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Placebo-Controlled Clinical Study of Membrane-Free Stem Cell Extract in Dogs with Osteoarthritis

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Abstract

Osteoarthritis (OA) is a chronic degenerative disease which commonly occurred in dogs. Although there are many treat options, they can only relieve symptoms, but not can modify the disease. Moreover, conventional treatment has limitations because of its adverse events. Therefore, it is necessary that development of Disease Modifying OA Drugs (DMOADs) with low adverse effects. To evaluate the effects of Membrane-Free Stem Cell Extract (MFSCE) on the clinical signs and cartilage regeneration of OA dogs. Thirty privately owned dogs with OA were separated into two groups; placebo treated group (n=6), MFSCE treated group (n=22). All dogs were injected MFSCE or placebo once a week for 4 weeks in intra-articular. To evaluate the symptoms, dogs and owners were visit hospital at day 0, 7, 14, 21, and 28. The effect of MFSCE on pain relief was evaluated using pain score and pain at palpation score. Both symptoms were improved in MFSCE treated group while no significant changes in placebo treated group after 4 weeks treatment. The effect of MFSCE on physical function improvement was evaluated using behavior score, standing score, walking score, lameness score, and weight bearing score. All symptoms were improved in MFSCE treated group while no significant changes in placebo treated group after 4 weeks treatment. The effects of MFSCE on joint structure were evaluated using radiographic score. Joint structure was improved in MFSCE treated group while no significant changes in placebo treated group after 4 weeks treatment. There were no changes on general symptoms, symptoms of lesion, adverse events or general symptoms that can be occurred by injection, hematological parameter, and urinal parameters. Overall, these results, MFSCE could be the first-in-class DMOAD.

Keywords: Osteoarthritis; Membrane-free stem cell extract; Adipose-derived stem cells; Anti-inflammatory drugs

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Introduction

Arthritis, defined as the disease affect to joints, is commonly occurred in both human and animals [1]. Clinical symptoms of arthritis are included swelling, deformities, pain and stiffness of joint [2]. Dogs show high prevalence of arthritis due to excessive exercise, injury, and genetic predisposition. It was reported that 25% of 77.2 million dogs has arthritis in United States [1]. Arthritis is separated *via* etiology to Osteoarthritis (OA), Rheumatoid Arthritis (RA), Psoriatic Arthritis (PsA), Ankylosing Spondylitis (AS) and Septic Arthritis (SA) [2]. It is reported that, OA, chronic joint degenerative disease, is most common disease among the arthritis in dogs [3-5]. Pathological symptoms of OA shown to be various symptoms such as progressive loss and destruction of articular cartilage, inflammation of synovium, and degeneration of ligaments and menisci of the knee etc., [6].

There are some treatment options to treat the OA in dogs [7]. Weight loss, exercise modification, and physical therapy can use as non-pharmaceutical therapy [8]. In the severe dogs, surgery can be used for treatment. For the pharmaceutical therapy, Non-Steroidal Anti-Inflammatory Drugs (NSAIDs) were used as standard therapy. However, NSAIDs has some limitations because of its adverse effects such as gastrointestinal ulceration [7]. There are other pharmaceutical options such as diacerein, corticosteroids, and hyaluronic acid. However, these pharmaceuticals also had limitations because they had also adverse effect and they been only relieving symptoms but not modifying disease [9]. Therefore, many studies are conducted to development of alternative products which enhanced efficacy and safety.

The stem cell, which considered for new therapeutic strategy of OA, defined as undifferentiated

multipotent cell [10,11]. Among the various stem cells, Adipose-Derived Stem Cells (ADSC), which divided from human adipose tissue, has been gotten attention because of its various advantages such as accessibility and abundance compared with other stem cells [12]. Recent studies have provided some evidence that ADSCs are effective in the treatment of OA. Kriston-Pál et al. [13] reported that intra-articular injection of allogenic ADSC with hyaluronan into OA dog shown the effect of lameness improvement and hyaline-type cartilage regeneration. However, there are some limitations such as instability of chondrocyte-like phenotypes, limited replicative lifespan, etc., [14-20]. To overcome these limitation, various studies in progressed.

To overcome limitation of stem cell therapy, we developed Membrane-Free Stem Cell Extract (MFSCE), which consist with 252 peptides using patented technology about removing the cellular membrane of ADSC and purifying the peptides. In the previous studies, we identified the anti-inflammatory effect and cartilage regenerative effect of MFSCE in Interleukin-1α (IL-1α) induced OA *in vitro* model using rat primary cartilage cells [21]. Moreover, MFSCE has shown the anti-inflammatory and regenerative effect in golf-injury patients [22]. However, the effect of MFSCE on OA in canines not yet identified. In the present study, we evaluated the effect of MFSCE on OA through change of clinical sign in dogs. We also evaluated the adverse effects of MFSCE through change of general sign, hematological sign and urinalysis in dogs.

