells and may have therapeutic utility to a greater extent than other types of MSCs. A previous study reported that canine AF-MSCs have great potential for neural precursor cell differentiation in vitro (Kim et al., 2014b). These AF-MSCs exhibit neural differentiation and express neuron cell markers, such as dopamine and tyrosine hydroxylase (Kakishita et al., 2000). In this case report, we describe the application of allogeneic AF-MSCs for the treatment of an acute lumbar SCI in a dog.

Case presentation

A 2-year-old male beagle weighing 9 kg suffered an acute SCI between the lumbar 3 and lumbar 4 vertebrae (L3, L4) due to trauma. The dog was unable to stand due to a loss of control of the hind limbs and hips, as well as symptoms indicating a loss of sensory and motor function. Treatment of this SCI by veterinarians began with surgical decompression and concurrent stem cell transplantation prior to secondary tissue injury.

The Surgical operations were performed by the Animal Medical Center of Chungnam National University (Daejeon, Republic of Korea). Following anesthetization with intravenous injections of ketamine at 6 mg/kg (Yuhan Co., Seoul, Republic of Korea), anesthesia was maintained with 2 % isoflurane (Ha Na Pharm Co., Ltd., Seoul, Republic of Korea). Immediately following surgical decompression, previously harvested allogeneic AF-MSCs were re-suspended in MSC serum-free media (Thermo Fisher Scientific, MA, USA) and 5×10^6 cells were injected directly into the injury site between L3 and L4 using a 22-gauge spinal cord needle.

The cells were collected and characterized as canine MSCs according to the methods described in previous studies (Choi et al., 2013). The culture condition for AF-MSCs was as follows: Dulbecco's modified Eagle medium (DMEM, Merck, MO, USA) containing 10 % fetal bovine serum (FBS, Thermo Fisher Scientific, MA, USA), 5 ng/ml fibroblast growth factor (FGF, Merck, MO, USA), 10 ng/mL epidermal growth factor (EGF, Merck, MO, USA), and 1 % 500 U/ml penicillin–5 mg/ml streptomycin (PS, Merck, MO, USA); cultivation was performed in an incubator at 38.5 °C and 5 % carbon dioxide (CO_2). Structural analysis of the AF-MSCs using a fluorescence microscope (TE2000-U, Nikon, Tokyo, Japan) revealed a fibroblast-like morphology and a monolayer with a spindle-shaped appearance. These AF-MSCs were subsequently transplanted into the injured site of the spinal cord (Fig. 1).

In order to assess functional recovery after the SCI, pelvic limb function was analyzed once a week for 15 weeks after AF-MSCs transplantation. As a positive control, a normal beagle dog (3 years old) of similar weight was used to compare and analyze the effects of the SCI regeneration treatment. A modified Olby scoring test, which is used to assess pelvic limb coordination in dogs when walking, was used to evaluate the recovery of hind limb motor function (Olby et al., 2001). Pelvic limb function can be evaluated by classifying the patient according to certain levels; 1 to 5 levels, from a minimum of 0 points to a maximum of 14 points. Immediately after SCI, the behavioral scores of the dog corresponded to 0 points, indicating complete hind limb paralysis without any pain sensation. The dog treated with AF-MSCs displayed significant recovery of pelvic limb function when compared to a normal dog without injury at 4 weeks after transplantation (Fig. 2).

To evaluate spinal structural changes, magnetic resonance imaging (MRI) analysis was performed for the SCI dog treated with AF-MSCs using a 0.25 Tesla MRI scanner (vet-MR Grande, Esaote, Italy) at one and 16 weeks after transplantation. MRI analysis was conducted under general anesthesia with ketamine (6 mg/kg, IV) and 2 % isoflurane in 99.9 % oxygen (2 L/min) as described. The images were taken in the transverse and dorsal planes using T1-weighted imaging (repetition time [TR] /echo time [TE] = 520/26 ms) and T2-weighted imaging (TR/TE = 800/26 ms) with a scan thickness of 4–5 mm at the transverse section of the spinal cord to assess the quality of the collagen matrix in the injured spinal sections. The MRI sections of the SCI lumbar spine region displayed the therapeutic effects of the AF-MSCs over time. Immediately after the injury, weighted images revealed a hyper-intense lesion signal at the site of injury (Fig. 3(A), (B)). After 16 weeks of treatment with AF-MSCs, T-weighted image signals of lesions in the AF-MSC-treated SCI dog revealed the promotion of axonal remyelination of lesions in the spinal cord (Fig. 3(C), (D)).

