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RESEARCH ARTICLE

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Clinical safety of computed tomography-guided injection of autologous muscle-derived mesenchymal stem cells in the intervertebral disc in dogs

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Abstract

Background: Pre-clinical randomized controlled animal trials have been conducted to evaluate the effect of mesenchymal stem cell (MSCs) transplantation on intervertebral disc (IVD) degeneration. MSCs can be obtained from different tissues, but systematic studies concerning the effects of muscle-derived MSCs injections on canine naturally degenerated IVD are still lacking. The aim of this study is the assessment of the clinical safety of this technique and its effects on the imaging features of the lumbosacral IVD.
Methods: Eight adult healthy Beagle dogs were used in this study. In the preliminary phase, viability of muscle-derived MSCs in presence of contrast medium was assessed. In the clinical assessment phase, MSCs were injected in the lumbosacral IVD by computed-tomography (CT) guidance, after the injection of contrast medium to assess the correct intradiscal needle position. Regular clinical examinations were performed and pre- and post-injections (CT) and magnetic resonance imaging (MRI) features of the IVD were assessed.
Results: The percentage of viability of MSCs in the presence of contrast medium ranged from 90 to 98%. $3x10^6$ MSCs were obtained from six dogs and injected in the IVD. No major or minor complications were reported during the procedure and no abnormalities were noticed during the clinical examinations. No statistically significant variations were noticed between the pre- and post-injections imaging features.
Conclusion: This technique is clinically safe and it is not associated with any progression of the IVD degeneration, detected by CT and MRI imaging.

Keywords: Autologous mesenchymal stem cells; Canine model; Intervertebral discs; CT-guided

Introduction

In dogs, degeneration of the intervertebral disc (IVD) is a common process, which can predispose to disc herniation[1,2]. Four distinct components are found in the healthy IVDs. The *nucleus pulpous* (NP) mainly composed of water is the central component of the IVD and is surrounded by a fibrous layer referred to as *annulus fibrous* (AF). The inner part of the AF, which is in contact with the NP, is a transition zone characterized by an increased cartilaginous/mucoid component. Finally, the adjacent cartilaginous vertebral endplates, which are strongly connected with the inner aspect of the AF[1]. The IVD is essentially not innervated and avascular. A limited blood supply and occasional nerve endings are present only in the outer layers of the AF[1].

This avascular and low cellular nature of the IVD predisposes it to degeneration, resulting in histological change of its matrix components and dehydration. As results, decreased IVD height, annular tears and finally bulging or herniation of the IVD are commonly seen[1]. The cervical or thoracolumbar spine is commonly affected in 3-7 years-old chondrodystrophic dogs, whereas the caudal cervical or lumbosacral spine is usually involved in older non-chondrodystrophic dogs[2,3]. Reduction of pain and spinal decompression is currently the aim of treatment, but recently in human medicine, there is an increased interest in new therapies focusing on the reparation of the degenerated disc[4]. Research is focusing on different regenerative options, such as intradiscal injection of chondrocyte-like cells, mesenchymal stem cell (MSCs) and notochordal cells. The lack of immunogenicity, as well as their anti-inflammatory properties, make MSCs potentially the ideal choice for treatment of immune-mediated or inflammatory disease or to replace damaged tissue such as the degenerated NP[5,6]. MSCs can be obtained by different tissues, such as bone marrow, adipose and synovial tissue, muscle, placenta, and umbilical cord blood[6]. However, the number of

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MSCs that is commonly isolated from adipose tissue or bone-marrow is quite low with even lower numbers in older donors[5]. Pre-clinical randomized controlled animal trials have been conducted to evaluate the effect of stem cell transplantation on IVD degeneration, predominantly with bone marrow or adipose-derived stem cells[6-9]. Few of these studies have been conducted on dogs and according to their results, intradiscal injection of MSCs is effective in arresting or slowing the NP degeneration process[6-8]. Moreover, the reported complication rate is low and involves histological changes of the IVD, such as ossification and formation of large osteophyte potentially results of cell leakage as described to occur in rabbits[7], with no systematic assessment of clinical effects. To authors' knowledge, only one study had been conducted on naturally degenerated IVD, using bone-marrow derived MSCs[10] and there are no studies concerning the injection of muscle-derived MSCs in naturally degenerated IVD in dogs. Therefore, the current study was focused on the CT-guided IVD injection of MSCs, derived from muscle. The specific aim was to assess the clinical safety of intradiscal injection of muscle-derived MSCs. We hypothesized that this technique was clinically safe.