Materials and Methods

Preparation of MFSCE

The MFSCE was provided from T-Stem Co., Ltd. (Changwon, Korea). The preparation was manufactured as previously described [23]. Human ADSCs were separated from human adipose tissue which donated from healthy female aged twenties. All donors had to complete blood test for check compatibility. ADSCs were cultured in 5% CO2 and 37°C condition. ADSCs were harvested at the passages of 5 to 7 to using for MFSCE. Cells membranes were removed using ultra sonication and centrifugation, and intracellular peptides, named MFSCE, were collected using filtration. The aqueous solution of MFSCE was further lyophilized and stored in powder form. Non-toxicity of MFSCE, the final product, was identified *via* 9 safety tests performed by the Good Laboratory Practice accreditation authority.

Experimental design

The study was conducted in compliance with the guidelines of the Institutional Animal Care and Use Committee. All owners were required to sign an informed consent form for the clinical trials before enrolling their animals in the study. The protocol was approved by the Animal and Plant Quarantine Agency of Korea.

Twenty-eight dogs with spontaneous occurred OA were enrolled in the study. To OA was evaluated using radiographic and clinical sign. Dogs that had other knee disease, treated with NSAIDs or corticosteroids within 14 days before enrolment, or pregnancy or likelihood of becoming pregnant during the study were excluded. For ethical reasons, dogs were analgesic treated or excluded from the study according to change of general symptoms or symptoms of lesion, and occurring infectious disease and adverse events. Characteristics of dogs which completed the study were shown in Table 1. There are no significant differences of characteristics between placebo and MFSCE groups at T0.

Dogs were separated into two groups; placebo treated group (n=6), MFSCE treated group (n=22). The MFSCE treated group was injected with 100 mg/2 mL of MFSCE (dissolved with phosphate buffer solution (PBS)) in intra-articular while placebo treated group was injected with 2 mL of PBS in intra-articular. All dogs were treated once a week for 4 weeks. To evaluate the symptoms, dogs and owners were visit hospital at day 0, 7, 14, 21, and 28. The overall study timeline was shown in Figure 1.

Evaluation of clinical efficacy

The clinical efficacy on OA was evaluated in 8 categories [24]; pain, standing, walking, pain at palpation, behavior, hindlimb lameness, hindlimb weight bearing and radiography.

Pain score was separated and evaluated by 4 clinical signs. 0= Absence of pain and any trouble at moving with active behavior; 1= Seems to be uncomfortable during rest and intermittent lameness but can put the legs on ground during walking with weak skin flare; 2= Abstinence to move and intermittent lameness at walking. Feel pain at palpation and insensitive to external stimuli with swelling around the joint; 3= cannot put the legs on ground with howling due to severe pain. Feel pain without palpation with severe swelling and flare around joint.

Pain at palpation score was separated and evaluated by 3 clinical signs. 0= Absence of pain symptoms; 1= Mild or moderate pain (allow the palpation but with uncomfortable behavior such as turn head, pull leg away, vocalizes or depress); 2= Severe pain (not allow the palpation).

Behavior score was separated and evaluated by 5 clinical signs. 0= Indifferent; 1= Friendly; 2= Nervous and submissive behavior; 3= Very nervous and try to move away; 4= Aggressive.

Standing score was separated and evaluated by 4 clinical signs. 0= Standing with perfect weight bearing condition; 1= Abnormal standing position with partial weight bearing condition; 2= Abnormal standing position with no weight bearing (use 3 legs); 3= Do not try to standing.

Walking score was separated and evaluated by 5 clinical signs. 0= Walking with perfect weight bearing condition; 1= Slight limp with partial weight bearing condition; 2= Severe limp with intermittent weight bearing; 3= No weight bearing condition (use 3 legs); 4= Cannot try to walking.

Hindlimb lameness score was separated and evaluated by 5 clinical signs. 0= Stands and walks normally; 1= Stands normally and slightly lame at walk; 2= Stands normally and severely lame at walk; 3= Abnormal stance and slightly lame at walk; 4= Abnormal stance and severely lame at walk.

Hindlimb weight bearing score was separated and evaluated by 5 clinical signs. 0= Normal at both rest and walk; 1= Normal at rest and favors affected limb at walk; 2= partial at both rest and walk; 3= partial at rest and no weight bearing at walk; 4= No weight bearing at rest and walk.

Radiography score was separated and evaluated by 5 clinical signs from X-ray. 0= No features of OA; 1= Doubtful decreasing of femoral muscle mass and increasing of patella angle; 2= Minimal decreasing of femoral muscle mass and increasing of patella angle; 3= Moderate decreasing of femoral muscle mass and increasing of patella angle; 4= Severe decreasing of femoral muscle mass and increasing of patella angle.

