Veterinary and Animal Science 10 (2020) 100152

Contents lists available at ScienceDirect

Veterinary and Animal Science

journal homepage: www.elsevier.com/locate/vas

# Clinical evaluation following the percutaneous transplantation of allogenic bone marrow-derived mesenchymal stem cells (aBM-MSC) in dogs affected by vertebral compression fracture

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ARTICLE INFO

*Keywords:*
Spinal cord injury
Mesenchymal stem cells
Canines
Neuronal defects
Vertebral compression fracture
Allogenic

ABSTRACT

Stem cell therapy has been extensively evaluated for its potential in managing neuronal diseases and disorders. The present study was performed to evaluate the therapeutic potential of allogenic bone marrow-derived mesenchymal stem cells (aBM-MSC) for the management of neural defects associated with vertebral compression fracture (VCF) in canine. Six clinical cases presented with the history of neural defects secondary to non-deviating VCFs were included in the present study. All the animals were subjected to detailed clinical, radiological, and haematological investigations and observations were recorded. The neurological defects in each case were graded based on routine neurological examination. The aBM-MSCs were isolated, cultured, and characterized as per ISCT criteria from the bone marrow collected from healthy dogs presented for elective surgery. The prepared cell suspension containing aBM-MSC at 3rd passage was utilized for transplantation in the clinical cases of VCF. Following the intraspinal administration of aBM-MSC, the dogs were treated with methylcobalamin and gabapentin orally throughout the study period. Improvement was evaluated on the basis of a detailed neurological examination. Significant improvement in locomotor status and sensory functions was observed in all the cases. Findings of the present study suggest that intraspinal administration of aBM-MSCs along with supportive therapy can be recommended as a therapeutic strategy for managing neural defects associated with non-deviating VCFs in canine patients.

| | |
|---|---|
| aBM-MSC | (Allogenic bone marrow-derived mesenchymal stem cell) |
| VCF | (Vertebral compression fracture) |
| SCI | (Spinal cord injury) |
| MSC | (Mesenchymal stem cell) |

## Presentation

Spinal cord injury (SCI) is a complex condition that is associated with loss of neuronal functions and results in paraplegia or paralysis (Veneruso et al., 2019). Unlike other tissues, damage to nerves can result in permanent loss of function since it has a limited capacity to repair damaged axons and to replace the lost neurons (David, López-Vales & Yong, 2012). Spinal disorders, either acute or chronic, can be caused by anomalies, degenerative conditions, neoplasia, inflammatory disease, external or internal trauma, or infarction (Braund, 2003). Spinal trauma can result either from exogenous or endogenous spinal injuries. Exogenous causes of spinal trauma include automobile-related injury, trauma from falling objects, falling from a height, and damage due to projectiles. Among these, the most common cause of spinal trauma in small animals is automobile-related injuries (Bagley, 2000). Exogenous spinal trauma often leads to vertebral fractures, subluxation or luxation depending on the position of the animal, type of force, and the point of impact. It is also greatly dependents on the inherent strength and weakness of the vertebral column (Bagley, 2000; Bali et al., 2009; Bruecker & Seim, 1993).

Although advancements in technology have improvised imaging techniques like computerized tomography (CT) and magnetic resonance imaging (MRI) to visualize damaged spinal cord, radiography and myelography still hold the position of most valuable diagnostic aids for

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https://doi.org/10.1016/j.vas.2020.100152
Received 15 January 2020; Received in revised form 14 October 2020; Accepted 14 October 2020
Available online 16 October 2020

localization the lesions in animals (Olby, Dyce & Houlton, 1994). Radiography is an invaluable diagnostic aid for spinal cord injury, and a good quality plain radiograph may provide the diagnosis in the majority of cases (Wheeler, 1989). Several therapeutic strategies have been proposed for managing spinal cord injury, including drugs, biological factors, and cellular therapy administered via different routes. Among them, stem cell therapy proved to be an advanced technique for repairing the damaged spinal cord. MSCs are multipotent stromal cells that have the potential for self-renewal and possess the capacity to differentiate into multiple cell lineages according to the specific conditions provided (Bhat et al., 2019). MSCs have been extensively studied for their utility as therapeutic agents in managing SCI (Vismara, Papa, Rossi, Forloni & Veglianese, 2017).