Materials and Methods

Animals

Eight healthy experimental adult dogs (4 intact females and 4 intact males, ranging from 2 to 8 years and from 14 to 18 kg) were initially included in this study with the approval of the University's Animal Care and Use Committee (nr. 1712). Two dogs out of eight were randomly chosen for the preliminary phase, whilst the definitive inclusion criteria for the clinical assessment phase were: the absence of orthopedic and neurological diseases based on history, general and neurological clinical examination; the number of $3x10^6$ MSCs obtained from muscular biopsies.

Muscle-derived MSCs culture

Microbiopsy specimens were obtained from *triceps brachii* muscles (long head) with a semi-automatic 16-gauge microbiopsy needle. For this purpose, dogs were slightly sedated by the intramuscular injection of 10 µg/kg bodyweight of medetomidine[a]. The sampling site was shaved and aseptically prepared. Each sample (approximately 15 to 20 mg of tissue) was collected through a skin incision made after application of local anaesthesia. Muscle-derived MSCs were then obtained and characterized as previously described[11]. Briefly, muscle samples were washed in phosphate buffer saline solution and cut into small pieces, which were placed individually into 24-multi-well dish pre-filled with culture medium. After 3 days of incubation in a CO2 incubator at 37 °C, the first cells started to appear around the muscle pieces. Appearance of a halo of cells indicated that the number of cells was sufficient for the isolation step. The cells are detached using tryspin, centrifuged and resuspended to allow for separation using a discontinuous Percoll density gradient (15%, 25% and 35%). After centrifugation at 200 g for 10 minutes, cells from 15–25% fraction were used for further expansion in a T-25 cm2 flask until observation of 80% confluence

Preliminary phase (viability of MSCs in contact with contrast medium)

Two healthy experimental adult dogs (dog 1 and dog 2, respectively intact female and intact male, weighing 12 kg) underwent muscular biopsy during this phase and their cells were treated as previously described[11]. Briefly, cells were treated with trypsine, centrifuged and counted. After centrifugation, the pellet was resuspended in an adequate volume of hypothermosol, in order to obtain a concentration of 1 million cells in 0.3 ml of hypothermosol. The cell suspension was divided into aliquots of 30 µl in Eppendorf tubes. Iohexol was added in increasing volumes to reach concentrations of 14.3%, 25%, 40%, and 50% in the final solution. Viability testing was made with trypan blue staining (dilution 1/5) in a Burker chamber and different concentration of iohexol[b] at varying contact times were tested.

Imaging procedures and CT-guided IVD injections of MSCs

A CT and MRI examination was performed before the injections. During the imaging procedures, the dogs were premedicated using 20 µg/kg of medetomidine[a] intramuscularly, followed by injection of 3-5 mg/kg of propofol[c] intravenously to allow for oro-tracheal intubation. When performing the CT procedure, anesthesia was maintained with a mixture of isoflurane in oxygen[d]. The MRI examination was performed using an equine standing system low-field magnet[e] of 0.27 Tesla magnetic field strength with dogs in sternal recumbency and hind limbs caudally extended. Sagittal GRE T1-w (TE 8, TR 24, thickness 1.25mm) and FSE T2-w (TE 87; TR 1815; thickness 3 mm) sequences, centered on the LS junction, were acquired. Immediately after, a CT scan examination[f], (helical acquisition mode with a slice thickness of 1 mm and a pitch of 0.8) of the lumbosacral region with dogs in sternal recumbency and cranially extended hindlimbs was performed. The CT-guided intradiscal injection of $3x10^6$ autologous MSCs was performed in aseptic conditions using a 22 gauge, 70 mm long, spinal needle[g] with a Quicke bevel. The needle insertion was followed under CT-guidance using routine biopsy software (10 short-ranging CT scans; Kv 120, mAs 50; slice thickness of 0.75 mm). To assess the correct position of the needle before the MSCs cell injection 0.1 ml of iohexol was injected (Figure 1).