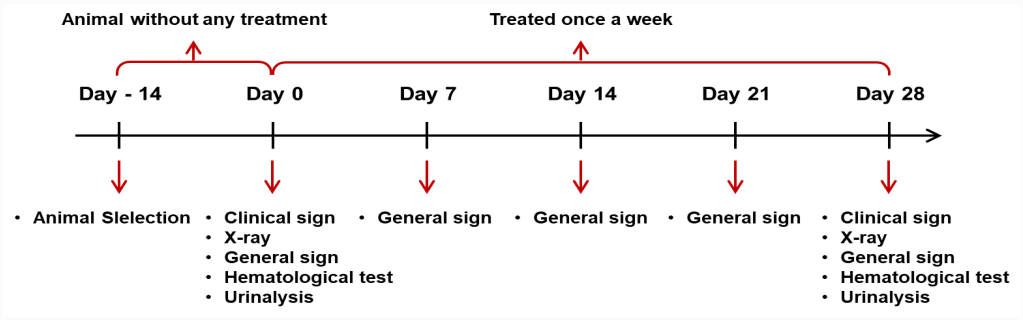

Figure 1: Study timeline of MFSCE treatment and clinical and general sign assessments.

Table 1: Characteristics of dogs which completed the study.

Characteristics	Control	MFSCE	p-value
Total number of subjects	6	22	NA
Male/Female	4/2	13/9	NA
Castrated male/Sterilized female/intact	4/0/2	8/3/2011	NA
Age at T0	6.83 ± 1.54	7.23 ± 0.93	0.842
Body weight at T0	5.40 ± 0.74	6.07 ± 1.34	0.802

Table 2: General symptoms evaluation criteria for evaluate the adverse effect in dogs.

Score	Clinical sign
1	Leave food
1	Eye boogers
1	Skin abnormalities
1	Alopecia
1	Rhinorrhea
2	Fever
2	Abnormalities of urination and defecation
3	Abnormalities of respiration
4	Inability of walking
5	Inability of standing
10	Death
Score	**Clinical sign**
0~5	Study continuation
6~9	Considered stop the study
≥ 10	Stop the study

Adverse effects

The adverse effects were evaluated in 6 categories; general symptoms, symptoms on lesion, infectious disease that can be occurred by injection, adverse events that can be occurred by injection, hematology, and urinalysis.

General symptom score was evaluated by presence/absence of symptoms as shown in Table 2. The progress of study was considered according to the general symptom scores; 0~5= Study continuation; 6~9= Consideration of stop the study; ≥ 10= Stop the study.

Symptoms on lesion score was evaluated by presence/absence of symptoms as shown in Table 3. Appropriate treatment was given according to the severity of each symptom.

Infectious disease score was evaluated by presence/absence of disease as shown in Table 4. Appropriate treatment was given according to the severity of disease, and progress of study was considered according to the infectious disease scores; 0~4= Study continuation; 5~7= Consideration of stop the study; ≥ 8= Stop the study.

Table 3: Clinical signs of lesion and treatment method to evaluate the adverse effect and treat appropriate treatment.

Clinical sign	Treatment
Flare	Disinfect the lesion Disinfect the lesion
Edema	Treat antibiotics and anti-inflammatory drugs in the case of severe edema
Pain	Treat antibiotics and anti-inflammatory drugs in the case of severe pain
Fever	Disinfect the lesion Treat antibiotics and anti-inflammatory drugs in the case of severe fever Disinfect the lesion
Swelling	Treat antibiotics and anti-inflammatory drugs if swelling is continued Incision and drainage in case of purulent swelling

Table 4: Adverse events evaluation criteria that can be occurred by injection in dogs.

Score	Infective disease	Treatment
1	Bacterial infection	Disinfect the lesion and treat antibiotics
1	Virus infection	Disinfect the lesion and treat antivirals
1	Fungal infection	Disinfect the lesion and treat antifungals
2	Allergic disease	Treat anti-histamines and analysis of the reason of allergy
3	Anaphylaxis	1) Check the vital sign
		2) Clear the airway
		3) Analysis the reason of anaphylaxis after be stable
		4) Consider stop the study

Score	Clinical sign
0~4	Study continuation
5~7	Considered stop the study
≥ 8	Stop the study

Adverse event score was evaluated by presence/absence of disease as shown in Table 5. Appropriate treatment was given according to the severity of symptoms, and progress of study was considered according to the adverse event scores; 0~4= Study continuation; 5~7= Study continuation carefully; 8~9= Consideration of stop the study; ≥ 10= Stop the study.

Hematological analysis was evaluated using two test methods,

Table 5: General symptoms evaluation criteria that can be occurred by injection in dogs.

Score	Clinical sign	Treatment
1	Behavioral abnormality	Check vital sign, treat stabilizer to severe dogs
1	Skin and hair abnormality	Education of owner, treat hair-restorer if alopecia was continued
1	Respiration abnormality	Check vital sign, clear the airway, ventilation
1	Loss of appetite	Education of owner, treat fluid with nutrients if symptoms continued
1	Vomiting	Analysis the reason and appropriate treatment
1	Diarrhea	Analysis the reason and appropriate treatment
4	Shock	Check vital sign, clear the airway, ventilation, treat electrolyte fluid, continuous monitoring
10	Mortality	Analysis the reason and consider stop the study

Score	Clinical sign
0~4	Study continuation
5~7	Study continuation carefully
8~9	Considered stop the study
≥ 10	Stop the study

Table 6: Hematological analysis and urinalysis parameter to evaluate the adverse effect in dogs.