This study aimed to report the clinical recovery following percutaneous aBM-MSCs therapy in six dogs with a vertebral compression fracture.

## History and presenting signs

The study was conducted in canines of different age, breed, and sex presented to Referral Veterinary Polyclinic with clinical signs of spinal cord injury (SCI). The dogs that had radiological evidence of non-deviating VCFs along with detectable neurological defects characterized by paraplegia, paraparesis or ataxia were included in the study. Among the six dogs evaluated (Table 1), case numbers 1, 2, and 6 were presented with paraplegia (but intact deep pain perception) and urinary incontinence. On the contrary, case number 3, even though presented with paraplegia and intact deep pain perception, lacked urinary incontinence. However, case numbers 4 and 5 were only presented with ataxia and paraparesis, comparatively milder form of neural deficit. It is important to note that none of the dogs had lost deep pain perception. All the selected dogs were subjected to detailed clinical, neurological, radiographic, and haematological investigations.

## Physical and laboratory evaluation

The study group contained six dogs of age ranging from 1 to 5 years. Among which five were male and one female of different breeds including, three mongrels (3/6), one German shepherd (1/6), one Labrador (1/6), and one Golden retriever (1/6). VCF was found to be commonly associated with grade 4 injuries (3/6), followed by grade 2 (2/6) and grade 3 (1/6). The blood smear prepared from the ear tip blood was examined for the presence of any haemoparasitic infection. Whole blood (1 ml) in EDTA was collected from each animal to evaluate different hematological parameters. Hematological screening did not reveal any significant finding, and none of the animals had any other systemic haemoprotozoal illness.

Radiographs were made on the day of presentation. Both lateral and ventro-dorsal views of the vertebral column were used to identify and pinpoint the exact location of VCF. In the present study, lumbar vertebrae were found to be involved in maximum number of cases (4/6) followed by thoracic vertebrae (2/6). VCFs were diagnosed in L1, L2, L3, and L7 lumbar vertebrae whereas T9 and T11 were the affected thoracic vertebrae.

All the dogs were subjected to a detailed neurological examination to localize the site and type of the lesion. Animals were evaluated for postural reactions (conscious proprioception, placing, and wheel barrowing), spinal reflexes (patellar reflex, flexor reflex, and perianal reflex), gait, bladder tone, defecation control, muscle atrophy, nociception (the presence of superficial and deep pain), and recovery scores were noted at 15 days interval. The observations were correlated with radiographic findings. The neurological deficits in all the patients were graded based on the classifications put forward by Wheeler and Sharp (1994).

## Treatment

Bone marrow was collected from healthy canine patients (screened for canine distemper, canine parvovirus enteritis, ehrlichiosis, and babesiosis), which were presented to the Referral Veterinary Polyclinic for elective surgical procedures with the consent of the pet owners. The animals were anesthetized with xylazine (1 mg/kg body weight) followed by ketamine (5 mg/kg body weight) intramuscularly. The site over the iliac crest was prepared aseptically, and approximately about 5 ml of bone marrow was collected using a 16-gauge bone marrow biopsy needle. The nucleated cells were isolated by density gradient technique using histopaque (10,771; Sigma). The cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 15% fetal bovine serum (FBS; 16,000,044: Invitrogen), along with gentamycin sulphate (50 μg/ml). Once the aBM-MSCs have attained 80–90% confluence, they were treated with trypsin (0.25%) and EDTA (0.001 M) for detaching the cells. MSCs at the 3rd passage were used for further study.

The MSCs were characterised as per the ISCT (International Society for Cellular Therapy) criteria and by following the method of Ansari et al. (2013). Briefly, growth characteristics of MScs were observed for their plastic attachment and fibroblastic growth, trilineage differentiation (adipogenic, chondrogenic and osteogenic) potential, expression of certain stem cell surface markers (CD73, CD90, CD 105 and CD34) by reverse transcription polymerase chain reaction (RT-PCR) and immunocytochemistry. The MSCs were also evaluated for expression of pluripotency markers like Oct4, Nanog, SOX2 by RT-PCR assay.