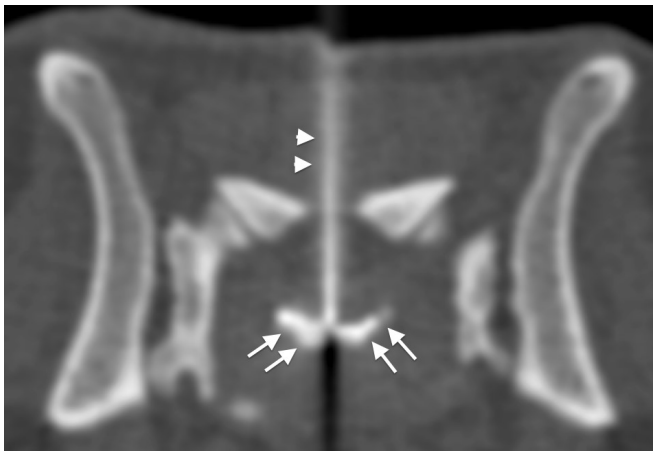

Figure 1. Transverse image (bone window) of the lumbosacral region of a dog of the injection group. After the insertion of the needle (arrowshead) in the intervertebral disc, 0.1 ml of contrast medium (arrows) was injected. The intradiscal distribution of the contrast medium confirms the correct position of the needle.

The injection of MSCs was followed again by the injection of 0.1 ml of contrast medium to allow the complete passage of MSCs from the needle to the IVD. During the procedures, the vital parameters (heart rate, respiratory rate, and oxygen saturation of hemoglobin) were carefully evaluated and recorded. A second control MRI and CT examination was performed in all dogs, eight weeks after the initial procedure

Clinical assessment

The clinical status of the dogs was evaluated performing a general and specific neurological clinical examination the day before the injections and was repeated 1, 3, 7, and 28 days after the procedure.

The general clinical and specific neurological examination focused on LS region, were performed according to a pre-established scheme based on the absence of clinical signs and presence of reflexes (Supplementary Table 1). The presence of the slightest detectable clinical signs was recoreded as 'present' and a reduction of a reflex was recorded as 'absent'. It was always performed by the same operator (MG). Any abnormalities or neurological deficits were evaluated and recorded. No other clinical or neurological examination was performed after 28 days, however all the dogs are constantly under surveillance by the person-in-charge of the experimental kennel.

Intervertebral disc imaging assessment

Pre-procedure and follow-up images of all dogs were evaluated and different types of measurements were performed blindly always by the same radiologist (AL). The images were evaluated using a free open-source software for DICOM imaging reviewing (OsiriX v.4.1.2 32-bit).

The following CT measurements (Supplementary Table 2) were performed using bone or soft tissue window (window width 2500 Hounsfield Units (HU) and window level of 500 HU and window width of 350 HU and window level of 40 HU, respectively):

1) Dorsal IVD width was calculated, measuring the width of the IVD between the dorsal aspect of the caudal endplate of the seventh lumbar vertebra (L7) and the dorsal aspect of the cranial endplate of the first sacral vertebra (S1) on sagittal reconstructed images (bone window). To avoid including dorsal or ventral spondylosis as point of reference, the dorsal aspect between L7 and S1 was defined as the dorsal point of the endplate, at the same level of the dorsal aspect of the mid-body of L7 and S1 (Figure 2).

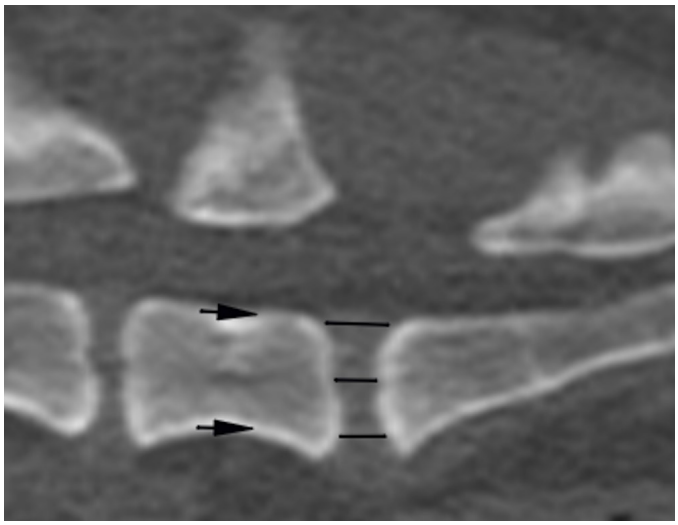

Figure 2. Reconstructed sagittal image (bone window) of the lumbosacral region of a dog of the control group, illustrating the measurements of the intervertebral disc. The dorsal width (most dorsal black line) is measured at the same level of the dorsal aspect of the mid-body (arrow) of the 7th lumbar vertebra. The ventral width (most ventral black line) is measured at the same level of the ventral aspect of the mid-body (arrow) of the 7th lumbar vertebra. The central width (central black line) is measured at the intermediate level between the dorsal and ventral width.