After two years, the dog died of pneumonia, and SCI tissue between

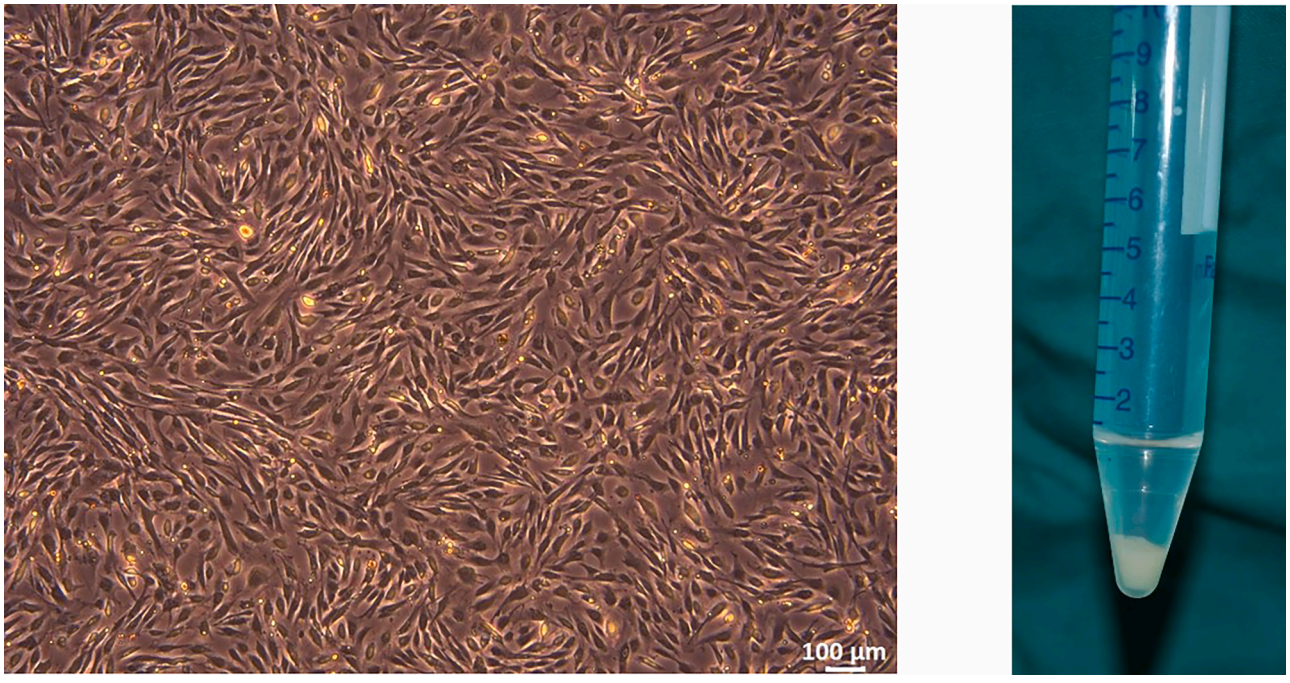

Fig. 1. Morphological characteristics of the amniotic fluid-derived mesenchymal stem cells (AF-MSCs) in culture. A photomicrograph indicating a typical fibroblast-like shaped cell (A). Magnification: ×100. After cell harvest, AF-MSCs were prepared for transplantation to the site of the spinal injury (B).