MSCs at the 3rd passage showed plastic adherence and fibroblast-like morphology and were successfully differentiated into adipogenic, chondrogenic and osteogenic lineages. The aBM-MSCs were positive for stem cell surface markers (CD76, CD90 and CD105) and negative for CD34 (negative marker) both in RT-PCR and immunocytochemistry. Similarly, MSCs also showed appreciable expression of pluripotency markers (Oct4, Nanog, Sox 2) as revealed by PCR and immunocytochemistry assays. Well-characterized MSCs at 3rd passage were used for clinical application in canine cases.

After describing the therapeutic protocol (Fig. 1) and the possible complications associated with stem cell therapy, written consent was obtained from all of the dog owners as per the guidelines. All the animals were anesthetized with xylazine (1 mg/kg body weight) followed by

**Table. 1**
Clinical data of the patients with VCF selected for stem cell therapy using canine aBM-MSCs.

| Sl No. | Breed | Age | Sex | Site of lesion | Duration | Etiology | Grading* |
|---|---|---|---|---|---|---|---|
| 1 | GSD | 3year | M | L3 vertebra | 2 months | Fall from height | Grade 4 |
| 2 | Mongrel | 4year | M | L2 vertebra | 2 months | Trauma to spine | Grade 4 |
| 3 | Labrador | 1year | F | T9 vertebra | 3 months | Fall from height | Grade 3 |
| 4 | GR | 1year | M | L7 vertebra | 1 month | Fall from height | Grade 2 |
| 5 | Mongrel | 5year | M | L1 vertebra | 2 months | Trauma to spine | Grade 2 |
| 6 | Mongrel | 1year | M | T11 vertebra | 1 month | Trauma to spine | Grade 4 |

GSD – German shepherd, GR – Golden retriever.

* Clinical grading system of neurological signs in dog suggested by Wheeler and Sharp (1994). **Grade 0** – Normal, **Grade 1** - Painful back, no neurological deficits, **Grade 2** - Ataxia and Paraparesis, **Grade 3** - Paraplegia with intact deep pain perception, **Grade 4** -Paraplegia with intact deep pain perception and urinary incontinence, **Grade 5** - Paraplegia with loss of deep pain perception.

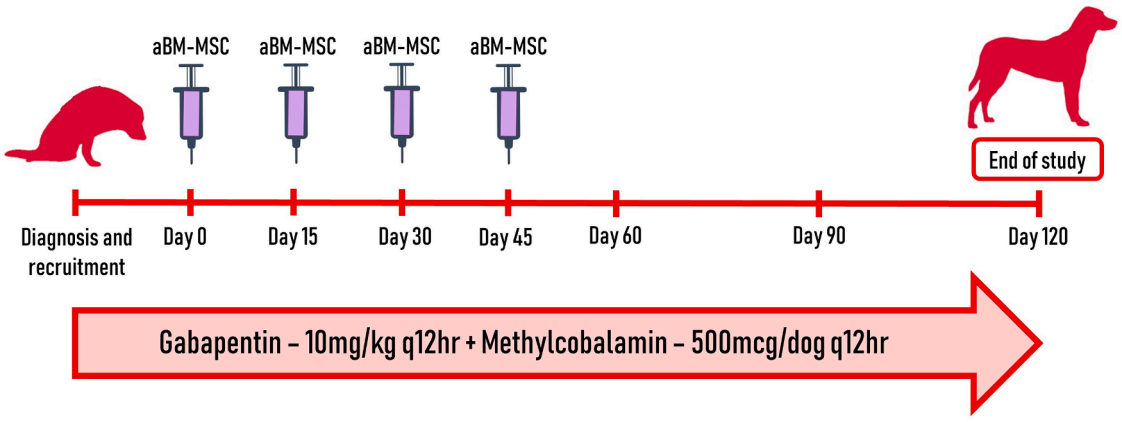


**Fig. 1. Treatment protocol.** Injection schedule for evaluating the therapeutic potential of canine aBM-MSC for managing neural deficit associated with VCF.

