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Anti-inflammatory effects of canine adipose-derived mesenchymal stem cells in a rat model of severe acute pancreatitis

급성췌장염랫드모델에서

개의 지방유래 중간엽 줄기세포의 항염증효과

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Anti-inflammatory effects of canine adipose-derived mesenchymal stem cells in a rat model of severe acute pancreatitis

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Abstract

Severe acute pancreatitis (SAP) is associated with systemic complications and high mortality rate in dogs. Mesenchymal stem cells (MSCs) have been investigated for their therapeutic potential in several inflammation models. The present study investigated the effects of canine adipose tissue-derived (cAT)MSCs in a rat model of SAP induced by retrograde injection of 3% sodium taurocholate solution into the pancreatic duct. cATMSCs labeled with dioctadecyl-3,3,3'-tetramethylindo-carbocyanine perchlorate (1×10^7 cells/kg) were systemically

administered to rats and pancreatic tissue was collected 3 days later for histopathological, quantitative real-time PCR, and immunocytochemical analyses. Greater numbers of infused cATMSCs were detected in the pancreas of SAP as compared to sham-operated rats. cATMSC infusion reduced pancreatic edema, inflammatory cell infiltration, and acinar cell necrosis, and decreased pancreatic expression of the pro-inflammatory cytokines tumor necrosis factor-α, interleukin (IL)-1β, -6, -12, -17, and -23 and interferon-γ, while stimulating expression of the anti-inflammatory cytokines IL-4 and IL-10 in SAP rats. Moreover, cATMSCs decreased the number of cluster of differentiation 3-positive T cells and increased that of Forkhead box P3-positive T cells in the injured pancreas. These results indicate that cATMSCs can be effective as a cell-based therapeutic strategy for treatment of SAP in dogs.

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CHAPTER III.

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Abbreviations

cAT-MSCs	Canine adipose tissue-derived mesenchymal stem cells
CCR	Chemokine receptor
CM	Conditioned medium
CM-DiI	Chloromethylbenzamidio-1, 1'-dioctadecyl-3,3,3'-tetramethylindo-carbocyanine perchlorate
cPBMC	Caine peripheral blood mononuclear cell
cRPS5	Canine ribosomal protein S5
MSCs	Mesenchymal stem cells
SAP	Severe acute pancreatitis
TCA	Sodium taurocholate

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CHAPTER I

Literature review

1. Severe acute pancreatitis and their treatment

SAP is an acute abdominal disease with a high prevalence rate, severe symptoms, complicated etiology and mortality rate of 20 to 30%. Although SAP's mechanism has not completely recovered, changes in the secretory pattern of pancreatic brain cells, intracellular activation of proteases, and the production of inflammatory mediators may be associated with SAP outbreaks. About 10% of patients with acute pancreatitis lead to necrosis of the pancreas and surrounding tissues leading to necrotic tissue infection, multiple organ failure and death (Xiao-Huang et al. 2014).

During AP, Local recruitment and activation of inflammatory cells induces the release of proinflammatory cytokines including interleukin (IL) 6, IL-8, IL-18 and tumor necrosis factor (TNF-a). These prominent inflammatory mediators play a pivotal role in the pathogenesis of SAP and AP-associated organ dysfunction. Until now, certain treatments for AP in clinical setting have had limited impact on patient outcomes. Failure of medication in clinical trials may be due to improper

intervention and a single pharmacological treatment strategy applied (Marie Louise Malmstrom et al. 2012).

Currently, clinical therapies of SAP are often conservative therapies, including the synthesis and secretion of pancreatic enzymes, antibiotic use, and nutritional support. But until now, there are no effective strategies for treating SAP. Therefore, new treatment strategies should be developed to reduce the complications and mortality of SAP (Kyung Nee Jung et al. 2014).

2. Mesenchymal stem cells (MSC)

Stem cell based therapies for the repair and regeneration of tissue and organs offer promising therapeutic solutions for various diseases. MSCs belong to adult stem cells with self-renewal and multilineage differ- entiation potentials. They have been used to treat a lot of diseases, including pulmonary injury, acute kidney failure, and myocardial infarction. Also, MSCs has been reported to act as immune regulators, which can inhibit inflammatory injury. All these advantages are beneficial to the cell replacement therapy for the treatment of various inflammatory diseases (Kyung Nee Jung et al. 2014).

3. The anti-inflammatory effects of MSCs

A deeper understanding of the mechanisms by which MSCs derived from veterinary species control inflammation and contribute to healing will benefit both humans and animals. Many veterinary species are currently being used as human disease models for cellular therapy (eg pigs for cardiovascular disease, chlorine for orthopedic lesions). MSC therapy is also increasingly used as a mainstream in a variety of accompanying animal diseases, including tendon, bone and cartilage damage in horses and arthritis, and arthritis in dogs. MSCs have been shown to interact with CD4 and CD8 lymphocytes and, when activated in the presence of inflammatory mediators, release mediators that down regulate inflammation (Danielle D Carried and Dori L Borjesson, 2013).

