A THESIS FOR THE DEGREE OF MASTER

Safety assessment of intravenous administration of allogenic canine adipose tissue-derived mesenchymal stem cells in 40 client-owned dogs

40마리의 개에서 동종 지방 조직 유래 개 중간엽 줄기 세포의 정맥 내 투여의 안전성 평가

2020년 2월

서울대학교 대학원

수의과대학 임상수의학(수의내과학) 전공

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지도교수 윤 화 영
이 논문을 수의학 석사 학위논문으로 제출함

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Safety assessment of intravenous administration of allogenic canine adipose tissue-derived mesenchymal stem cells in 40 client-owned dogs

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Abstract

To evaluate the side effects of mesenchymal stem cell (MSC) transplantation by intravenous infusion of allogenic MSCs in dogs and to examine the long-term safety including tumorigenesis. The study conducted a retrospective analysis of various clinical assessments such as physical examination, blood tests, and radiography and monitored the formation of neoplasms, during the 6-month follow-up period in 40 client-owned dogs that received intravenous infusion of adipose-tissue derived MSCs (AT-MSCs) for the treatment of various underlying diseases between 2012 and November 2018. No significant side effects of the MSC therapy were detected by clinical assessment, blood tests, and radiographic examination in the 6-month follow-up period after the first MSC treatment, and no new neoplasms were observed during this period. This study is the first to evaluate the long-term (≥ 6 month) safety aspects and risk of tumorigenesis for intravenous allogenic AT-MSC infusion. These

results suggested that the allogenic AT-MSC infusion does not have a tumorigenic potential in dogs and confirmed that MSC therapy can be a useful and a relatively safe therapeutic approach in canines.

Key words: Cell therapy, Dogs, Intravenous injection, Mesenchymal stem cells, Side effects, Tumorigenesis

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1. Introduction

Mesenchymal stem cell (MSC) therapy has been widely studied for many years for its therapeutic potential and clinical applications in various diseases. The self-renewal capacity of MSCs and their ability to differentiate into cells of various lineages, such as the myoblasts, fibroblasts, bone, tendon, ligament, and adipose tissue, make them an ideal candidate for applications in regenerative medicine. MSCs can also regulate excessive immune responses and play a role in regulating the expression of mediators of immune responses, and such functions may prove useful in the immune-mediated diseases (Wei *et al*. 2013). Another benefit of MSC therapy is the convenient isolation and expansion of MSCs.

Various studies have investigated the therapeutic potential and safety aspects of MSC therapy in veterinary medicine. Applications of MSC therapy in dogs have been reported for conditions such as inflammatory bowel disease (IBD), pemphigus, refractory atopic dermatitis, chronic osteoarthritis, and intervertebral disk disease (IVDD) (Black *et al*. 2008, Han *et al*. 2015, Perez-Merino *et al*. 2015, Kim *et al*. 2016, Villatoro *et al*. 2018). In cats, the safety and efficacy of MSC therapy have been reported for conditions such as gingivostomatitis, chronic enteropathy, chronic kidney disease (CKD), and acute kidney injury (AKI) (Quimby *et al*. 2013, Webb *et al*. 2015, Quimby *et al*. 2016, Rosselli *et al*. 2016, Arzi *et al*. 2017).

However, no study, to the best of my knowledge, has yet investigated the long-term safety aspects of the MSC therapy such as the risk of tumorigenesis, in dogs. This study, thus, aims to evaluate the side effects of MSC transplantation by the intravenous (IV) infusion of allogenic MSCs in dogs, and to examine the long-term safety including tumorigenesis. We conducted a retrospective analysis of various clinical assessments, such as physical examination, blood tests, and radiography, and monitored the formation of neoplasms, during the follow-up period, for this purpose.

2. Materials and Methods

2.1 Study population

A total of 40 client-owned dogs which received the IV infusion of adipose-tissue derived MSCs (AT-MSCs) for treatment of various underlying diseases in the Seoul National University (SNU) veterinary medical teaching hospital (more than two times) with appropriate follow-up, between 2012 and 2018, were included in this study. The signalment data are summarised in Table 1. The mean age of the dogs was 11.2 years, and the mean body weight was 6.12 kg. In this study, dogs with a variety of diseases were included, and the details are summarised in Table 2. These dogs were assessed before transplantation and at each post-treatment follow-up point by performing clinical evaluations such as history taking, physical examination, blood analysis, thoracic radiography, ultrasonography, and the risk of tumorigenesis. All treatment and examination processes were conducted with written consent from the owner.