ketamine (5 mg/kg body weight) intramuscularly. The site of stem cell injection was decided on the basis of neurological examination and radiography. The prepared cell suspension of aBM-MSC containing 1 × $10^6$ cells/ml (1 ml) was injected just cranial to the site of lesion. The animals were positioned in ventral recumbency in such a way that the vertebral column is flexed dorsally. The spinal needle (26 gauge) was inserted slightly caudo-lateral to the spinous process of the fractured vertebrae. It was then directed through the interarcuate space cranio-ventrally at 30°−45° angle (Fig. 2). The needle was inserted until it penetrated the spinal cord parenchyma which was evident from the sudden jerk produced by the anaesthetized animal. After the tip has entered parenchyma, half of the stem cells were injected slowly into the spinal cord. The remaining half was injected while withdrawing the needle from the spinal cord parenchyma so that the stem cells are deposited at multiple layers. The dog received routine analgesia when recovered from anaesthesia and was observed for possible adverse effects for a period of 24 h. The stem cell therapy was repeated every 15 days till complete recovery to a maximum of four doses. Supportive therapy included oral administration of methylcobalamin at the dose of 500 µg/dog q12 h along with gabapentin 10 mg/kg body weight q12 h throughout the treatment period.

## Follow-up

Recovery in each case was scored using the scale (on a scale of 0 to 14) suggested by Olby et al. (2001) (Fig. 3). The recovery can occur in 5 stages in dogs with spinal cord injuries and can be further subdivided based on recovery patterns. Scores were assigned to each patient by a panel of two scientists based on the history and gait analysis. For a dog to be considered as fully weight-bearing, it should bear its full weight with extended joints for a minimum of two steps. Complications like infection, neuropathic pain, or deterioration of neurological function if any, were recorded during each visit. Survey radiographs were taken in every visit to identify any radiographic evidence of healing/complications.

All animals exhibited significant improvement in the neurological function within the study period (Fig. 4, 5, and 6). Out of the four animals having paraplegia, three animals exhibited complete weight-bearing and became ambulatory following the stem cell therapy. Even though the remaining dog did not bear weight, it showed significant improvement in the recovery score. The two animals that had grade 2 injury became completely normal following the therapy. None of the subjects had complications like infection, neuropathic pain, and deterioration of neurologic function.

The therapy with stem cells resulted in the improvement of spinal reflexes like patellar reflex and flexor reflex. Appreciable improvement was also observed in pain perception (nociception), leading to the sudden return of superficial pain perception. Postural reactions such as conscious proprioception, visual/tactile placing, and wheelbarrowing were associated with delayed recovery. All the cases presented with urinary incontinence regained voluntary control over urination.

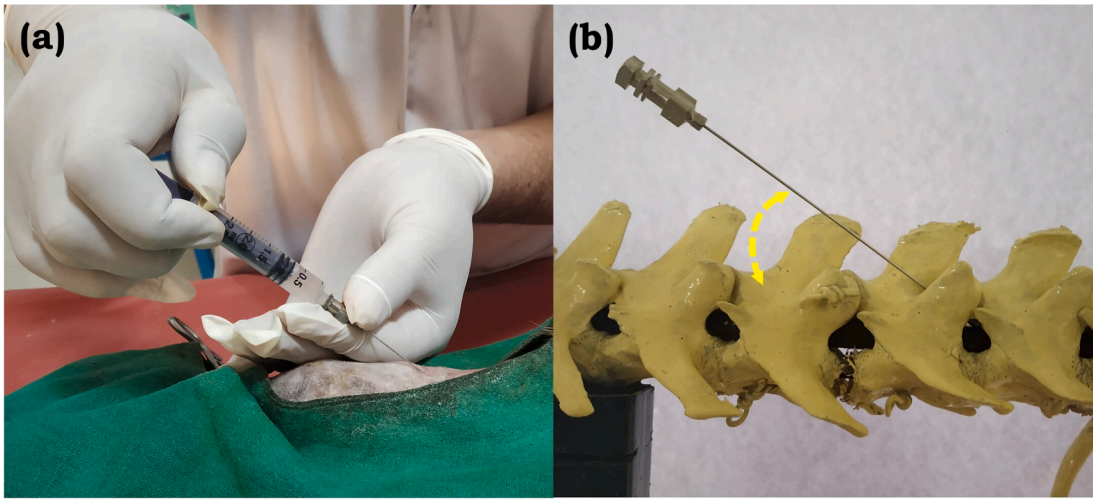


**Fig. 2. Site of percutaneous injection.** a) Following the entry of needle tip into the spinal cord parenchyma, canine aBM-MSCs was injected slowly. b) Positioning the spinal needle in between the vertebrae of canine skeleton at 30°−45° angle.