Category	Test	Parameter
Hematological analysis	Hematological test	WBC, RBC, HGB, HCT, MCV, MCH, MCHC, RDW, PLT, PCT, RET, WBC diff. cont.
	Blood biochemical test	AST, ALB, ALT, TBIL, ALP, TG, BUN, Ca, CRE, IP, GLU, Na, CHO, K, TP, Cl, CRP
Urinalysis	Urine strip test	Blood, Bilirubin, Urobilinogen, Ketones, Protein, Nitrite, Glucose, pH, Specific gravity, Leucocytes

hematological test and blood biochemical test, as shown in Table 6. In hematological test, White Blood Cell (WBC), Red Blood Cell (RBC), Hemoglobin (HGB), Hematrocrit (HCT), Mean Corpuscular Volume (MCV), Mean Corpuscular Hemoglobin Concentration (MCHC), Red Blood Cell Distribution Width (RDW), Platelet (PLT), Plateletcrit (PCT), Reticulocyte Hemoglobin Equivalent (RET), and WBC differential count were evaluated. In blood biochemical test, Aspartate Aminotransferase (AST), Albumin (ALB), Alanine Aminotransferase (ALT), Total Bilirubin (TBIL), Alkaline Phosphatase (ALP), Triglyceride (TG), Blood Urea Nitrogen (BUN), Calcium (Ca), Creatinine (CRE), Inorganic Phosphorus (IP), Glucose (GLU), Sodium (Na), Total Cholesterol (CHO), Potassium (K), Total Protein (TP), Chloride (Cl), C-Reactive Protein (CRP) were evaluated.

Urinalysis was evaluated using strip test as shown in Table 6. Blood, bilirubin, urobilinogen, ketones, protein, nitrite, glucose, pH, specific gravity, and leucocytes were evaluated.

Statistical analysis

Statistical analysis was performed using GraphPad PRISM statical package ver. 2.00 (Graph Pad software inc., USA). All data were expressed as mean ± standard deviation. All groups were compared using a paired Student's *t*-test. Significance was accepted for p values of <0.05.

Results

The effect of MFSCE on pain relief

To identify the effect of MFSCE on the OA, we evaluated the pain relief effect *via* behavioral and symptomatic changes. Pain score of MFSCE treated group was significantly decreased after 28 days treatment (0.41 ± 0.11) compared with 0 day (2.09 ± 0.13) while no changes was shown in placebo treated group (1.50 ± 0.22 at 0 day and 1.67 ± 0.21 at 28 day) as shown in Figure 2.

We also evaluated the pain relief effect for palpation *via* behavioral changes in response to palpation. Pain at palpation score of MFSCE treated group was significantly decreased after 28 days treatment (0.18 ± 0.08) compared with 0 day (1.41 ± 0.11) while no changes was shown in placebo treated group (1.00 ± 0.02 at 0 day and 1.00 ± 0.02 at 28 day) as shown in Figure 3.

The effect of MFSCE on physical function improvement

To identify the effect of MFSCE on the OA, we evaluated the physical function improvement effect *via* behavioral changes. Behavior score of MFSCE treated group was significantly decreased after 28 days treatment (0.91 ± 0.15) compared with 0 day (2.05 ± 0.23) while no changes was shown in placebo treated group (1.50 ± 0.22 at 0 day and 1.50 ± 0.22 at 28 day) as shown in Figure 4.

Next, we evaluated the physical function improvement effect *via* behavioral changes in the situation at standing. Standing score of MFSCE treated group was significantly decreased after 28 days