Lymphocyte proliferation. MSCs derived from all tissue sources have potent immunomodulatory capacity in vitro. Autologous and allogeneic MSCs are non-immunogenic, and completely unmatched MSCs do not induce leukocyte proliferation in the absence of in vitro activation. MSC is also an anti-inflammatory. The ability of MSCs to inhibit the proliferation of T lymphocytes stimulated in vitro is well documented for MSCs obtained from nonhuman primates, dogs, chickens, rabbits, pigs, sheep, and horses. Lymphocyte proliferation in vitro is suppressed to a maximum at MSC: lymphocyte ratios of 1: 1, 1: 5 or 1:10. One proposed mech-

anism for this inhibition of lymphocyte proliferation is the MSC-induced T-cell-cycle arrest at G0, which is thought to be regulated at the molecular level by a decrease in lymphocyte cyclin D levels. MSC reduces the expression of the activation markers (CD25, CD38 and CD69) in T cells and prevents activation and proliferation.

Pretreatment of MSC with IFNγ, an intervening mediator in inflammatory conditions, enhances MSC's ability to reduce lymphocyte proliferation. Also, in one in vitro study, heterologous porcine BM-MSC did not stimulate human lymphocyte proliferation; Rather, they inhibited lymphocyte proliferation in a dose-dependent manner after stimulation.

Both cell-cell contact and soluble factors are thought to play an important role in the inhibition of lymphocyte proliferation by MSCs. Toll-like receptors on the surface of MSC and FAS-ligand-dependent interactions, intracellular adhesion molecule 1 and vascular cell adhesion molecule 1 are thought to be involved in cell-cell immunosuppression but not in aqueous cells. MSC supports the idea that MSCs also inhibit lymphocyte proliferation in transwell analysis where the cells are physically separated from lymphocytes, producing MSCs with soluble factors related to immunomodulatory activity. In addition, media prepared previously from cultures of activated MSCs (defined as MSCs exposed to proinflammatory mediators) inhibit lymphocyte proliferation.

Although this work has not been extensively extended from veterinary species to MSCs, MSC interactions with other immune cells have been extensively studied in both humans and rodents. Human MSCs reduce the proliferation of CD4 + and CD8 + T cells, induce a conversion to the Th2 phenotype, and suppress Th17 differentiation and function. Human and rodent MSCs regulate dendritic cell (DC) development in monocytes and impair DC function. Damaged DC function includes modulation of MHCII and T cell assisted stimulation molecule expression, down regulation of cytokine production and prevention of DC homing to the lymph nodes. The downstream effects of this change include the limitation of the ability of DC to stimulate T cell responses. Similar to the effects on T lymphocytes, human MSC inhibits cell cycle progression by inhibiting B cell proliferation in a dose-dependent manner.

Mediator production. Soluble immunosuppressive factors that have been shown to be produced or expressed by MSCs from veterinary species include TGFβ1, hepatocyte growth factor, PGE2, indoleamine 2,3-dioxygenase (IDO), nitric oxide (NO), vascular endothelial growth factor And IL6. Mediator production by MSC in veterinary species has not been thoroughly tested, and mediators are reported in the literature. The absence of an arbitrator means that the MSC has not produced it and its production has not yet been decided. Some mediators are produced constitutively, while others are secreted after MSC activation by cytokines or mediators found in inflammatory environments. Mediators produced by MSCs downregulate

inflammation and stimulate angiogenesis.

The specific mediator from MSC produces may vary depending on the species and tissue source. Horses MSCs produce differentiated mediators according to the MSC tissue supply. MSCs extracted from peripheral plasma (BM- and CB-MSC) produce NO, but not MSCs extracted from solid tissue (CT and AT-MSC). It is not known whether this difference in mediator production in vivo confers in vivo functional differences. There is implicit evidence that the time to heal for speech lesions is reduced when the horse CB-MSC is compared to other tissue-derived MSCs. I speculate that NO production by horse CB-MSC causes increased angiogenesis in vivo. Species-related variability was described in the production of NO and IDO by MSC. Human MSCs produce high levels of NO and NO, but mouse MSCs produce high levels of NO and do not produce IDO. MSCs in humans and mice have been reported to induce T cells (CD4 + CD25 + FoxP3 +) that inhibit the immune response of T cells and promote T cell tolerance to the antigen. Induction or specific production of T-regulated cells is indicated by the presence of TGF [beta] l and IL10 in the microenvironment. The study is progressing structurally compared to the inducible production of TGFβ1 by human MSCs.