| Stage | Score | Neurological status |
|---|---|---|
| Stage 1 | 0 | No pelvic limb movement and no deep pain sensation |
| | 1 | No pelvic limb movement with deep pain sensation |
| | 2 | No pelvic limb movement but voluntary tail movement |
| Stage 2 | 3 | Minimal non-weight-bearing protraction of pelvic limb (movement of one joint) |
| | 4 | Non-weight-bearing protraction of pelvic limb with no more than one joint involved less than 50% of the time |
| | 5 | Non-weight-bearing protraction of pelvic limb with no more than one joint involved greater than 50% of the time |
| Stage 3 | 6 | Weight-bearing protraction of pelvic limb less than 10% of the time |
| | 7 | Weight-bearing protraction of pelvic limb 10-50% of the time |
| | 8 | Weight-bearing protraction of pelvic limb greater than 50% of the time |
| Stage 4 | 9 | Weight-bearing protraction 100% of time with reduced strength of pelvic limb - Mistake greater than 90% of the time |
| | 10 | Weight-bearing protraction 100% of time with reduced strength of pelvic limb - Mistake 50-90% of the time |
| | 11 | Weight-bearing protraction 100% of time with reduced strength of pelvic limb - Mistake less than 50% of the time |
| Stage 5 | 12 | Ataxic pelvic limb gait with normal strength, but mistakes made greater than 50% of time |
| | 13 | Ataxic pelvic limb gait with normal strength, but mistakes made less than 50% of time |
| | 14 | Normal pelvic limb gait |

**Fig. 3. Score card for evaluating recovery.** Recovery scoring was performed based on the scale suggested by Olby et al. (2001) which is illustrated in the figure given below.

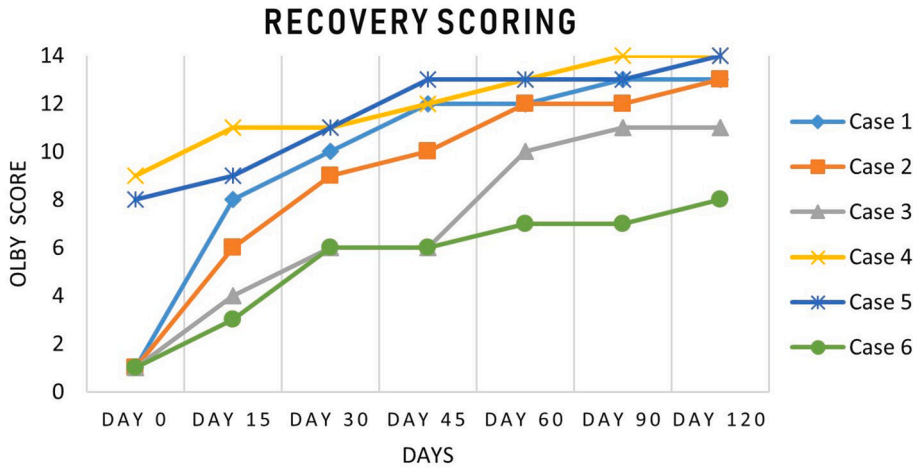


**Fig. 4.** Recovery in each case was scored using the scale (on a scale of 0 to 14) suggested by Olby et al. (2001). Scoring done on days 0, 15, 30, 45, 60, 90, and 120 to keep the track of improvement in each case.

## Discussion

Vertebral fracture or luxation can result in lack of nociceptive response in the pelvic limbs, which indicates poor prognosis for functional recovery (Bagley, 2000). The dogs that have lost deep pain reflex for greater than 48 h are not suitable for treatment due to less chance of