2.2 Isolation and characterisation of canine AT-MSCs

Canine adipose tissue was obtained from healthy beagle dogs < 2 years old (free of infection by canine distemper virus, parvo virus, and corona virus) under a protocol approved by the Institutional Animal Care and Use Committee (SNU-170724-5) of SNU. The MSCs were isolated and characterised as previously described (Yang et al., 2018). Briefly, tissue samples were washed with phosphate-buffered saline (PAN Biotech, Aidenbach, Germany) containing penicillin (100 U/mL) and streptomycin (100 g/mL), then cut into small pieces. Afterward, they were digested at 37 °C with collagenase type IA (1 mg/mL; Sigma-Aldrich, St. Louis, MO, USA) for 1 h. After incubation, Dulbecco's modified Eagle's medium (PAN Biotech) containing 10% foetal bovine serum (PAN

Biotech) was added to stop enzymatic activity. Following centrifugation at 1200 × g for 5 min, the supernatant was discarded. Then, the pellet was filtered through a 70-μm Falcon cell strainer (Fisher Scientific, Waltham, MA, USA) and incubated in Dulbecco's modified Eagle's medium containing 10% foetal bovine serum at 37 °C in a humidified atmosphere of 5% CO_2 After 48 h, the cultures were washed five times with phosphate-buffered saline to remove non-adherent cells and centrifuged again. Then, the cells were incubated with fresh medium, which was changed every 48 h until cells reached 70–80% confluence, after which they were repeatedly sub-cultured under standard conditions. In addition, the cells were determined to be free of mycoplasma using mycoplasma PCR detection kit (Cellsafe Co. Ltd., Suwon, Korea).

Before their use in this study, the cells were characterised by immunophenotyping and multilineage differentiation. For immunophenotyping, the isolated cells were evaluated by flow cytometry using fluorescein isothiocyanate (FITC)-, phycoerythrin (PE)-, and allophycocyanin (APC)-conjugated antibodies against the following proteins: cluster of differentiation (CD)29-FITC, CD31-FITC, and CD73-PE (BD Biosciences, Franklin Lakes, NJ, USA); and CD44-FITC, CD45-FITC, and CD90-APC (eBioscience, San Diego, CA, USA). Then, the cells were analysed using a FACSAria II system (BD Biosciences). Multilineage differentiation was evaluated using StemPro Adipogenesis Differentiation, StemPro Osteogenesis Differentiation, and StemPro Chondrogenesis Differentiation kits (all from Gibco/Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions; followed by oil red O staining, alizarin red staining, and alcian blue staining, respectively.

2.3 Administration of canine AT-MSCs

All dogs were pre-treated with intravenous chlorpheniramine (0.5 mg/kg) and dexamethasone (0.5 mg/kg) for each MSC therapy. Immunophenotypic characterisation of the canine AT-MSCs were performed by flow cytometry analysis, and their differentiation potential was assayed.

Allogenic AT-MSCs were administered into the cephalic or saphenous vein through a catheter. Cells were resuspended in 5–10 mL of 0.9% normal saline, and 5 × 106 cells/kg were administered, and the infusion of the MSCs was performed slowly for 30 min.

2.4 Clinical assessment

Each dog underwent a physical examination before and after the IV allogenic MSC infusion. The physical examination included the following components: blood pressure, body temperature, respiratory rate, pulse rate, thoracic auscultation, presence of neurological signs, and swelling of the four limbs. This study mainly included dogs from the outpatient department, and hence, we conducted the history taking from the owners to estimate the changes in appetite or vitality, pain response, oedema, or fever during the next visit.