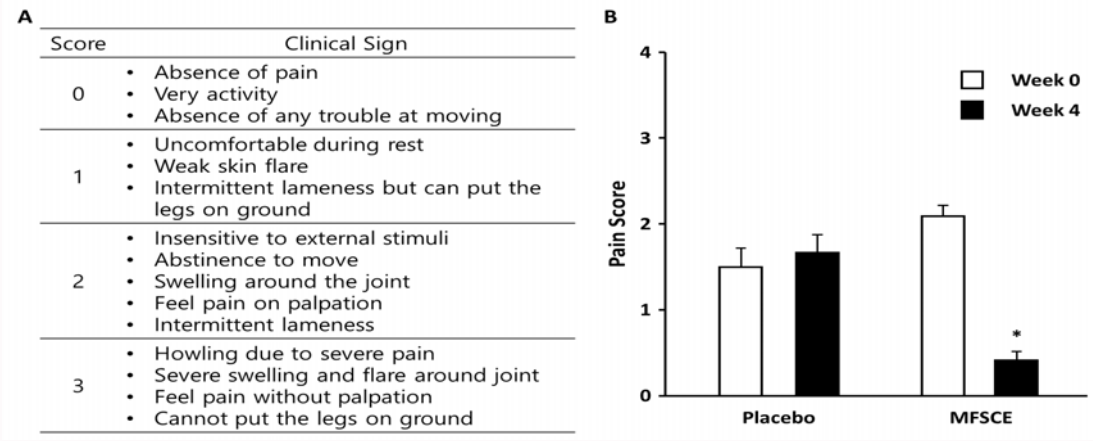

Figure 2: Clinical sign criteria for evaluate pain (A) and changes of pain score after Membrane-Free Stem Cell Extract (MFSCE) treatment (B). Placebo or MFSCE was injected in intra-articular once a week for 28 days (Placebo: n=6; MFSCE: n=22). The data are expressed as the mean ± standard deviation of the mean (*P<0.05).

A

Score	Clinical Sign
0	• Absence of pain symptoms
1	• Mild or moderate pain (allow the palpation but with uncomfortable behavior such as turn head, pull leg away, vocalizes or depress)
2	• Severe pain (not allow the palpation)

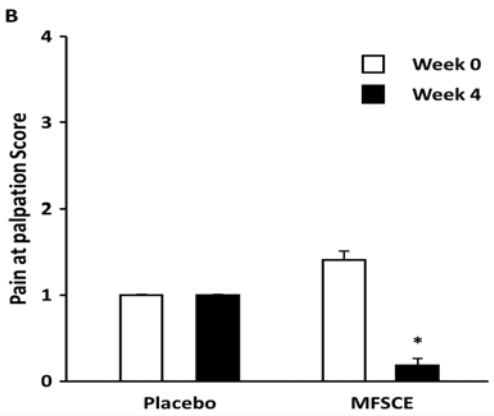

Figure 3: Clinical sign criteria for evaluate pain at palpation (A) and changes of pain at palpation score after Membrane-Free Stem Cell Extract (MFSCE) treatment (B). Placebo or MFSCE was injected in intra-articular once a week for 28 days (Placebo: n=6; MFSCE: n=22). The data are expressed as the mean ± standard deviation of the mean (*P<0.05).

A

Score	Clinical Sign
0	• Indifferent
1	• Friendly
2	• Nervous, submissive behavior
3	• Very nervous, tries to move away
4	• Aggressive

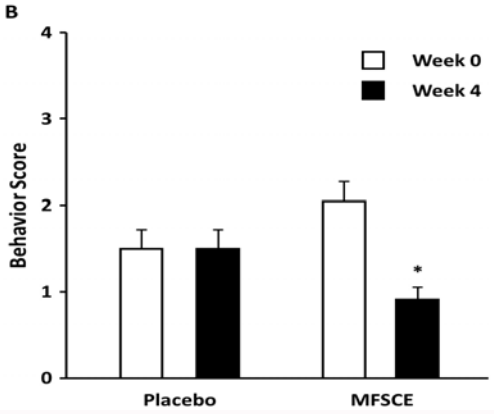

Figure 4: Clinical sign criteria for evaluate behavior (A) and changes of behavior score after Membrane-Free Stem Cell Extract (MFSCE) treatment (B). Placebo or MFSCE was injected in intra-articular once a week for 28 days (Placebo: n=6; MFSCE: n=22). The data are expressed as the mean ± standard deviation of the mean (*P<0.05).

A

Score	Clinical Sign
0	• Standing with perfect weight bearing condition
1	• Abnormal standing position with partial weight bearing condition
2	• Abnormal standing position with no weight bearing (use 3 legs)
3	• Do not try to standing

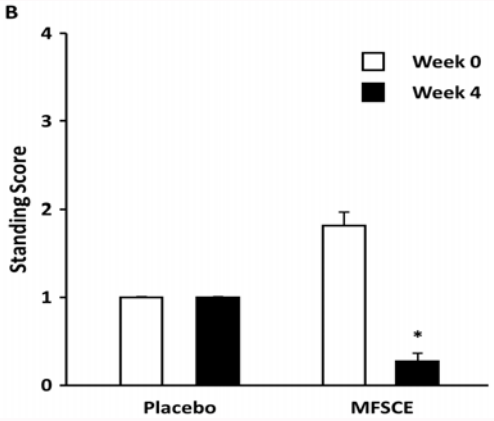

Figure 5: Clinical sign criteria for evaluate standing (A) and changes of standing score after Membrane-Free Stem Cell Extract (MFSCE) treatment (B). Placebo or MFSCE was injected in intra-articular once a week for 28 days (Placebo: n=6; MFSCE: n=22). The data are expressed as the mean ± standard deviation of the mean (*P<0.05).

treatment (0.27 ± 0.10) compared with 0 day (1.82 ± 0.16) while no changes was shown in placebo treated group (1.00 ± 0.01 at 0 day and 1.00 ± 0.01 at 28 day) as shown in Figure 5.