Production of TGFβ1 by veterinary species depends on species, tissue type and MSC activation status. Dog BM-MSC, pig BM-MSC and horses AT, BM, CT and CB-MSC do not increase TGFβ1 production after exposure to activated lymphocytes. The dog BM-MSC produces TGFβ1 at levels insufficient to inhibit

lymphocyte proliferation. Conversely, dog AT-MSC increases TGFβ1 production after exposure to lymphocytes and rabbit BM-MSC increases TGFβ1 production after IFNγ pretreatment in vitro. So far, studies on veterinary species have failed to demonstrate that MSC production of TGFβ1 shifts T lymphocytes to the T-modulated phenotype.

The role of specific mediators on lymphocyte proliferation or function can be assessed through the measurement of chemical blockade of individual mediators and corresponding lymphocyte proliferation in MSC-lymphocyte co-cultures (mixed lymphocyte responses). Although it has not yet been elucidated in veterinary species, it has been shown that blocking TGFβ1 produced by human BM-MSC reduces the production of T-regulated cells and significantly inhibits T cell proliferation, thus TGFβ1 plays an important role in MSC function have. Blocking of PGE2 or IDO produced by open BM- or AT-MSC restores lymphocyte proliferation, indicating that both lymphocytes function in regulating MSC-lymphocyte interactions. Also, our experience shows that PGE2 is a major mediator in inhibiting lymphocyte proliferation by horse AT, BM, CT, and CB-MSC, because lymphocyte proliferation is restored by blocking PGE2

MSC immunoregulation is stimulated by proinflammatory mediators, IFNγ and TNFα. MSCs derived from IFNγ-receptor knockout mice can not inhibit the lymphoproliferative response. At baseline, unstimulated lymphocytes and MSC also do not produce this proinflammatory mediator. However, activated

lymphocytes secrete IFNγ and TNFα to stimulate MSCs. While AT-MSC reduces TNFα production and increases IFNγ production by lymphocytes, horse BM-, AT-, CB- and CT-MSC decrease both TNFα and IFNγ. Pig BM-MSC reduces IFNγ and IL2 production by lymphocytes. Whether such a reduction in the mediator is associated with a decrease in the total number of lymphocytes present in the culture or with a functional change in the lymphocyte phenotype and the induction of T-regulatory cells is not known.

The relevance of these mediators in vivo is not well understood in veterinary medicine. Few studies have examined mediators in body fluids or tissues, and the dynamics of inflammation and the overlapping of many mediators make interpretation of mediator concentration difficult at the point of isolation. In the horse model of osteoarthritis, PGE2 levels were significantly decreased in synovial fluid after BM-MSC treatment. The authors noted that after one week of MSC administration, the difference was due to decreased swelling and inflammation. Human MSCs exposed to joint fluid from osteoarthritis and rheumatoid arthritis in vitro increase IL-6 and IDO mRNA expression and inhibit lymphocyte proliferation.

There is still some discrepancy in the literature on species-specific identification of inflammatory mediators produced by MSCs or inhibited by MSCs. The diversity of results can be attributed to the methodology by which researchers measure cytokine production. Test kits and reagents are not always specific to veterinary species and have been adopted in human or laboratory animal methodology.

Through large collaboration and communication through the veterinary and stem cell research communities, all researchers have access to the tools that are most appropriate for their research.

4. Therapeutic potential of MSCs in severe acute pancreatitis

MSC has been shown to improve the inflammatory response of the liver, kidneys and lungs and has the potential for clinical applications in myocardial infarction, Crohn's disease and organ transplantation. Several inflammatory cytokines have been implicated in the pathogenesis of SAP, including proinflammatory cytokines (IL-1, IL-6, TNF-α) and anti-inflammatory cytokines (IL-4, IL-10). Therefore, MSCs have the potential to treat pancreatitis due to the anti-inflammatory effects of MSCs. In addition, MSCs can be induced to differentiate into different cell types including insulin-secreting cells and endothelial cells. Thus, it is possible to promote pancreatic regeneration and recovery of microvascular endothelial cells of the pancreas with MSCs.

CHAPTER II

Isolation of canine mesenchymal stem cell from adipose tissues

1. Introduction

Cell therapy is a promising application in the field of veterinary regenerative medicine and surgery, separating and characterizing stem cells in a variety of areas of interest.

The source of abundant and accessible stem cells is fatty tissue. This cell, called adipocyte-derived stromal cell (ASC), is a fibroblast-like cell capable of multiple differentiation and is found in other species. Several groups have demonstrated that human mesenchymal cells in epilepsy-blood vessel fraction (SVF) of subcutaneous adipose tissue (PLA) cells are capable of differentiation in multiple lines, including muscle cells, in the presence of lineage-specific inducible mediators (N.M Vieira et al. 2010).