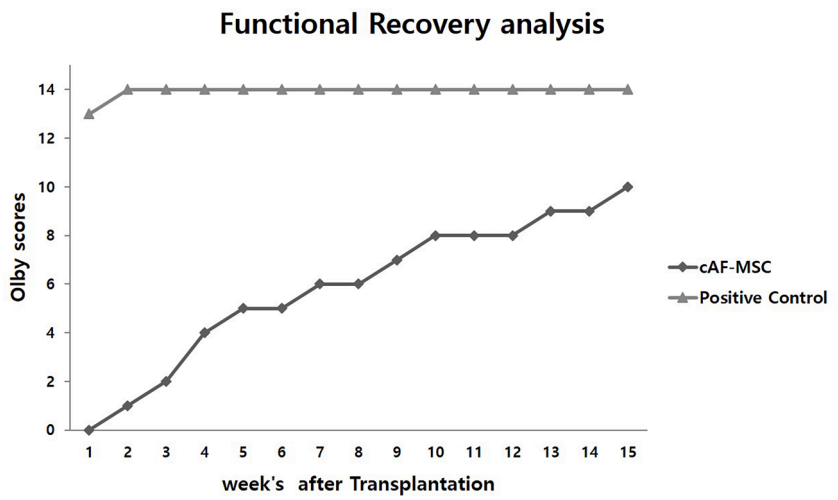

Fig. 2. Analysis of functional recovery of pelvic limb function following stem cell transplantation. Recovery scores were evaluated every single day during a 4-week period. Functional recovery testing was based on the Olby scoring system, which yields a score on a scale of 0 to 14. A score of 0 indicates no pelvic limb movement and no deep pain sensation and a score of 14 indicates a normal pelvic limb gait.