We also evaluated the physical function improvement effect *via* behavioral changes in the situation at walking. Walking score of MFSCE treated group was significantly decreased after 28 days treatment (0.45 ± 0.13) compared with 0 day (2.23 ± 0.24) while no changes was shown in placebo treated group (1.17 ± 0.17 at 0 day and 1.33 ± 0.21 at 28 day) as shown in Figure 6.

Next, we evaluated the physical function improvement effect *via*

A

Score	Clinical Sign
0	• Walking with perfect weight bearing condition
1	• Slight limp with partial weight bearing condition
2	• Severe limp with intermittent weight bearing
3	• No weight bearing condition (use 3 legs)
4	• Cannot try to walking

B

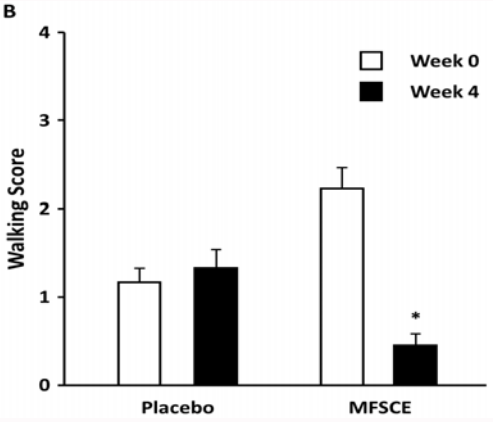

Figure 6: Clinical sign criteria for evaluate walking (A) and changes of walking score after Membrane-Free Stem Cell Extract (MFSCE) treatment (B). Placebo or MFSCE was injected in intra-articular once a week for 28 days (Placebo: n=6; MFSCE: n=22). The data are expressed as the mean ± standard deviation of the mean (*P<0.05).

A

Score	Clinical Sign
0	• Stands and walks normally
1	• Stands normally, slightly lame at walk
2	• Stands normally, severely lame at walk
3	• Abnormal stance, slightly lame at walk
4	• Abnormal stance, severely lame at walk

B

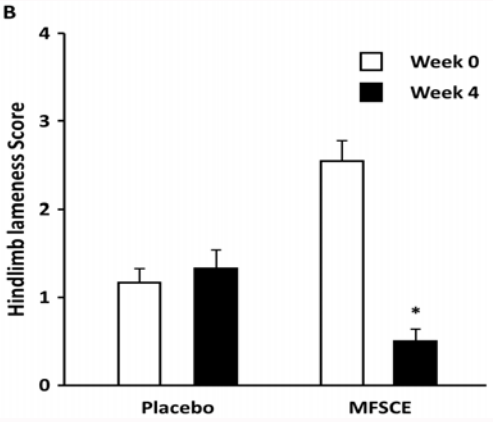

Figure 7: Clinical sign criteria for evaluate lameness (A) and changes of lameness score after Membrane-Free Stem Cell Extract (MFSCE) treatment (B). Placebo or MFSCE was injected in intra-articular once a week for 28 days (Placebo: n=6; MFSCE: n=22). The data are expressed as the mean ± standard deviation of the mean (*P<0.05).

A

Score	Clinical Sign
0	• Normal at both rest and walk
1	• Normal at rest, favors affected limb at walk
2	• Partial at both rest and walk
3	• Partial at rest, no weight bearing at walk
4	• No weight bearing at rest and walk

B

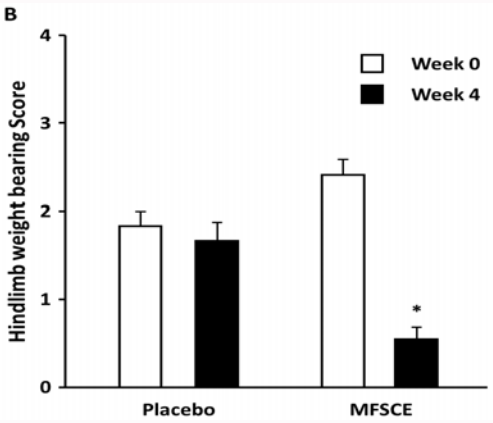

Figure 8: Clinical sign criteria for evaluate weight bearing (A) and changes of weight bearing score after Membrane-Free Stem Cell Extract (MFSCE) treatment (B). Placebo or MFSCE was injected in intra-articular once a week for 28 days (Placebo: n=6; MFSCE: n=22). The data are expressed as the mean ± standard deviation of the mean (*P<0.05).

behavioral changes of hindlimb which has the lesion. Lameness score of MFSCE treated group was significantly decreased after 28 days treatment (0.50 ± 0.14) compared with 0 day (2.55 ± 0.23) while no changes was shown in placebo treated group (1.16 ± 0.17 at 0 day and 1.33 ± 0.21 at 28 day) as shown in Figure 7.