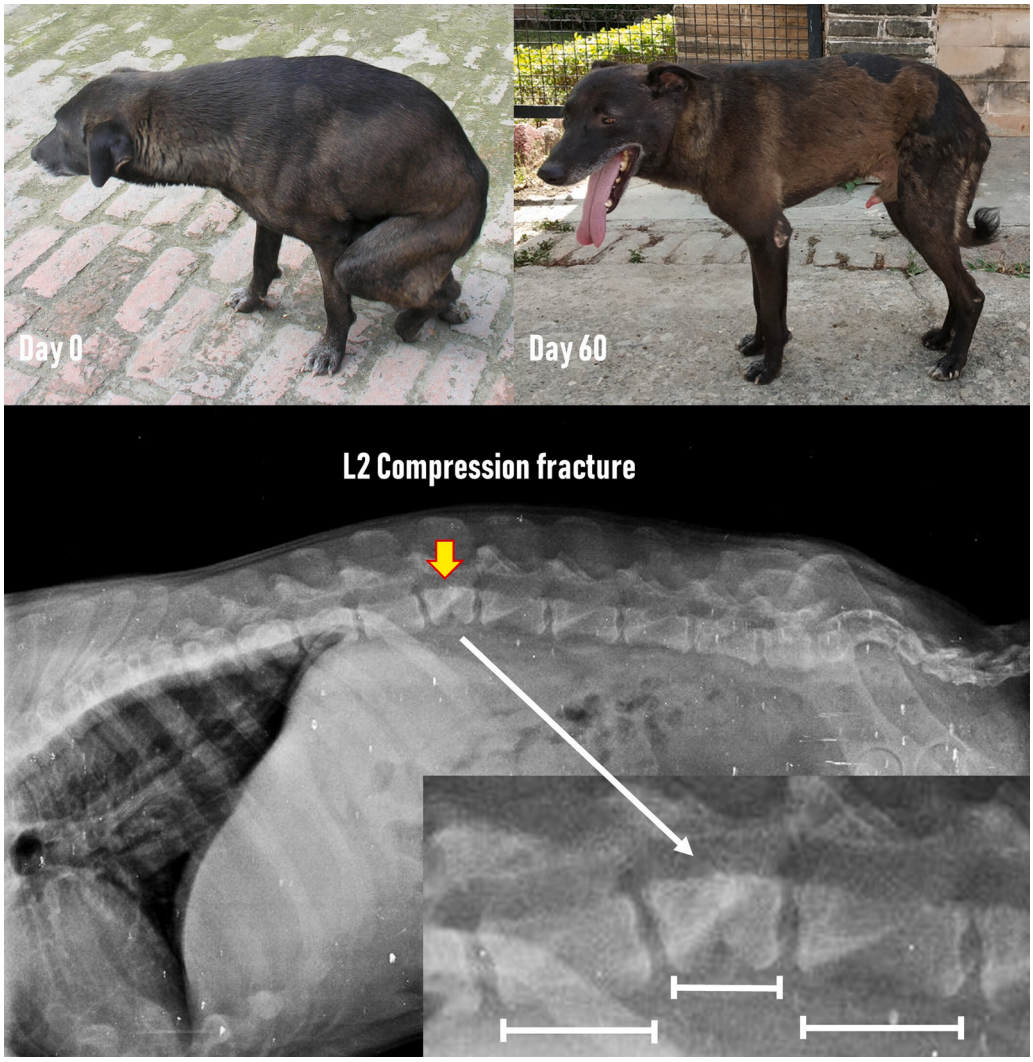


**Fig. 5. A dog presented with non-deviating L2 (second lumbar) compression fracture (yellow arrow). Day 0** – Animal was completely paraplegic with grade 4 clinical signs (Paraplegia with urinary incontinence, intact deep pain perception). Day 60 - Complete recovery with fully weight-bearing hind limbs.

regaining locomotive function in such cases (Knecht, 1972). None of the dogs in our study had a grade 5 level of SCI characterized by paraplegia with loss of deep pain perception. This might have been the reason for the promising results recorded in our study. No significant variation in haematological parameters was observed during treatment and all the animals presented were free from systemic haemoprotozoal illness. Bali et al. (2009) reported higher incidence of vertebral fracture and luxation at the thoracolumbar region (T13) in dogs. On the contrary, our study reported VCF more commonly in lumbar vertebrae than the thoracic vertebrae. It suggests that VCF can occur at any region of the vertebral column depending upon the site of injury.