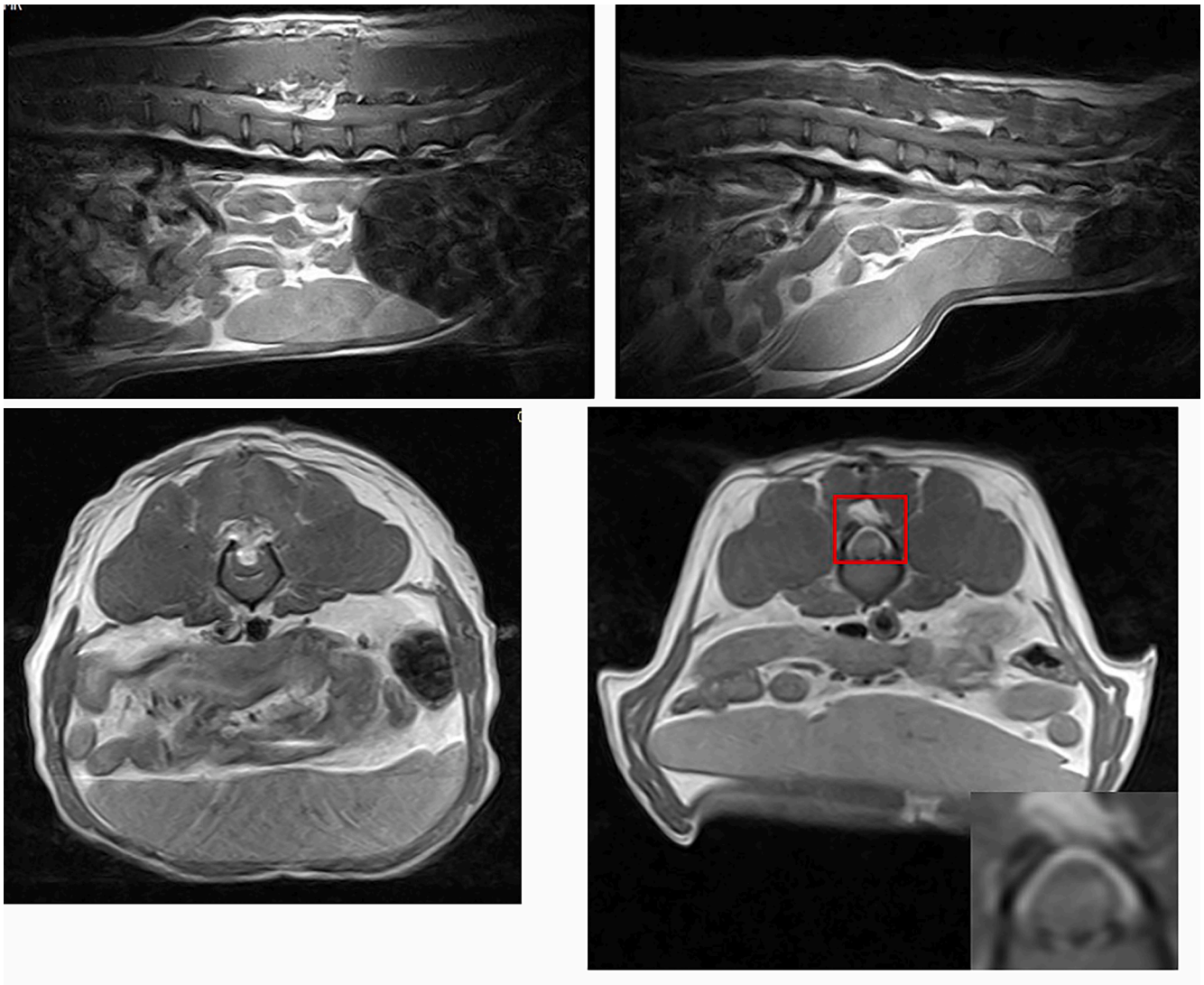

Fig. 3. Representative magnetic resonance imaging (MRI) scan images of the spinal cord injury (SCI) region. After injury, the dog had clear signals indicating an SCI lesion (A, B). Approximately 16 weeks after injury and stem cell treatment in the spinal cord (C, D). The sagittal (A, C) and transverse (B, D) sections of the lumbar region are shown, and the remyelinated vertebrae are indicated by red box.

L3 and L4 was collected and histopathological and immunohistochemical analysis were performed. Spinal cord tissue was fixed in 4 % formaldehyde solution (Thermo Fisher Scientific, MA, USA). Fixed tissue samples were processed using routine histopathological methods. Briefly, tissues were trimmed, embedded in paraffin, and sectioned using a microtome (Microm, Walldorf, Germany). For histopathological assessment, sections (4 μm-thick) were mounted on glass slides and stained with hematoxylin & eosin (H&E). The stained sections were observed using a fluorescence microscope (TE2000-U, Nikon, Japan). For immunohistochemical evaluation, the sections were deparaffinized in xylene three times for 5 min each and washed twice with 100 % and 95 % ethanol for 10 min each, respectively. Tissue sections were then placed in 10 mM sodium citrate buffer at pH 6.0 for the microwave antigen unmasking process. To inactivate the endogenous peroxidase enzyme, the sections were incubated with 3 % hydrogen peroxide. Blocking was followed by incubation with 3 % bovine serum albumin (BSA, Merck, MO, USA) in 1 × tris buffered saline (TBS, Bio–Rad, CA, USA) containing 0.1 % Tween®-20 (Thermo Fisher Scientific, MA, USA) as blocking solution. The sections were incubated overnight with a neuron-specific marker, rabbit anti-nestin antibody (1: 200, Abcam, MA, USA) and rabbit anti-bIII-tubulin antibody (1: 200, Abcam). After washing in 1 × phosphate buffered saline (PBS, Merck, MO, USA) with 0.1 % Tween®-20, sections were incubated with secondary immunoglobulin rabbit antibody (1: 200, Abcam) for 40 min before final washing. 4′.6-Diamidino-2-phenylindole (DAPI, Life Technologies, CA, USA), which has a high affinity for DNA, was used for nuclear staining. The immunostained sections were observed using a confocal laser-scanning microscope (LSM5 live configuration Vario Two VRGB), and were processed with electronics and computer modules (Real Time control system), and standard software (system configuration, ReUse function, acquisition, *Z*-function multi-tracking, presentation, image operation, fast-focusing system, etc.). Histological and immunohistochemical examination revealed that damage to the spinal cord segments was ameliorated in the stem cell-transplanted dog, which is in agreement with the MRI findings (Fig. 4). H&E staining of transverse sections of the spinal cord indicated a clear distinction between gray and white matter in the AF-MSC-treated dog. After stem cell transplantation, the expression of nerve-specific markers, nestin and β3-tubulin was confirmed in the tissue of the injured spinal region, indicating that nerve cells were regenerated.