Weight bearing score of MFSCE treated group was also significantly decreased after 28 days treatment (0.55 ± 0.14) compared with 0 day (2.41 ± 0.18) while no changes was shown in placebo treated group (1.83 ± 0.17 at 0 day and 1.67 ± 0.21 at 28 day) as shown in Figure 8.

A

Score	Clinical Sign
0	• Normal at both rest and walk
1	• Normal at rest, favors affected limb at walk
2	• Partial at both rest and walk
3	• Partial at rest, no weight bearing at walk
4	• No weight bearing at rest and walk

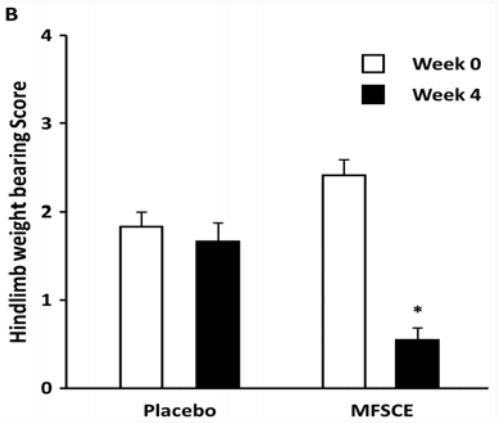

Figure 9: Clinical sign criteria for evaluate radiography (A) and changes of radiographic score after Membrane-Free Stem Cell Extract (MFSCE) treatment (B). Placebo or MFSCE was injected in intra-articular once a week for 28 days (Placebo: n=6; MFSCE: n=22). The data are expressed as the mean ± standard deviation of the mean (*P<0.05).

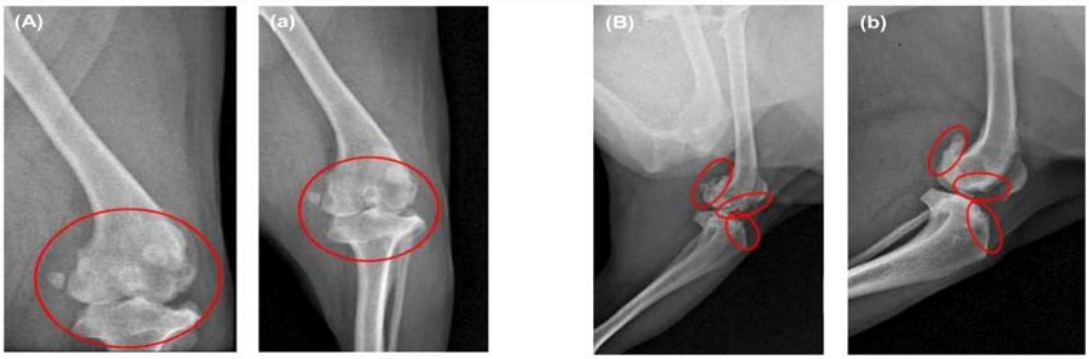

Figure 10: Representative results of changes of joint structure after Membrane-Free Stem Cell Extract (MFSCE) treatment. Radiography of OA at 0 day (A, B) was significantly improved 28 days after MFSCE treatment (a, b).

The effect of MFSCE on joint structure

To identify the effect of MFSCE on the OA, we evaluated the joint structure using X-ray. Radiographic score of MFSCE treated group was significantly decreased after 28 days treatment (1.27 ± 0.13) compared with 0 day (2.73 ± 0.15) while no changes was shown in placebo treated group (2.00 ± 0.37 at 0 day and 2.00 ± 0.37 at 28 day) as shown in Figure 9.

Adverse effect of MFSCE

There were no changes on general symptoms, symptoms of lesion, adverse events or general symptoms that can be occurred by injection, hematological parameter, and urinal parameters. Therefore, MFSCE has no adverse effects.

Discussion

This is the first study that demonstrated the treatment effect of MFSCE on OA without adverse effects in dogs. All clinical symptoms related with OA such as pain, physical function and radiography were significantly improved after 28 days treatment. Even so, no changes in any general symptoms, adverse events, hematological parameters, and urinalysis parameters were seen after 28 days treatment.

In the present study, we evaluated the various clinical symptoms with reference of Western Ontario and McMaster Universities (WOMAC) osteoarthritis index for identify the effect of MFSCE on OA. WOMAC which is consist with 24 questions for evaluate the pain, stiffness, and physical functioning of joint, is the most widely used index in clinical studies to evaluate the symptoms of OA [25-28]. Pain is the one of main symptom of OA [29]. Pain in OA is occurred by activation of pain-sensing afferent neurons within the joint [30-35]. Nociceptors in the OA joint may be stimulated by various stimuli including physical/mechanical or chemical stimuli such as inflammatory mediators such as Cyclooxygenase-2 (COX-2) and Prostaglandin E2 (PGE2) [36,37]. In the present study, MFSCE showed the pain relief effect through reducing the pain score and pain at palpation score. Furthermore, in the previous study, we identified that MFSCE was decreased COX-2 and PGE2 levels in IL-1α induced osteoarthritis *in vitro* model using rat primary chondrocytes [21]. Therefore, it was suggested that MFSCE may has pain relief effect through decreasing the COX-2 and PGE2 levels.