2.5 Blood tests

Data from the complete blood count (CBC) and serum biochemistry profile were assessed to determine the adverse reactions to the allogenic MSC infusion. Parameters for CBC included red blood cell, white blood cell, platelet, neutrophil, lymphocyte, monocyte, and eosinophil counts, packed cell volume, and haemoglobin levels. The serum was assayed for sodium, potassium, chlorine, alkaline phosphatase, alanine aminotransferase, aspartate aminotransferase, gamma glutamyltransferase, total bilirubin, ammonia, total protein, albumin, triglycerides, total cholesterol, glucose, blood urea nitrogen, creatinine, phosphorus, and calcium levels. The CBC and serum biochemistry profile were compared between the values obtained for the parameters before and after the MSC treatment. The specific parameters of the blood test varied depending on the underlying disease of the dogs.

2.6 Thoracic radiography and abdominal ultrasonography

Radiographic examination (EVA-HF525, Comed, Gyeonggi-do, Korea) was performed to assess any adverse effects of the allogenic MSC infusion such as pulmonary oedema, haemorrhage, and pulmonary thromboembolism. Right lateral and ventral dorsal thoracic views for both the inspiratory and expiratory phase were obtained. Ultrasonographic evaluation (ProSound Alpha 7, Hitachi-Aloka Medical, Ltd., Tokyo, Japan) of the entire abdomen was also performed to assess the risk of tumorigenesis of the MSC therapy. After MSC infusion, it were observed whether there were any new neoplasms in the abdomen and whether there were neoplastic changes of the abdominal organs, including liver, spleen, and abdominal lymph nodes. Image analysis was conducted by the veterinarians of the relevant department, and we compared whether there was a significant change before and after the injection of the MSCs.

3. Results

3.1 Characterization of canine AT-MSCs

Cells isolated from canine adipose tissue were characterised by immunophenotyping. Flow cytometry analysis showed that the cells had a high expression of stem cell markers such as CD29, CD44, CD73, and CD90. In contrast, the cells did not express CD34, and CD45 (Fig. 1A). We also determined the differentiation potential of canine AT-MSCs into adipocytes, osteocytes, and chondrocytes (Fig. 1B).

3.2 No changes were observed between the various clinical parameters tested before and after the MSC therapy

In most of the dogs, no notable clinical changes were observed between the various parameters assayed before and after the MSC treatment (Table 3). The physical examination carried out in the hospital found no changes in injection sites. Also, there were no changes in the systolic blood pressure, pulse rate, respiratory rate, and body temperature, following the infusion of allogenic AT-MSCs. The auscultation of cardiac murmurs and lung sounds did not indicate any changes, and no swellings, or vomiting, or other neurological signs were observed. However, in 3 out of the 314 injections (40 dogs), the symptoms of pain, flare, and oedema were observed in the leg to which the MSC infusion was performed, and the dogs had to visit the hospital again. These dogs were diagnosed with vasculitis, and the symptoms improved within a week after providing the appropriate treatment. The CBC and serum biochemistry profiles showed no specific changes before and after the MSC infusion. The side effects of the MSC treatment such as pulmonary thromboembolism, pulmonary oedema, and haemorrhage were not observed in the dogs. In addition, the radiographs also showed no

significant changes before and after the MSC infusion.

3.3 No risk of tumorigenesis was observed after MSC therapy

To assess the risk of tumorigenesis of the MSC therapy, we evaluated whether any growth of new neoplasms occurred after the first MSC treatment by conducting physical examination, blood analysis, and imaging (radiography and/or ultrasonography). Neoplasms were not identified within the follow-up period (at least 6 months) in all injections (Table 3).

4. Discussion

This study evaluated the side effects of MSC transplantation by the IV infusion of allogenic MSCs in dogs, and examined the long-term risk of tumorigenesis, based on physical examination, blood tests, and radiography. Apart from the 3 injections, among the total of 314 injections (40 dogs), which showed mild vasculitis, no significant side effects of the MSC therapy were detected in 6 months after the first MSC treatment. The risk of tumorigenesis of MSC therapy which was evaluated by conducting a long-term monitoring of neoplasms detected no new neoplasm within the 6-month follow-up period.

The safety of IV administered MSCs has been assessed in numerous studies. In a study which involved human patients with a chronic inflammatory condition, treatment with the human umbilical cord blood-derived MSCs (hUBC-MSCs) was reported to be safe and efficient (Mehling et al. 2015). Another study which performed the intravenous administration of allogenic AT-MSCs in cats with a chronic kidney disease reported no significant side effects (Quimby et al. 2013, 2016). Another two studies performed on dogs with inflammatory bowel disease and refractory atopic dermatitis, respectively, the IV infusion of allogenic canine AT-MSCs shows no systemic side effects during the follow-up period of 6 weeks and 6 months, respectively, (Perez-Merino et al. 2015, Villatoro et al. 2018).