2) Ventral IVD width was calculated measuring the width of the IVD at the ventral aspect between the caudal endplate of L7 and the ventral aspect of the cranial endplate of S1 on sagittal bone reconstructed images. To avoid including dorsal or ventral spondylosis as point of reference, the ventral aspect between L7 and reference, the ventral aspect between L7 and S1 was defined as the ventral point of the endplate at the same level of the ventral aspect of the mid-body of L7 and S1 (Figure 2).

3) Central width of the IVD was calculated measuring the width at mid-point between the previously measured dorsal and ventral IVD width, between the caudal endplate of L7 and the cranial endplate of S1 on sagittal bone reconstructed images (Figure 2).

4) Degree of IVD calcification was evaluated for each dog measuring the HU on transverse images and reconstructed sagittal images, both on bone windows. On transverse images the region of interest (ROI) included the entire IVD (Figure 3).

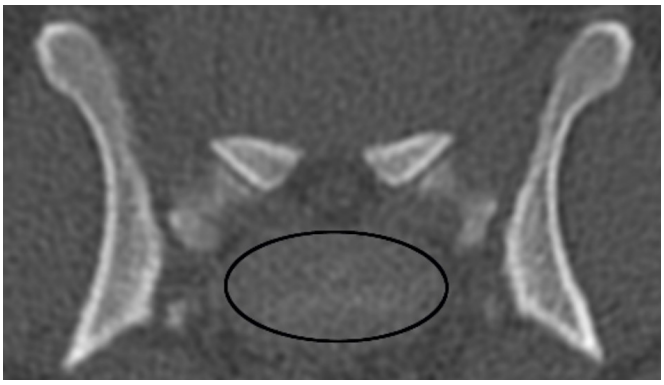

Figure 3. Transverse image (bone window) of the lumbosacral region of a dog of the injection group, showing the region of interest to calculate the Hounsfield Units of the intervertebral disc. The region of interest (IVD) is delimitated in the black circle.

On reconstructed sagittal images, the ROI was centered at the central aspect of the IVD and extended from the most cranial aspect to the most caudal aspect of the IVD. The most dorsal and ventral aspect of this region corresponded to the dorsal and ventral lines that were drawn to calculate the dorsal and ventral IVD width (Figure 4).

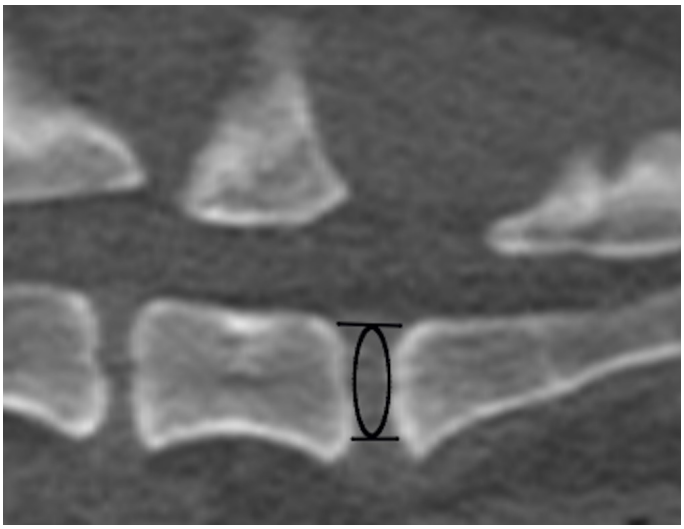

Figure 4. Reconstructed sagittal image (bone window) of the lumbosacral region of a dog of the control group. The region of interest to calculate the Hounsfield Units of the intervertebral disc is drawn between the lines used to calculate the dorsal and ventral width and extended from the caudal endplate of the 7th lumbar vertebra to the cranial endplate of the sacrum.