In humans, ASC for autologous transplantation is relatively rapidly separated from adipose tissue by collagenase digestion. I have recently shown that ASCs in human subcutaneous fat can differentiate in adipocytes, osteogenic cells, chondrocytes and

myeloid lineages and produce human muscle proteins in vitro and in vivo.

Successful transplantation of canine adipose-derived stem cells (cACSs) in dogs has been reported by Li et al. And Black et al. However, this copy lacked the full character of the managed cell population. Here, I report the isolation, characterization and possibility of multiple regression differentiation of cASC in abdominal fat tissue as routine surgery.

2. Material and Methods

2.1 Adipose tissue isolation

Canine adipose tissue was obtained from a healthy dog < 1 year old during routine spay surgery at Seoul National University Veterinary Medicine Teaching Hospital (SNU VMTH) and MSCs were isolated as previously described (Vieira NM et al, 2010). Briefly, the tissue was washed three times with phosphate-buffered saline (PBS; PAN Biotech, Aidenbach, Germany) containing 100 U/ml penicillin and 100 g/ml streptomycin, then cut into small pieces and digested for 1 h at 37℃ with collagenase type IA (1 mg/ml; Sigma-Aldrich, St. Louis, MO, USA). The enzymatic activity was inhibited with Dulbecco's Modified Eagle's Medium (DMEM; PAN Biotech) containing 10% fetal bovine serum (FBS; PAN Biotech).

After centrifugation at 1200 x g for 5 min, the pellet was filtered through a 70-µm Falcon cell strainer (Fisher Scientific, Waltham, MA, USA) to remove debris and then incubated in DMEM containing 10% FBS at 37℃ in a humidified atmosphere of 5% CO2.

2.2 Cell culture

After 48 h, cultures were washed with PBS to remove non-adherent cells and incubated with fresh medium, which was changed every 48 h until cells reached 70%–80% confluence, after which they were repeatedly subcultured under standard conditions.

2.3 Flow cytometery

Cells were characterized for the expression of several stem cell markers by flow cytometry using fluorescein isothiocyante (FITC)-, phycoerythrin (PE)-, or allophycocyanin (APC)-conjugated antibodies against the following proteins: cluster of differentiation (CD)29-FITC, CD31-FITC, CD34-PE, and CD73-PE (BD Biosciences, Franklin Lakes, NJ, USA); and CD44-FITC, CD45-FITC, and CD90-APC (eBiosciences, San Diego, CA, USA). Cells were analyzed using a FACSAria II system (BD Biosciences). Cellular differentiation was evaluated using the

StemPro Adipogenesis Differentiation, StemPro Osteogenesis

2.4 Differentiation

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.

3. Results

3.1 Characterization of cATMSCs

The cells showed fibroblast-like shapes when cultured on plastic culture plates. They were identified by the expression of known MSC markers. The cATMSCs were shown to have excellent multilineage plasticity. Cell type–specific staining showed that the cATMSCs successfully differentiated into adipocytes, osteoblasts, and chondrocytes, when they were induced in vitro by adipogenic, osteogenic, and chondrogenic media, respectively.

Cells isolated from canine adipose tissue were identified by immunophenotyping and multilineage differentiation. The cells had a high expression of known MSC markers such as CD29, CD44, CD73, and CD90, and did not express CD31,

CD34, or CD45 (Fig. 1A). The cATMSCs had multilineage plasticity, as demonstrated by their potential for adipogenic, osteogenic, and chondrogenic differentiation (Fig. 1B).

4. Discussions

Zuk et al. were the first to describe the isolation and characterization of human stem cells derived from adipose tissue. These cells were able to differentiate into adipogenic, osteogenic, chondrogenic, and myogenic lineages when exposed to inductive media.

Human ASCs are usually obtained from fat tissue that is discarded after liposuction cosmetic surgery. Adipose tissue can be harvested in large quantities with minimal morbidity in several regions of the body and, on average, 100 ml of human adipose tissue yields about 1×10^{6} stem cells. In dogs, the adipose tissue can be collected by a simple adapted liposuction surgery, through biopsies or in routine veterinary surgery procedures because I could isolate cASCs from just 100 μl of adipose tissue.

Adipose tissue-derived MSCs have the advantage that they are easier to isolate and can be obtained in a larger quantities than other types of MSC.