In this study, Olby scale was used to evaluate the clinical improvement. Even in cases that exhibit significant behavioral improvement following stem cell therapy, it is difficult to get evidence of spinal cord regeneration in histopathological or MRI evaluation (Ryu et al., 2009). Similarly, for the diagnosis of VCF in canines, plain radiography is the simplest and most efficient diagnostic tool. Identifying the point of insult in the spinal cord can be done by combining radiography and neurological examination in situations where the advanced imaging techniques like MRI are unavailable. Different sources of stem cells have been utilized for the treatment of canine spinal cord injury like bone marrow-derived MSCs (Bhat et al., 2019), adipose-derived stem cells (Ryu et al., 2009), neural stem cells (Kim et al., 2010), canine exfoliated deciduous teeth-derived stem cells (Prado et al., 2019), immature dental pulp stem cells (Feitosa et al., 2017), and umbilical cord blood-derived MSCs (Lim et al., 2007). Stem cell therapy can be performed using autologous, allogenic or xenogenic transplantation strategies (McMahill, Borjesson, Sieber-Blum, Nolta & Sturges, 2015). Although autologous transplantation may be associated with better distribution ratio of MSCs on the injured lesion, transplantation of allogenic MSCs was associated with similar functional recovery following SCI. Therefore, both autologous and allogenic MSC transplantation could be considered as major therapeutic strategy for treating SCI (Jung et al., 2009).

Stem cells can be transplanted via routes like intravenous, intrathecal, and parenchymal techniques (Veneruso et al., 2019). Takahashi et al. (2011) conducted a study to identify the optimal route of stem cell transplantation among intralesional, intrathecal, and intravenous injections in managing SCI. Based on factors like cell viability and patient safety, it was concluded that intralesional administration of stem cells gave a better result in SCI. In the present study, the same route of administration was used. The cells were injected at multiple layers. The advantage of this technique is that it can be performed without any imaging techniques like fluoroscopy. It may also guarantee the presence of stem cells at different layers, making it available on a broader area. Rather than going for acute transplantation of stem cells in SCI, superior results are obtained when transplanted in the later stages of injury (Karimi-Abdolrezaee, Eftekharpour, Wang, Morshead & Fehlings, 2006). Following the SCI, the inflammatory process sets in, and the