Discussion

Damage to the central nervous system is a problem that can occur in

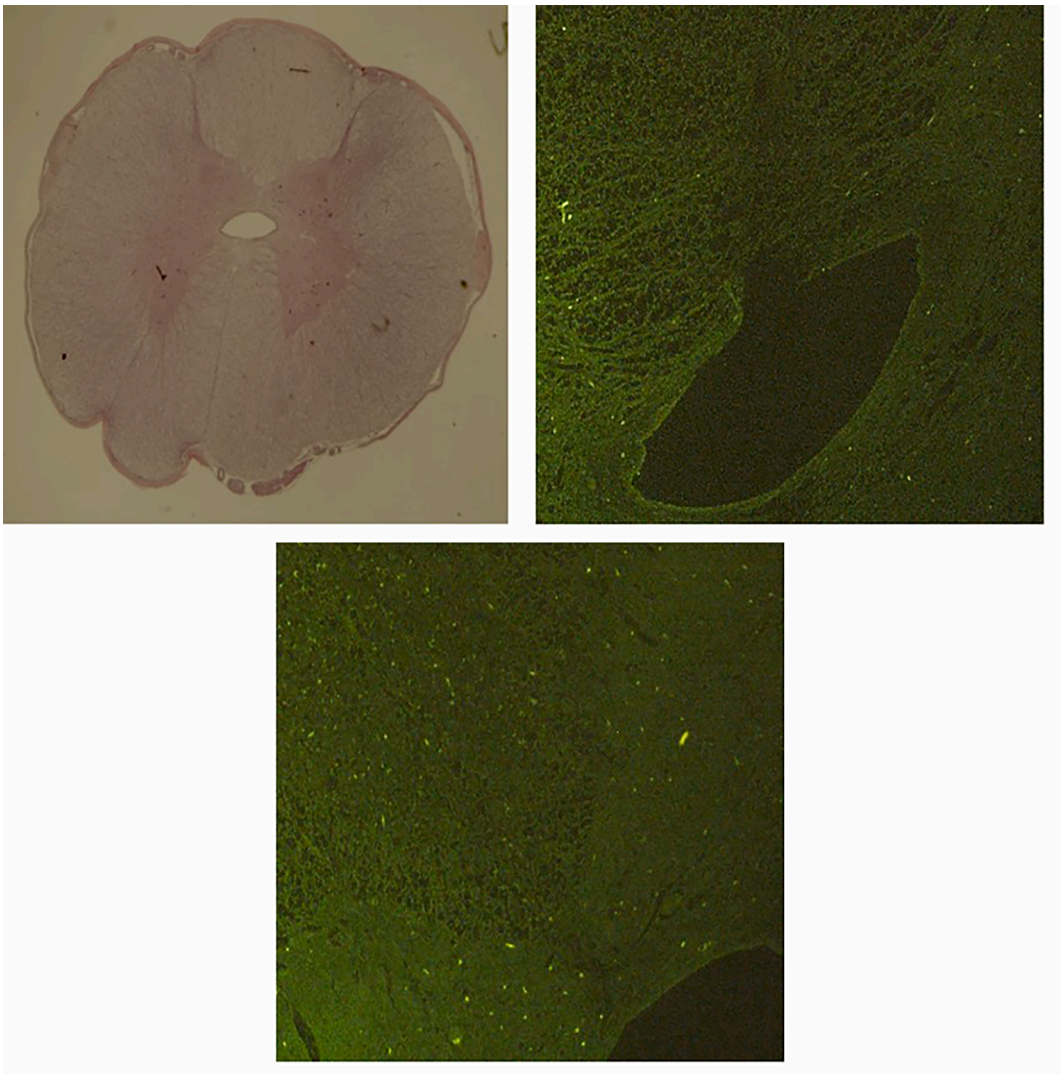

Fig. 4. Histological and immunohistochemical evaluation. Hematoxylin and eosin (H&E) staining of the transverse section of the spinal cord between L3 and L4 indicates a clear distinction between gray and white matter (A). Magnification: ×40. Expression of neuron-specific marker, nestin (B) and β3-tubulin (C). Magnification: ×100.

humans as well as in animals that share a living environment with humans (Granger & Carwardine, 2014). The process of treating these injuries is time-consuming and expensive and can lead to reduced performance, disability or euthanasia (Saadoun & Jeffery, 2021). In this study, allogeneic AF-MSCs were used to treat a dog with a damaged spinal cord. Despite the presence of an acute spinal cord injury between L3 and L4, the ataxic pelvic limb gait was almost normal and regeneration of the damaged spinal cord was observed approximately 16 weeks after stem cell transplantation. Although it is difficult to confirm that the clinical symptoms improved solely due to stem cell treatment, it can be inferred that AF-MSCs were effective in treating the SCI, considering the distinct changes before and after stem cell transplantation and the extension of the dogs lifespan.