In OA dogs, decreased physical function due to pain and structural change is commonly occurred [29]. Because of these functional impairments resulting in a poor quality of life, improve the physical function and structural dysfunction in OA is one of main therapeutic target [38]. In the present study, MFSCE not only shown the physical function improve effect through reducing the behavior score, standing score, walking score, hindlimb lameness score and hindlimb weight bearing score, but also shown the structural dysfunction improve effect through reducing radiography score. The loss of articular cartilage is the pathological feature of OA which is appeared as a reduction of joint space in radiographs [29]. Although the pathogenesis of OA was not fully understood, some molecular mechanism which related with cartilage formation or destruction was

identified in OA. Matrix Metalloproteinase (MMP) is the proteolytic enzymes which are occurred cartilage destruction through degradation of collagen, aggrecan, and various proteoglycans in OA [39-41]. It was reported that *MMP-3* and *MMP-13* were increased in OA [42]. *MMP-13* is the main proteinase which is directly related with degradation of collagen, aggrecan and proteoglycans in OA cartilage [43], while *MMP-3* can help the *MMP-13* to degrade cartilage components [44]. In the previous study, it was identified that MFSCE decreased *MMP-3* and *MMP-13* gene and protein level in IL-1α induced OA *in vitro* model [21]. Furthermore, we also identified that MFSCE inhibited the nuclear factor kappa B (NF-κB) and Mitogen-Activated Protein Kinases (MAPKs) signaling pathway which are regulated the MMP levels [21,45]. Therefore, it was suggested that MFSCE may inhibit the progress of OA through inhibition of MMPs. Another molecular mechanism which related with cartilage formation is SRY-type high-mobility group box-9 (*SOX-9*) which is control chondrogenesis [46]. *SOX-9* expressed in pre-chondrogenic mesenchyme and fully differentiated chondrocytes [47]. It was reported that the expression level of *SOX-9* was lower in OA chondrocytes [48,49]. Moreover, overexpression of *SOX-9* in explant cultures of OA articular cartilage increased collagen and proteoglycan expression level similar with normal cartilage [50,51]. Therefore, it was considered that increasing *SOX-9* expression is important treat target for cartilage regeneration [52]. In the previous study, MFSCE increased *SOX-9* gene and protein level in IL-1α induced OA *in vitro* model [21]. Therefore, it was suggested that MFSCE may promote cartilage regeneration through increasing *SOX-9* level. In conclusion, MFSCE may improve the OA through not only inhibit the progress of OA but also increase the cartilage regeneration.

Many researches for the Development Disease-Modifying OA Drug (DMOAD) were progressed; however, it was still not developed because OA has complex pathogenic mechanism [53]. It was reported that, stem cell can promote cartilage regeneration by various mechanisms [54]. It also has been reported that transplanted stem cells can replace the damaged cartilage through differentiate to target cell [55]. Recently, it was identified that stem cells can be affect to cartilage regeneration through release the paracrine molecules such as growth factor and thrombospondin [56]. Furthermore, stem cells can modulate the immune response through release the cytokines [57]. However, although the stem cell had attention as a new DMOAD, there are still some limitations [38]. To treat OA using stem cells, it has to be attached on the lesion, and has to be differentiated and proliferated. However, these processes are affected by various factors such as cell condition, growth condition, amount of stem cell and other endogenous factors [54]. Moreover, stem cell can be differentiated unexpected cell because of its multi-potentiality [58,59]. Because of these various limitations, stem cell therapy has been difficulties to development. In the present study, we evaluated the cartilage regenerative effect using radiography. As shown in Figure 10, joint structure was improved and cartilage was regenerated 28 days after MFSCE treatment. We suggested that MFSCE may act as paracrine factor released from stem cell to regulate cartilage regeneration and immune response. Moreover, we identified that MFSCE did not shown any toxicity performed at the GLP institution (data not shown). Therefore, MFSCE may alternate the stem cell therapy because it overcomes the limitations of stem cell therapy.

In this study, we found that 4 weeks treatment of MFSCE not only improve clinical sign of OA but also regenerate cartilage with no significant adverse effects. In addition, previous studies have confirmed the mechanism of action of MFSCE on OA, and the non-toxicity. Therefore, MFSCE could be the first-in-class DMOAD.

Author Contributions

Conceptualization, Y.S.K., J.O.L. and T.H.K.; methodology, S.E.H. and G.S.K.; formal analysis; Y.S.K., J.O.L. and T.H.K.; writing-original draft preparation, Y.S.K., J.O.L. and T.H.K.; writing-review and editing, S.E.H.; supervision, G.S.K.; project administration, Y.S.K., J.O.L. and T.H.L. All authors have read and agreed to the published version of the manuscript.

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