5) The size of the IVD and its protrusion within the vertebral canal was evaluated as ratio, using the "A index"[12]: area of disc herniation *100 / area of spinal canal, measured on transverse images (soft tissue window) (Figure 5).

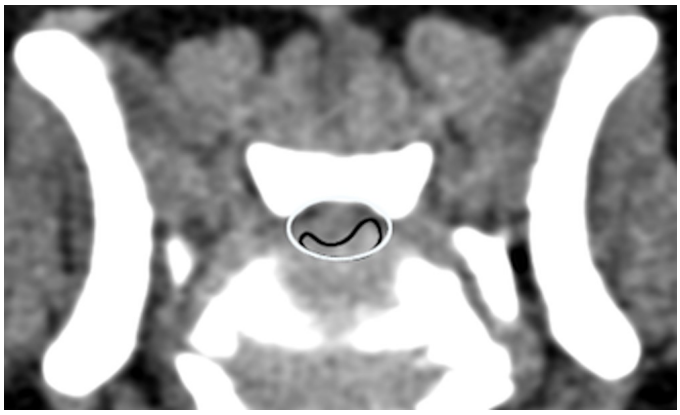

*Figure 5. Transverse image (spine window) of the lumbosacral region of a dog of the injections group. The "A index" is calculated as the ratio between the area of disc herniation (black, crescentic-like shaped, region of interest) * 100 / area of spinal canal (white, oval-shaped region of interest).*

6) The Hounsfield Units of the discal material protruding within the vertebral canal was calculated from the ROI, which was drawn for the "A index".

The following MRI measurements were performed (Supplementary Table 2):

1) Signal to noise ratio (SNR) was calculated on sagittal T1-w and T2-w images, dividing the difference in signal intensity (SI) between the ROI drawn on the LS IVD and the background just dorsal to the LS space, by the standard deviation of the signal from the background.

2) Contrast to noise ratio (CNR) was calculated on sagittal T1-w and T2-w images, measuring the difference in SNR between the LS IVD and the 6th-7th lumbar IVD.

3) Intervertebral disc degeneration on T2-w sagittal images was classified using a previous published classification system for staging the lumbar intervertebral disc degeneration[13] (Supplementary Table 3).

4) Intervertebral disc degeneration on T2-w sagittal images was also classified using the Pfirrmann grade of image[14] (Supplementary Table 4).

Each measurement was repeated three times and the average measurement was taken into account for statistical analysis. Statistically significant changes between the pre- and post-injections imaging parameters were analyzed performing a linear model with a mixed procedure, using commercially available software (R version 3.1.3.). For all analyses, P-value ≤ 0.05 was considered statistically significant.

Results

Preliminary phase

From muscular biopsies 4.000.000 and 360.000 cells (respectively dog 1 and dog 2) were obtained and diluted in 1.2 ml and 0.1 ml of hypothermosol. The percentage of viable cells varied from 90 to 98% in all tested conditions. Table 1 gives the percentages of viable MSCs at different concentrations of iohexol at increasing contact times from dog 1.

Muscle-derived MSCs culture and CT-guided IVD injections

The number of $3x10^6$ cells was not obtained in 2/8 dogs. These dogs were excluded from the study. The included dogs were 3 intact female dogs and 3 intact male dogs, (ranging from 4 to 8 years and from 14 to 18 kg). A total of $3x10^6$ cells in 0.2 ml of FRS Hypothermosol were injected in the LS IVD by CT-guidance in each dog. No particular resistance was encountered during the injection.

Clinical assessment

No major or minor complications were reported during the procedure. A total of 24 clinical examinations were performed in the days following the procedure and revealed no abnormalities. No statistical differences between the examinations before and after the injections were identified. Three months after the beginning of the project, one dog had to be euthanized because of sudden clinical degradation from a pre-existing splenic tumor, which was unrelated to the current study. Unfortunately, due to the emergency of the situation, the dog's body was discarded and no histopathology examination of the injected IVD was possible. The other five dogs were alive and did not show any particular symptoms, lameness or neurological disease.