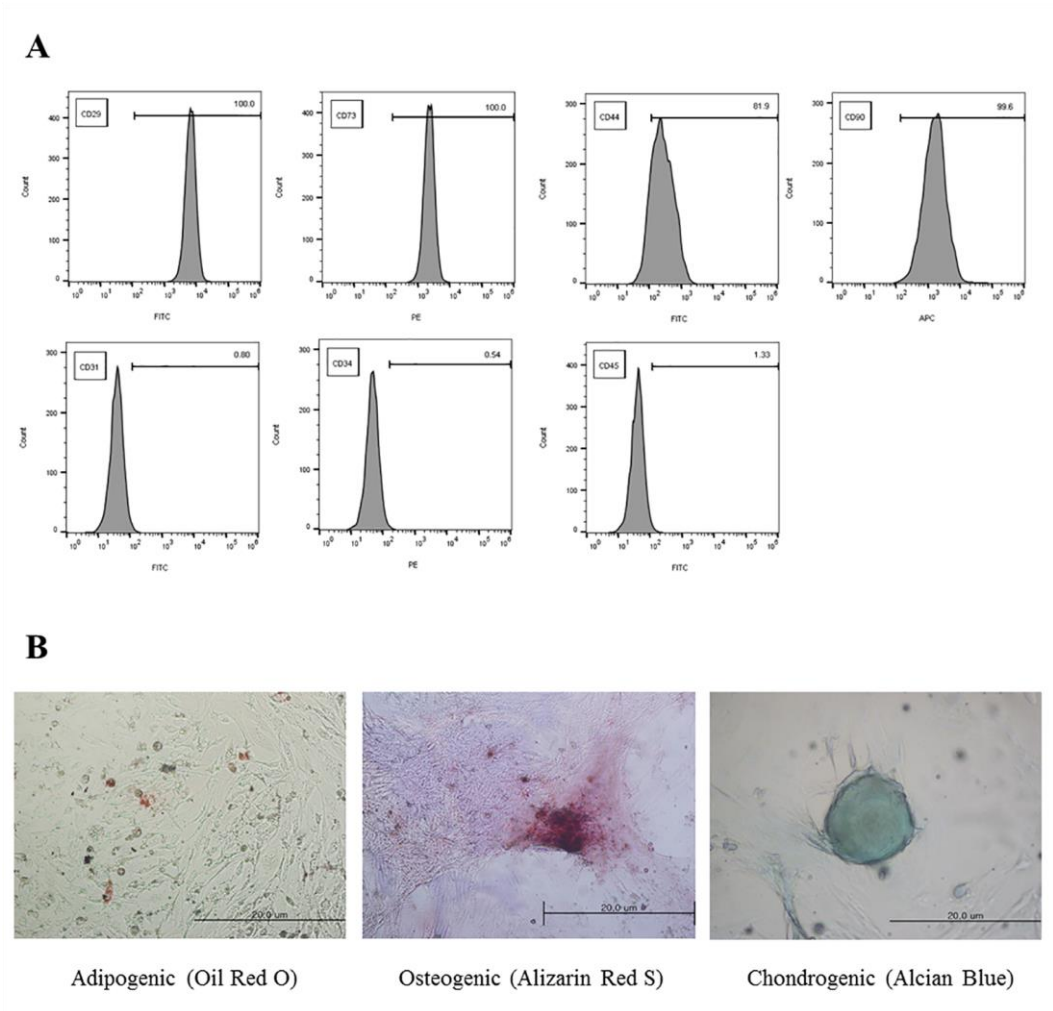

Figure 1. Identification of mesenchymal stem cells (MSCs) isolated from canine adipose tissue (A) immunophenotypic analysis by flow cytometery. (B) Adipogenic, osteogenic, and chondrogenic differentiation of canine adipose tissue-derived (cAT)-MSCs. 200X. Scalebar = 20 µm (B).

CHAPTER III

Therapeutic effects of canine ATMSCs on SAP in the rat model

1. Introduction

Acute pancreatitis is a common disease in dogs. Although most cases are self-limiting and fully reversible, some progress to severe acute pancreatitis (SAP), which leads to systemic complications such as multi-organ failure and diffuse intravascular coagulation (Cook A et al., 1993; Manfield C C, 2012). Mortality rates among dogs with SAP are 27%–42% (Hess RS et al., 1998). To date, there are no effective treatment strategies, which requires a better understanding of the pathophysiology of SAP. A breed predisposition has been reported for acute pancreatitis that deteriorates into SAP, implying that the disease is related to hereditary mutations, including those that cause auto-activation of trypsin resulting in pancreatic edema, death of acinar cells (Whitecomb DC et al., 1996), and an inflammatory response mediated by cytokines such as tumor necrosis factor (TNF)-α, interleukin (IL)-1β, -6, -12, -4, and -10, interferon (IFN)-γ released by macrophages and T cells (Pandiyan P et al., 2007; Tsuda H et al., 2014). Overproduction of these inflammatory cytokines can lead to systemic manifestations, multi-organ failure, or

death (Norman J. et al., 1998)