Many researchers are optimistic that advances in stem cell therapy will make the repair of degenerative damage and the treatment of incurable diseases possible (Wei et al., 2013). The study of stem cell-based regenerative therapy has been ongoing in numerous clinical trials over the last 30 years. Bone marrow is the original source from which MSCs are derived, and it is still the most studied tissue, with the advantage of autologous transplantation along with adipose tissue-derived MSCs (Strioga et al., 2012). Recently, MSCs derived from perinatal tissues, such as umbilical cord blood, Wharton's jelly, amniotic fluid, and amniotic membranes, have been considered as new sources of stem cells (De Coppi et al., 2007)

The potential of amniotic stem cells as a novel source of stem cells for therapeutic use has been previously reported (Kakishita et al., 2000). Amniotic stem cells originating from fetal tissue are at an intermediate stage between pluripotent embryo and multipotent adult stem cells, and express MSC markers as well as pluripotency makers, such as OCT4, SOX2, KLF4, and C-MYC (Prusa et al., 2003). In addition, they exhibit low immune reaction and tumorigenic properties and have minimal ethical considerations (Smith & Jeffery, 2006). These advantages of AF-MSCs have been reported in many studies and clinical applications in a variety of injuries and diseases (Prusa & Hengstschlager, 2002).

Conclusion

Stem cell transplantation may promote neuronal reconstruction and repair motor function in a canine model of SCI, indicating that mesenchymal stem cells originating from amniotic fluids could be applied as a cell therapy for neurodegenerative diseases. The application of stem cell therapy using AF-MSCs should be encouraged for the treatment of tissue damage in clinical practice in dogs. Improvements in the applicability of cell therapy, together with further studies related to molecular pathology and repair mechanisms, are necessary for the design of improved treatment plans for cell therapy in companion animals. Further studies on the molecular pathological mechanisms of AF-MSCs for spinal regeneration and repair are needed to explore the most beneficial pathways for nerve regeneration and increase the efficiency of cell therapy in dogs.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author.

Ethics statement

The AF-MSC treatment was carried out in accordance with the conditions of "The Guide for the Care and Use of Laboratory Animals" by the Ethical Committee of the Chungnam National University (Approval no. 202206-CNU-077).

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

References

Alhadlaq, A., & Mao, J. J. (2004). Mesenchymal stem cells: Isolation and therapeutics. *Stem Cells and Development, 13*(4), 436–448.

Choi, S. A., Choi, H. S., Kim, K. J., Lee, D. S., Lee, J. H., Park, J. Y., Kim, E. Y., Li, X., Oh, H. Y., Lee, D. S., & Kim, M. K. (2013). Isolation of canine mesenchymal stem cells from amniotic fluid and differentiation into hepatocyte-like cells. *In Vitro Cellular and Developmental Biology - Animal., 49*, 42–51.

De Coppi, P., Bartsch, G., Jr., Siddiqui, M. M., Xu, T., Santos, C. C., Perin, L., Mostoslavsky, G., Serre, A. C., Snyder, E. Y., Yoo, J. J., Furth, M. E., Soker, S., & Atala, A. (2007). Isolation of amniotic stem cell lines with potential for therapy. *Nature Biotechnology, 25*(1), 100–106.

Gaggi, G., Di Credico, A., Guarnieri, S., Mariggiò, M. A., Di Baldassarre, A., & Ghinassi, B. (2022). Human mesenchymal amniotic fluid stem cells reveal an unexpected neuronal potential differentiating into functional spinal motor neurons. *Frontiers in Cell and Developmental Biology, 10*, Article 936990.

Galende, E., Karakikes, I., Edelmann, L., Desnick, R. J., Kerenyi, T., Khoueiry, G., Lafferty, J., McGinn, J. T., Brodman, M., Fuster, V., Hajjar, R. J., & Polgar, K. (2010). Amniotic fluid cells are more efficiently reprogrammed to pluripotency than adult cells. *Cellular Reprogramming, 12*(2), 117–125.

Ghavami, S., Shojaei, S., Yeganeh, B., Ande, S. R., Jangamreddy, J. R., Mehrpour, M., Christoffersson, J., Chaabane, W., Moghadam, A. R., Kashani, H. H., Hashemi, M., Owji, A. A., & Łos, M. J. (2014). Autophagy and apoptosis dysfunction in neurodegenerative disorders. *Progress in Neurobiology, 112*, 24–49.

Granger, N., & Carwardine, D. (2014). Acute spinal cord injury: Tetraplegia and paraplegia in small animals. *Veterinary Clinics of North America: Small Animal Practice, 44*(6), 1131–1156.

Hu, H. Z., Jeffery, N. D., & Granger, N. (2018). Somatosensory and motor evoked potentials in dogs with chronic severe thoracolumbar spinal cord injury. *The Veterinary Journal, 237*, 49–54.