However, there are many conflicting reports regarding the tumorigenic potential of MSCs. It was reported that murine bone marrow-derived MSCs undergoes a malignant transformation in vitro and in vivo (Tolar et al. 2007, Jeong et al. 2011). On the other hand, another study reported that human MSCs are not susceptible to malignant transformation in long-term in vitro culture (Bernardo et al. 2007). Further, various studies reported that the administration of hUCB-MSCs in immunodeficient mice do not result in the formation of tumours, implicating that hUCB-MSCs lacks tumorigenicity in vivo (Wang et al. 2013, Park et al. 2016). However, studies have rarely monitored

the long-term risk of tumorigenesis of the allogenic MSC infusion in dogs and our study suggests that allogenic canine AT-MSCs do not have a tumorigenic potential.

This study, however, has a few limitations. The sample size used in the study is small as only a limited number of dogs satisfied the inclusion criteria. In addition, an even longer period of monitoring is necessary to assess the long-term safety aspects of the MSC therapy. Despite the limitations, observations from this study are important because it is the first study to assess the long-term safety of IV infusions of allogenic AT-MSCs in client-owned dogs.

5. Conclusions

In conclusion, the IV administration of canine allogenic AT-MSCs in the client-owned dogs treated for various diseases did not show any side effects as observed by the physical examination, blood tests, and radiography, in most of the injections. Further, the neoplasms were not observed during the period of at least 6 months following the MSC transplantation, suggesting a minimal risk of tumorigenesis associated with the MSC therapy. Thus, in canine subjects, allogenic MSC infusion proved to be a useful and a relatively safe therapeutic approach.

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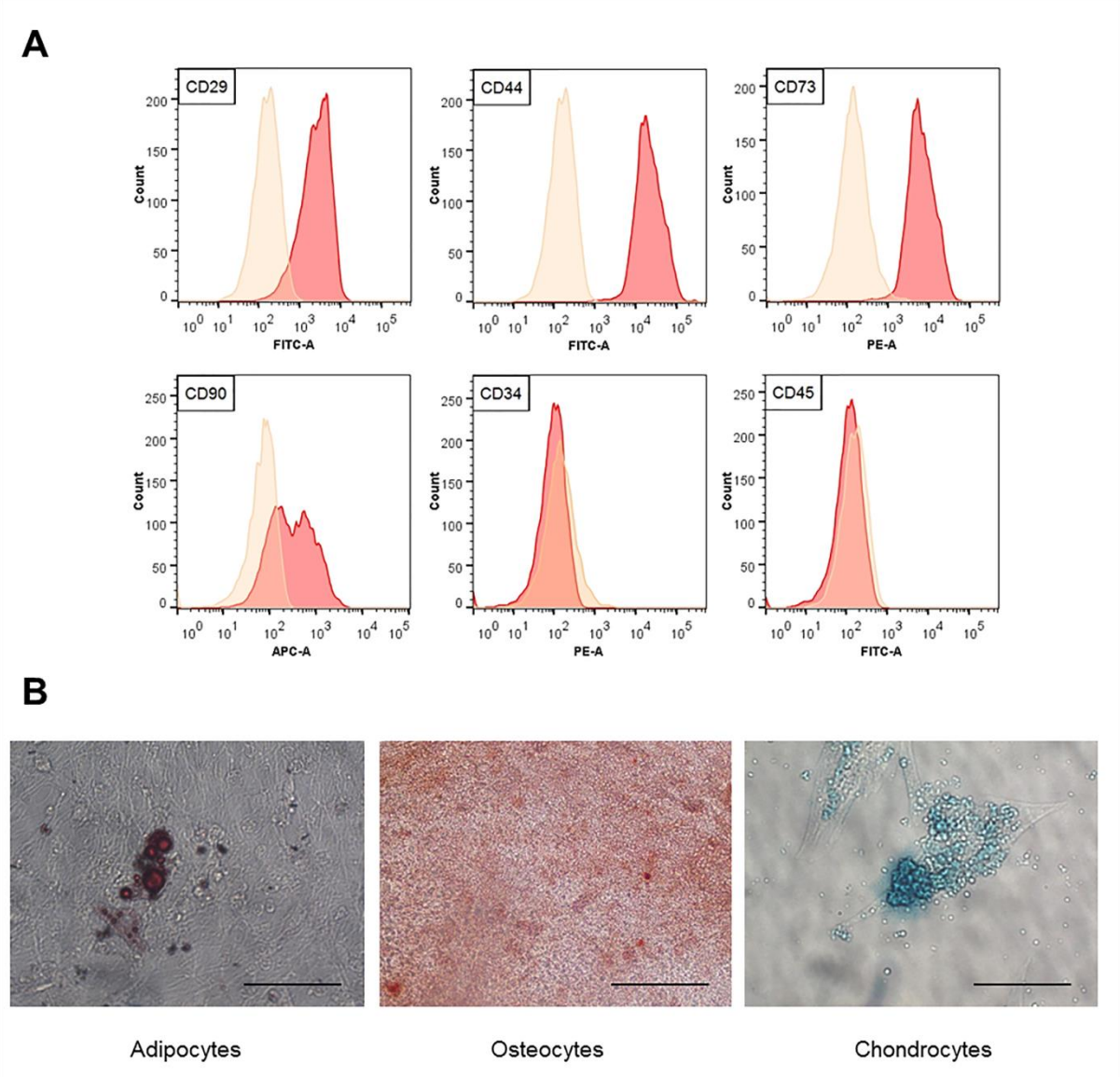