MSCs have recently been investigated for their therapeutic potential in the treatment of SAP. Previous studies have shown that MSCs regulate immune responses in inflammatory bowel disease, sepsis, encephalomyelitis, and arthritis models (Augello A et al., 2007; Gonzalez-Rey E et al., 2009; Gonzalez MA et al., 2009; Zappia E. et al., 2005). In addition, human MSCs have been reported to mitigate SAP by suppressing inflammation in a rodent model (Jung KH et al., 2011; Jung KH et al., 2015; Meng HB et al., 2013). Although canine MSCs can differentiate into multilineage cells (Li H et al., 2009; Penha EM et al., 2014), few studies have focused on their immunomodulatory effects.

Therefore, the present study investigated the therapeutic effects of canine adipose tissue-derived (cAT)MSCs in a rat model of SAP as well as their modulation of host immune response.

2. Materials and Methods

2.1 Animal experiments and cell transplantation

Male Sprague-Dawley rats (Nara Biotech, Seoul, Korea) weighing 190–220 g were used for experiments. All experimental procedures involving animals were approved by the Institutional Animal Care and Use Committee of SNU (protocol no. SNU-150820-1). Rats were randomly divided to the following four groups:

SHAM+PBS (n = 4), SHAM+MSC (n = 4), SAP+PBS (n = 8), and SAP+MSC (n = 8). SAP was induced by retrograde injection of TCA (Sigma-Aldrich) into the pancreatic duct as previously described (Hua J et al., 2013). Briefly, rats were anesthetized and laparotomized at the midline. After exposing the first loop of the duodenum and the pancreas, the proximal common bile duct was clipped with a microclamp. A needle catheter was inserted through the middle common bile duct via the duodenal papilla and ligated, and a solution of 3% TCA in saline (1 ml/kg) was injected into the common bile-pancreatic duct over a 60-s period. The micro-clamp and ligature were then removed and the abdomen closed. Rats in SHAM groups were also anesthetized and laparotomized at the midline, but their abdomens were closed without additional manipulations. After surgery, cATMSCs labeled with chloromethylbenzamido-1,1'-dioctadecyl-3,3,3'-tetramethylindocarbocyanine perchlorate (CM-DiI; Invitrogen, Carlsbad, CA, USA) were administered via the tail vein to rats in the MSC groups (1×10^7 cells/kg in 200 μl PBS), while an identical volume of PBS was administered via the same route to PBS groups. Rats were sacrificed 3 days after these procedures. Blood samples were centrifuged to obtain serum, which was stored at −80°C until use. Pancreatic tissue was collected and washed in cold PBS. After weighing the pancreas, half of the tissue was fixed in 10% formaldehyde and embedded in paraffin while the other half was stored at −80°C until use.

2.2 Histological analysis

Paraffin-embedded tissue samples were cut into 4-μm sections that were stained with hematoxylin and eosin and examined under a light microscope. A total of 20 randomly selected fields per group were scored in a blinded manner. Pancreatic acinar cell injury was scored on a scale from 0 to 4 based on the degree of edema (0 = absent, 1 = expanded interlobar septa, 2 = expanded interlobular septa, 3 = expanded interacinar septa, 4 = expanded intercellular septa), infiltration [0 = 0–1 white blood cells (WBCs)/high-power field (HPF), 1 = 2–10 WBCs/HPF, 2 = 11–20 WBCs/HPF, 3 = 21–30 WBCs/HPF, 4 = > 30 WBCs/HPF], and necrosis (0 = absent, 1 = 1–4 necrotic cells/HPF, 2 = 5–10 necrotic cells/HPF, 3 = 11–15 necrotic cells/HPF, 4 = >15 necrotic cells/HPF).

2.3 Measurement of serum amylase, lipase, and inflammatory cytokine levels

Serum amylase and lipase activities were measured using the EnzyChrom α-Amylase Assay and QuantiChrom Lipase Assay kits (both from BioAssay Systems, Hayward, CA, USA) according to the manufacturer's instructions. Serum IFN-γ and IL-10 levels were measured using rat-specific Quantikine enzyme-linked immunosorbent assay kits (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions.