In 't Anker, P. S., Scherjon, S. A., Kleijburg-van der Keur, C., Noort, W. A., Claas, F. H., Willemze, R., Fibbe, W. E., & Kanhai, H. H. (2003). Amniotic fluid as a novel source of mesenchymal stem cells for therapeutic transplantation. *Blood, 102*(4), 1548–1549.

Kakishita, K., Elwan, M. A., Nakao, N., Itakura, T., & Sakuragawa, N. (2000). Human amniotic epithelial cells produce dopamine and survive after implantation into the striatum of a rat model of Parkinson's disease: A potential source of donor for transplantation therapy. *Experimental Neurology, 165*(1), 27–34.

Kassem, M., Kristiansen, M., & Abdallah, B. M. (2004). Mesenchymal stem cells: Cell biology and potential use in therapy. *Basic and Clinical Pharmacology and Toxicology, 95*(5), 209–214.

Kim, E. Y., Lee, K. B., & Kim, M. K. (2014a). The potential of mesenchymal stem cells derived from amniotic membrane and amniotic fluid for neuronal regenerative therapy. *BMB Reports, 47*(3), 135–140.

Kim, E. Y., Lee, K. B., Yu, J., Lee, J. H., Kim, K. J., Han, K. W., Park, K. S., Lee, D. S., & Kim, M. K. (2014b). Neuronal cell differentiation of mesenchymal stem cells originating from canine amniotic fluid. *Human Cell, 27*(2), 51–58.

Kim, H., Zahir, T., Tator, C. H., & Shoichet, M. S. (2011). Effects of dibutyryl cyclic-AMP on survival and neuronal differentiation of neural stem/progenitor cells transplanted into spinal cord injured rats. *PloS One, 6*(6), e21744.

Olby, N. (1999). Current concepts in the management of acute spinal cord injury. *Journal of Veterinary Internal Medicine, 13*(5), 399–407.

Olby, N. J., De Risio, L., Muñana, K. R., Wosar, M. A., Skeen, T. M., Sharp, N. J., & Keene, B. W. (2001). Development of a functional scoring system in dogs with acute spinal cord injuries. *American Journal of Veterinary Research, 62*(10), 1624–1628.

Prado, C., Fratini, P., de Sá Schiavo Matias, G., Bocabello, R. Z., Monteiro, J., Dos Santos, C. J., Jr., Joaquim, J. G. F., Giglio, R. F., Possebon, F. S., Sakata, S. H., & Miglino, M. A. (2019). Combination of stem cells from deciduous teeth and electroacupuncture for therapy in dogs with chronic spinal cord injury: A pilot study. *Research in Veterinary Science, 123*, 247–251.

Prusa, A. R., & Hengstschlager, M. (2002). Amniotic fluid cells and human stem cell research: A new connection. *Medical Science Monitor, 8*, RA253–RA257.

Prusa, A. R., Marton, E., Rosner, M., Bernaschek, G., & Hengstschlager, M. (2003). Oct-4-expressing cells in human amniotic fluid: A new source for stem cell research? *Human Reproduction, 18*(7), 1489–1493.

Saadoun, S., & Jeffery, N. D. (2021). Acute traumatic spinal cord injury in humans, dogs, and other mammals: The under-appreciated role of the dura. *Frontiers in Neurology, 12*, Article 629445.

Smith, P. M., & Jeffery, N. D. (2006). Histological and ultrastructural analysis of white matter damage after naturally occurring spinal cord injury. *Brain Pathology, 16*, 99–109.

Strioga, M., Viswanathan, S., Darinskas, A., Slaby, O., & Michalek, J. (2012). Same or not the same? Comparison of adipose tissue-derived versus bone marrow-derived mesenchymal stem and stromal cells. *Stem Cells and Development, 21*(14), 2724–2752.

Toreih, A. A., Sallam, A. A., Ibrahim, C. M., Maaty, A. I., & Hassan, M. M. (2018). Intercostal, ilioinguinal, and iliohypogastric nerve transfers for lower limb reinnervation after spinal cord injury: An anatomical feasibility and experimental study. *Journal of Neurosurgery. Spine, 30*(2), 268–278.

Wei, X., Yang, X., Han, Z. P., Qu, F. F., Shao, L., & Shi, Y. F. (2013). Mesenchymal stem cells: A new trend for cell therapy. *Acta Pharmacologica Sinica, 34*(6), 747–754.

Yu, W. Y., & He, D. W. (2015). Current trends in spinal cord injury repair. *European Review for Medical and Pharmacological Sciences, 19*(18), 3340–3344.