Figure 1 Mesenchymal stem cells isolated from canine adipose tissue were characterised. (A) Immunophenotype analysis by flow cytometry. (B) Adipogenic (oil red O staining), osteogenic (alizarin red S staining), and chondrogenic (alcian blue staining) differentiation of canine adipose tissue–derived mesenchymal stem cells. Bars = 200 µm.

Table 1. Signalment factors of the dogs enrolled in the study

Patients characteristics	**Treated dog**
Age (years)	11.2 ± 3.13
BW (kg)	6.12 ± 6.55
Breeds	
Poodle	5
Maltese	10
Pomeranian	1
Shih tzu	7
Cocker spaniel	3
Yorkshire terrier	3
Mixed	2
Miniature Pinscher	1
White terrier	1
Schnauzers	3
Afghan Hound	2
Pekingese	1
Old English Sheepdog	1
Sex	
Castrated male	19
Intact male	2
Intact female	6
Spayed female	13

BW: body weight

Table 2. Underlying diseases of the dogs enrolled in the study

Disease type	Details (n*)
Urinary disease	Acute kidney injury (2), Chronic Kidney Disease (12), Fanconi's syndrome (1)
Cardiovascular disease	Chronic Valvular Heart Disease (5)
Neurological disease	Hydrocephalus (1), Granulomatous Meningoencephalitis (1)
Hepatobiliary disease	Chronic Hepatitis (3), Liver Cirrhosis (1), Hepatocutaneous syndrome (1)
Gastrointestinal disease	Inflammatory Bowel disease (1), Protein-Losing Enteropathy (1)
Endocrine disease	Diabetes Mellitus (4)
Tumour	Lymphoma (3), Hepatocellular Carcinoma (1)
Immune-mediated disease	Immune-mediated haemolytic anaemia (1), Immune-mediated polymyositis (1), Pemphigus foliaceus (1)

*Number of dogs

Table 3. Safety evaluation of intravenous administration of canine allogenic adipose tissue-derived MSCs