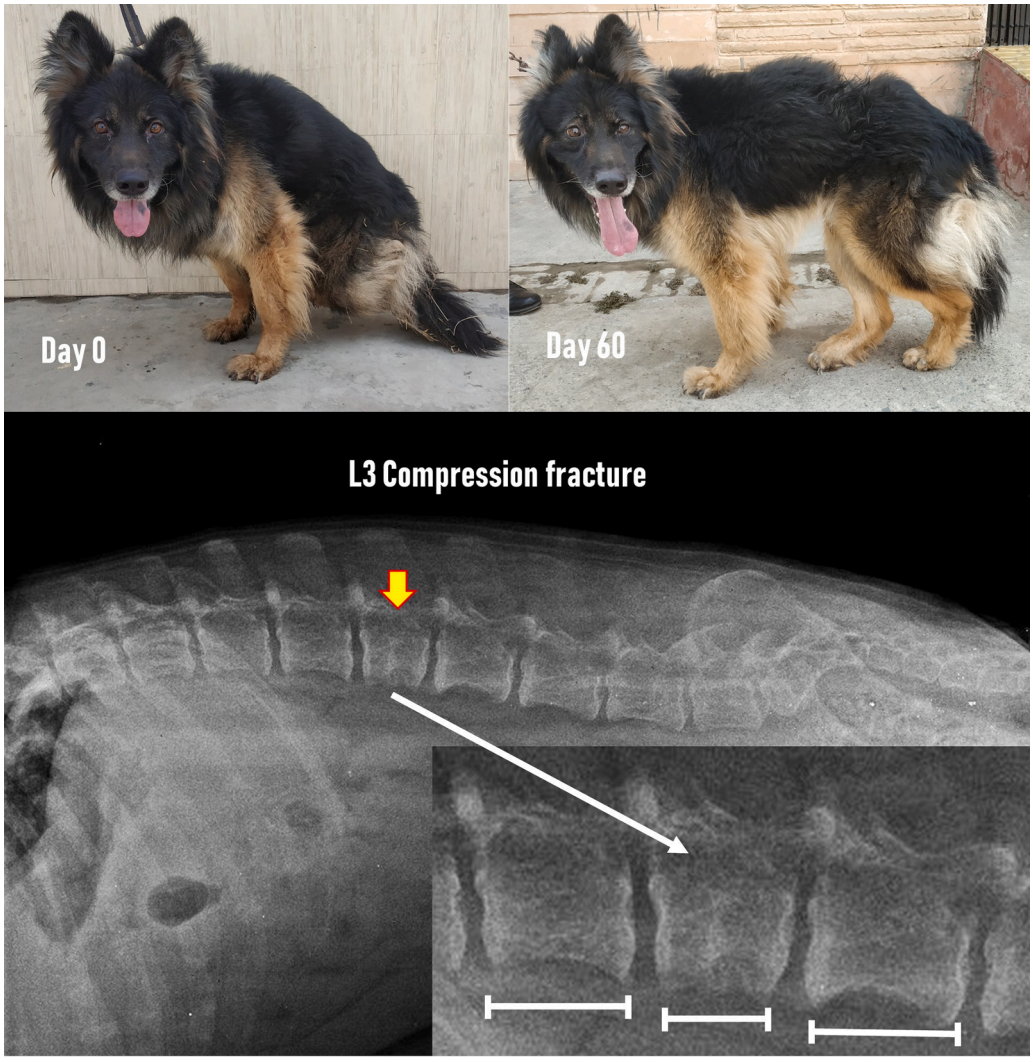


**Fig. 6.** A dog presented with non-deviating L3 (third lumbar) compression fracture (yellow arrow). Day 0 – Animal was completely paraplegic with grade 4 clinical signs (Paraplegia with urinary incontinence, intact deep pain perception). Day 60 - Complete recovery with fully weight-bearing hind limbs.

initial proinflammatory response induces secondary tissue damage. The major detrimental stages of this inflammatory response tend to occur in the initial two weeks following SCI. Hence it is advised to perform stem cell transplantation after this period to provide a supportive tissue environment for both endogenous and transplanted stem cell populations (David et al., 2012). In our study, cases were brought for treatment when this phase has already passed.

Our study suggested that percutaneous transplantation of canine aBM-MSCs has the therapeutic potential for improving the neurological status of canine patients with neural deficits secondary to VCFs. The findings from this study is significant since dogs are considered to be the translational model for human diseases (McMahill et al., 2015). The present study was performed on a small number of dogs, therefore making it difficult to establish the efficacy of stem cell transplantation. The study also lacked a control group that is critical in studies involving diseases with possibility of spontaneous recovery. But to resolve these limitations, we recruited cases that were presented 1–3 months following the initial insult and were found refractory to medical managements using steroids and other supportive therapy so that the possibility of spontaneous recovery is minimized. A second phase of the study should be conducted with a larger population and a control group to confirm the results obtained in this study.

In summary, all the animals under treatment showed progressive improvement in neurological function. Some of the cases even showed complete ambulation following a single dose of stem cell. The findings point towards the possible therapeutic potential of aBM-MSCs in SCI. The present study suggests that the percutaneous transplantation of canine aBM-MSCs can be considered as a feasible and safe therapeutic strategy for managing neural defects associated with VCF in canine patients. However, several questions are yet to be answered including the standardization of selected cases, efficient route of administration, optimum frequency of administration, and dose of cells. For this purpose, a large scale randomized clinical study should be conducted from which all the above parameters can be standardized. This will ensure that stem cell therapy using canine aBM-MSCs can be made available to the canine patients affected with VCF even at the field level.

**Funding**

Authors received no funding/grant support for this study.

**Availability of data and materials**

The figures supporting the conclusions of this article are included within the article.

## Ethics approval

The patient was treated according to current guidelines. Written consent was obtained from the owner before performing the therapy. The bone marrow samples were collected from healthy client owned canine patients after informed consent. Written consent was obtained from the owner before performing the therapy. All procedures performed are in accordance with the ethical standards of the institution at which the studies were conducted.

## Declaration of Competing Interest

None of the authors has a financial or personal relationship with other people or organizations that could inappropriately influence or bias the content of the paper.

## Acknowledgements

The authors are thankful to Director, ICAR-Indian Veterinary Research Institute, Izatnagar, Bareilly, India for providing necessary research facilities to carry out this work.

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