2.4 RNA extraction and quantitative real-time PCR

Approximately 100 mg of pancreas tissue was homogenized and RNA was extracted using the Easy-BLUE Total RNA Extraction kit (Intron Biotechnology, Sungnam, Korea). cDNA was synthesized using LaboPass M-MuLV Reverse Transcriptase (Cosmo Genetech, Seoul, Korea) according to the manufacturer's instructions. Samples were assayed in duplicate in 10 µl AMPIGENE qPCR Green Mix Hi-ROX with SYBR Green dye (Enzo Life Sciences, Farmingdale, NY, USA) using 1 µl cDNA and 400 nM forward primer and reverse primers (Cosmo Genetech). The cycling conditions were 95℃ for 2 min, followed by 40 cycles at 95℃ for 5 s and 60℃ for 25 s. Expression levels were normalized to that of glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

2.5 Canine peripheral blood mononuclear cell (cPBMC) isolation

The blood of a healthy canine donor was obtained from SNU VMTH and diluted with an equivalent volume of PBS, then layered over Ficoll-Paque PLUS (GE Healthcare Life Sciences, Uppsala, Sweden) in conical tubes. After centrifugation at 400 × *g* for 30 min, the cell layer was collected and washed twice with PBS. cPBMCs were resuspended in Roswell Park Memorial Institute (RPMI)-1640 medium (PAN Biotech) containing 10% FBS.

2.6 Rat splenocyte isolation

Splenocytes were isolated from rats as previously described. Briefly, rat spleens were removed and cut into small pieces in a cell strainer with PBS. The tissue was crushed using the plunger from a 1-ml syringe. The homogenized cell suspension was transferred to a tube and the cell pellet was obtained by centrifugation and resuspended in Red Blood Cell Lysis Buffer (Sigma-Aldrich). After two washes with PBS, splenocytes were resuspended in RPMI-1640 containing 10% FBS.

2.7 Mixed lymphocyte reaction (MLR)

Isolated canine PBMCs and rat splenocytes were used in the MLR. cATMSCs were treated with 25 μg/ml mitomycin C (Sigma-Aldrich) for 1 h at 37℃. After five washes, cATMSCs were seeded in a 96-well plate at 1×10^4, 1×10^3, and 1×10^2 per well to determine cATMSC-to-cPBMC ratio dependency. After 6 h, mitomycin C-treated cATMSCs were attached, and cPBMCs stimulated with 2 μg/ml concanavalin A (ConA; Sigma-Aldrich) were added to each well of cATMSCs cultured at 1×10^5/well. After 5 days, cPBMC proliferation was assessed with the BrdU Cell Proliferation Assay kit (BioVision, Milpitas, CA, USA) according to the manufacturer's instructions. For MLR with CM, cATMSCs were seeded in 6-well plates at 3×10^5/well, and the medium was changed after 24 h. After 5 days, the CM was harvested and centrifuged to remove debris. The MLR was carried out in CM as described above. The procedure was repeated using rat splenocytes.

2.8 Immunocytochemistry

Paraffin sections were cut at a thickness of 4 µm for immunolabeling. Sections were deparaffinized and rehydrated, and antigen retrieval was carried out in 10 mM citrate buffer. Sections were washed and blocked with blocking buffer containing 5% normal goat serum (Gibco/Life Technologies) and 0.3% Triton X-100 (Sigma-Aldrich) for 1 h, then incubated overnight at 4℃ with mouse monoclonal anti-CD3 (1:100) and anti-Forkhead box (Fox)P3 (1:100) antibodies (both from Santa Cruz Biotechnology, Santa Cruz, CA, USA). After three washes, the sections were incubated with secondary antibody (1:200; Santa Cruz Biotechnology) for 1 h at room temperature in the dark, then washed three times and mounted in Vectashield mounting medium containing 4',6-diamidino-2-phenylindole (Vector Laboratories, Burlingame, CA, USA). The samples were visualized with an epifluorescence microscope. Immunoreactive cells were counted in 20 random fields per group.

2.9 Statistical analysis

Data are expressed as mean ± standard deviation. Group means were compared by one-way analysis of variance and an unpaired Student’s t test using the GraphPad Prism v.6.01 software (GraphPad Inc., La Jolla, CA, USA). $P < 0.05$ was considered statistically significant.

3. Results

3.1 Histological analysis and assessment of pancreatic markers after cAT-MSCs infusion

Pancreatic tissue samples from SAP groups exhibited edema, inflammatory cell infiltration, and necrosis of acinar cells compared to those from SHAM animals. The severity of pancreatic injury was markedly diminished in the SAP+MSC as compared to the SAP+PBS group ($P < 0.001$; Fig. 2A). After cATMSC infusion, serum amylase and lipase activities—important markers of pancreatic function—were decreased by 45% (Fig. 2B) and 32% (Fig. 2C), respectively, as compared to the SAP+PBS group. In addition, the pancreas-to-body weight ratio, a measure of pancreatic edema, was reduced in the SAP+MSC relative to the SAP+PBS group ($P < 0.05$; Fig. 2D).