Intervertebral disc imaging assessment

No statistically significant changes in imaging parameters were noticed pre- and post-injections. One dog underwent only CT examination, because of the presence of a metallic implant negatively affecting the quality of MRI images. This dog had received a gastric stimulator in a previous experiment and the metallic electrodes were still present in the gastric wall. The same dog underwent the second CT examination seven weeks after the procedure, instead of eight weeks as the remaining dogs.

All the dogs were considered having a degenerated IVD based on a previously published classification system for staging the lumbar intervertebral disc degeneration [13] and the Pfirrmann grade of image[14] with a grade ranging from two (only one dog) to four-five (all the other dogs).

Discussion

In this study, we evaluated the clinical safety and the effects on CT and MRI IVD features of CT-guided injections of muscle-derived MSCs in IVDs of dogs affected by natural disc degeneration. According to the results of this study, this technique is clinically safe and it is not associated with any progression of the IVD degeneration, detected by CT and MRI imaging. In human medicine, there has been an increased interest in new therapies based on the intradiscal injections of MSCs cell as regenerative therapy of the IVD. Therefore, many pre-clinical randomized controlled animal trials have been conducted and reported complication rates were 2.7%, all occurring in rabbit models[7]. These complications were related to the osteophyte formation, which was hypothetically attributed to leakage of the MSCs[15]. Recently, the clinical safety of bone-marrow derived lumbosacral IVD injection in dogs affected by natural IVD degeneration was assessed[10]. However, to the authors' knowledge, the effects of muscle-derived MSCs has not been assessed. Therefore, for the prospective application of this technique in the daily veterinary activity, it was essential to evaluate systematically the clinical effects. As inclusion criteria, we decided to inject only dogs when $3x10^6$ of autologous MSCs were obtained. This choice was empirical. In literature, indeed the number of injected cells into the IVD of different animal models is quite various, varying from $2x10^4$ to 10^7[7]. Recently it was shown that best IVD regenerative results were obtained when 10^6 autologous cells

were injected[16]. Moreover, in a veterinary study evaluating the accuracy of CT-guided intradiscal injections the administered dose varied between 0.05 ml to 0.4 ml despite a target dose of 0.5 ml[17]. Therefore, we considered appropriate the injection of $3x10^6$ MSCs diluted in 0.2 ml with 0.1 ml of contrast medium. However, further studies are needed to evaluate this aspect. Two dogs (one female and one male) failed to have a sufficient number of cells at the day of injection and one dog from the viability test phase had a low number of cells after culture. The reason for this slow growth was not particularly analyzed, but we realized that cell growths seems to be impacted by the initial explant procedure and that carefully selecting the muscle piece may positively affect the growing speed of the cells.

In our study, we evaluated the clinical safety by performing general and specific neurological examination. This latter aimed to specifically evaluate the lumbosacral region and follows a pre-established scheme. The same clinical examination allowed us to examine the site of muscle biospy, and no adverse effect was noticed. The examination was designed based on a simple yes/no scale as can be seen in the supplementary table 1. The slightest suspicion of a clinical sign was considered as present and a slight reduction or slowing of a reflex was considered as 'reflex absent'. Like this, the sensitivity of the clinical exam increased. No modification was detected in any of the dogs at any time point therefore, no statistical analysis was performed. Indeed, the first clinical examination was done 24 hours after the injection, and probably slight transitory clinical signs had disappeared by then.

To rule out other possible non-clinical complications, such as osteophyte formations, and to compensate for the absence of histological examinations, which could be considered the biggest limitation of our study, we evaluated the effects on CT and MRI IVD features, comparing pre- and post-injections results. In human, common evaluated parameters are changes in disc height, NP rehydration on T2 weighted MRI images, and histologic disc degeneration grade. These parameters are also considered as the objective outcome of disc regeneration. Indeed, it seems that MSCs replicate and differentiate toward NP cells when injected into a degenerated site, stimulating endogenous NP cells to proliferate and to repopulate the intervertebral disc[7]. Direct consequences are therefore, the increase of the disc height and secondary reduction of disc protrusion within the vertebral canal and increase water content, visible as increase T2 hyperintensity on MRI imaging[7,9]. Therefore, in our study we decided to include imaging parameters aiming at evaluating both the morphology and size of the IVD and its histological grade. To evaluate size and morphology we chose IVD width and "A index" measured on CT scan images, while to evaluate histological composition/grade we measured the IVD HU and discal protrusion HU on CT scan images and SNR, CNR and two different IVD classification systems. In many pre-clinical randomized controlled animal trials, the name discal height is used. However, even if we performed essentially the same measurement type, we considered more appropriate to name it width, considering the canine skeleton orientation and the veterinary terminology. The "A index" is a well-established index in human medicine to measure discal herniation size[12] and it has already been used in veterinary medicine to measure for instance the size of discal herniation pre- and post-intradiscal injection of ozone in dogs[16]. All the other assessed measurements aimed to evaluate the changes in composition of the IVD or protruded discal material. Given the absence of histological examinations, we staged IVD on MRI images using two different classification systems[13,14], both having a good correlation with histological results. However, our values should not be considered true cut-off values, but only a way to compare pre- and post-injections results. *Inter*- and *intra*-observer repeatability, CT-acquisition algorithms and the use of a low-field MRI machine are all factors that could negatively influence these measurements.