Case	Signalment	Underlying disease	Complication after MSC administration	Number of MSC administration	Follow-up period from MSC therapy (Months)	Subsequent tumorigenesis
1	11yr MC Poodle	DM	-	3	14	-
2	12yr FS Maltese	CKD	-	3	16	-
3	13yr FS Pomeranian	DM	-	4	11	-
4	9yr IF Shih-tzu	Chronic hepatitis	-	5	39	-
5	9yr FS Cocker spaniel	CKD	-	2	6	-
6	6yr IF Maltese	Protein losing enteropathy	-	5	25	-
7	13yr MC Yorkshire terrier	CKD, Proteinuria	-	10	21	-
8	6yr MC Cocker spaniel	AKI	-	4	6	-
9	12yr MC Mixed	CVHD	-	8	33	-
10	15yr FS Poodles	DM	-	3	6	-
11	11yr MC Mixed	DM	Vasculitis	5	15	-
12	10yr MC Shih-tzu	CVHD	-	3	35	-
13	13yr MC Maltese	CKD	-	7	20	-
14	9yr FS Poodles	Fanconi syndrome	-	11	29	-
15	16yr FS Miniature pinscher	CKD	-	2	6	-
16	8yr FS Maltese	CVHD	-	4	8	-
17	14yr IF Maltese	Hydrocephalus	-	3	12	-
18	8yr MC White terrier	AKI	-	5	8	-
19	14yr MC Maltese	CKD, Proteinuria	-	6	7	-
20	14yr FS Schnauzer	CKD	-	4	6	-
21	13yr IM Cocker spaniel	CKD	-	2	6	-
22	12yr MC Maltese	Hepatocutaneous syndrome	-	24	18	-
23	7yr IF Afghan hound	Immune mediated polymyositis	-	3	7	-
24	12yr FS Shih-tzu	Hepatocellular Carcinoma	-	51	28	-
25	2yr MC Yorkshire terrier	GME	-	5	6	-
26	12yr MC Shih-tzu	CKD	-	3	6	-
27	13yr MC Schnauzer	Chronic Hepatic Failure	-	4	6	-
28	10yr MC Afghan hound	Multicentric lymphoma	-	16	7	-
29	16yr FS Schnauzer	Chronic Hepatic Failure	-	9	10	-
30	11yr MC Yorkshire terrier	CKD	-	19	20	-

31	15yr FS Maltese	CVHD	Vasculitis	13	16	-
32	7yr FS Poodle	Inflammatory Bowel Disease	Vasculitis	3	14	-
33	13yr MC Pekingese	CVHD	-	2	10	-
34	15yr MC Shih-tzu	Multicentric lymphoma	-	2	7	-

MC: Male Castrated, FS: Female Spayed, IM: Intact Male, IF: Intact Female

DM: Diabetes Mellitus, CKD: Chronic Kidney Disease, AKI: Acute kidney injury, CVHD: Chronic Valvular Heart Disease, GME: Granulomatous meningoencephalitis, IMHA: Immune-mediated Hemolytic Anemia

국문 초록

40마리의 개에서 동종 지방 조직 유래 개 중간엽 줄기 세포의 정맥 내 투여의 안전성 평가

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줄기세포 치료는 여러 질환에서 치료 효과와 임상적 적용에 대해 수 년 동안 연구되어 왔으며, 최근 수의학에서도 그에 관련된 연구들이 발표되었다. 하지만, 개에서 종양 형성능을 포함한 장기적 안전성을 평가한 보고는 거의 없다. 본 연구에서는 개에서 동종 지방유래 중간엽 줄기 세포의 정맥 내 투여의 부작용을 평가하고 종양 형성을 포함한 장기 안전성을 조사하고자 하였다. 2012년에서 2018년 사이에 기저 질환 치료를 위해 서울대학교 동물병원 내과에 내원한 40마리의 개에서 정맥 내로 줄기세포 투여 후 신체검사, 혈액검사, 영상검사 등의 다양한 임상적 평가가 이루어졌으며, 신생 종양 형성 여부를 6개월의 추적 기간 동안 조사하였다. 첫 번째 줄기세포 투여 후 6 개월의 추적 기간 동안 임상 평가, 혈액 검사 및 방사선 검사 결과 심각한 부작용은 발견되지 않았으며, 이 기간 동안 새로운 신생물은 관찰

되지 않았다. 본 연구는 정맥 내 동종 지방유래 중간엽 줄기세포 주입에 대한 6개월 이상의 장기적 안전성 및 종양 발생 위험성을 평가한 첫 연구이다. 결과는 동종 지방유래 중간엽 줄기세포 주입 시 개에게 종양 형성능이 확인되지 않았으며 줄기세포 치료는 개에서 유용하고 비교적 안전한 치료 방법이 될 수 있음을 시사하였다.

주요어: 개; 부작용; 세포 치료; 정맥 내 투여; 종양 형성; 중간엽 줄기세포

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