According to the results of our study, no difference between pre- and post-injections were found in the IVD imaging features. This result confirms the safety of this technique and can be related to the possible absence of regenerative process, induced by the injections of MSCs. These results were in agreement with a recently published study, where the effects of bone-marrow derived MSCs were assessed[10]. However, in human literature the histological changes compatible with a slowdown or interruption of the IVD degeneration process were not always associated with favorable imaging outcomes, such as changes of discal height or increase IVD T2 hyperintensity[9]. For the mentioned reasons, our study aimed to assess exclusively the safety and our results should not be interpreted as lack of efficacy of MSCs intradiscal injection. Further studies are needed to clarify better this aspect. It could be hypothesized that a failure of injection into the IVD could have negatively influenced the results of our study. However, the correct intradiscal position of the needle was verified with injection of contrast medium. Moreover, the viability of MSCs in presence of contrast medium was tested during the preliminary phase. The last post-injection examinations and the last imaging examinations were performed 28 days and 8 weeks after the injection, respectively. According to the literature, the timing of last control examination is quite varying, ranging from 4 to 24 weeks[7]. For instance, in a human study evaluating the effect of cell number on artificially induced canine disc degeneration, follow-up MRI examinations were performed at 4, 8, and 12 weeks after the injections, revealing changes of MRI T2 hyperintensity already at 4 weeks[18]. In the study reporting the osteophyte formation as complications, radiological and MRI examinations were performed 3 and 9 weeks after the injections and the histological examination was performed immediately after [15]. Therefore, we considered 8 weeks a good timing for the last control examination in our study. Moreover, even if the study design of our project was not focused on the long-term complications and no other examinations have been performed, five out six dogs were alive and do not show any particular symptoms while writing this manuscript, more than 12 months after the injections. The remaining dog had to be euthanized, but for reasons completely unrelated to the current study. It is regrettable that no histological examination was performed in any of the dogs. The initial study design did not include euthanasia of the dogs. One dog had to be euthanized because of the sudden degradation of a preexisting splenic tumor. Unfortunately, the euthanasia had to be done by the staff from the emergency department and we were not able to do histological examination of the injected disc. Besides, the absence of histological examination, other obvious limitations of our study include the overall low number of dogs and the heterogeneity of the studied population. Cell survival after injection was not evaluated in the present study, but a very recent study has demonstrated that stem cells injected into canine intervertebral discs survive for three weeks[19].

In conclusion, to the authors' knowledge this is the first study evaluating systematically the safety of CT-guided injections of muscle-derived MSCs in IVD of dogs affected by natural disc degeneration. It should be considered as preliminary research and further studies are needed to assess the efficacy of this technique as potential treatment for IVD degeneration in veterinary medicine.

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List of Abbreviations

AF	Annulus Fibrous
CNR	Contrast to Noise Ratio
CT	Computer Tomography
IVD	Intervertebral Disc
L7	Seventh Lumbar Vertebra
LS	Lumbosacral
MCSs	Mesenchymal Stem Cells
MRI	Magnetic Resonance Imaging
NP	Nucleus Pulpous
ROI	Region Of Interest
S1	First Sacral Vertebra
SNR	Signal To Noise Ratio

Potential Conflicts of Interests

Author Didier Serteyn is the co-inventor of a patent owned by Liege University. and CSO of the Spin-Off company in charge to develop the technlonogy for future clinical trials. Other authors have no conflicting interests.

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