3.2 Detection of systemically infused cATMSCs in rat pancreas

The capacity of infused cATMSCs to migrate to the injured pancreas was evaluated by fluorescence microscopy and reverse transcription PCR analysis. CM-DiI-labeled cATMSCs were visible by their red fluorescence. There was a greater number of CM-DiI-labeled cATMSCs in the pancreatic tissue of the SAP+MSC than of the SHAM+MSC group, whereas no fluorescent cells were detected in the PBS groups (Fig. 3A). Consistent with this observation, the mRNA level of canine-specific ribosomal protein S5 (cRPS5) was higher in the SAP+MSC than in

the SHAM+PBS group (Fig. 3B), confirming the presence of cATMSCs.

3.3 Effect of cATMSCs on inflammatory response

The mRNA expressions of pro- and anti-inflammatory cytokines in pancreatic tissue were evaluated by quantitative real-time PCR. The levels of TNF-α, IL-1β, -6, -12, -17, and -23 and IFN-γ were markedly increased after SAP induction. cATMSC infusion resulted in the downregulation of these pro-inflammatory cytokines ($P < 0.05$ or $P < 0.01$; Fig. 4A). Conversely, the expression of the anti-inflammatory cytokines IL-4 and -10 were decreased by SAP induction, but were increased in the SAP+MSC group as compared to the SAP+PBS group ($P < 0.05$; Fig. 4A). Furthermore, serum levels of IFN-γ and IL-10 were decreased by 30% and 21%, respectively, in the SAP+MSC relative to the SAP+PBS group (Fig. 4B).

3.4 T cell regulation by cATMSCs

I next evaluated the effect of cATMSCs on T cells, which release inflammatory cytokines. cATMSCs suppressed the proliferation of cPBMCs treated with ConA in a cATMSC:cPBMC ratio-dependent manner. At 1:10 and 1:100 ratios, cPBMCs proliferation was markedly suppressed ($P < 0.001$ and $P < 0.05$, respectively) as compared to cells grown without cATMSCs. However, this was not observed at a 1:1000 ratio. To assess the effects of soluble factors from cATMSCs on T cells, CM from cATMSC cultures was used as described above. The proliferation of

cPBMCs stimulated with ConA in CM was suppressed ($P < 0.05$) relative to cells treated with control medium (Fig. 5A). cATMSCs also inhibited the proliferation of rat splenocytes stimulated with ConA in a cATMSCs:rat splenocyte ratio-dependent manner. This effect was apparent at a 1:10 ratio and in CM ($P < 0.001$ and $P < 0.05$, respectively), but was not observed at 1:100 and 1:1000 ratios relative to splenocytes grown without cATMSCs or in control medium (Fig. 5B). In addition, a quantitative analysis of T cells detected in pancreatic tissue by immunocytochemistry revealed that the percentage of $CD3^+$ T cells was decreased ($P < 0.05$; Fig. 5C) whereas that of $FoxP3^+$ regulatory T cells was increased ($P < 0.01$; Fig. 5D) in the SAP+MSC as compared to the SAP+PBS group.

4. Discussion

Recent studies suggest that the anti-inflammatory function of MSCs can be applied to the treatment of SAP (Kua J et al., 2014; Jung KH et al., 2011; Jung KH et al., 2015; Meng HB et al., 2013). Most of these studies used MSCs isolated from human umbilical cord or bone marrow in animal SAP models; however, the therapeutic effects of canine MSCs have never been evaluated in an SAP model, which is necessary for their application to the treatment of SAP dogs. Adipose tissue-derived MSCs have the advantage that they are easier to isolate and can be obtained in a larger quantities than other types of MSC.

Intravenous administration of cATMSCs significantly mitigated SAP: not only were serum amylase and lipase activities reduced, but histopathological manifestations including pancreatic edema, inflammatory cell infiltration, and acinar cell necrosis were improved. In addition, infused CM-DiI-labeled cATMSCs were detected more frequently in injured than in normal pancreas, indicating that they had migrated to the injured organ where they decreased and increased the levels of pro- and anti-inflammatory cytokines, respectively. The finding that the numbers of $CD3^+$ T cells and $FoxP3^+$ T cells were reduced and increased, respectively, in damaged pancreatic tissue after cATMSC administration suggests that the migrated cATMSCs suppressed inflammation by blocking T cell infiltration and inducing the proliferation of FoxP3+ regulatory T cells.

It is critical to determine whether infused cells reach the sites of damage in MSC-based therapies. Previous studies have shown that injected MSCs can migrate to injured lung, liver, kidney, and colon (Gonzlez MA et al., 2009; Hu J et al., 2013; Jung KH et al., 2009; Moodley Y et al., 2009; Tsuda H et al., 2014). In the present study, the use of CM-DiI facilitated the tracking of injected MSCs (Kehoe O et al., 2014; Yu X et al., 2013). A large number of systemically administered CM-DiI-labeled cATMSCs were detected in the injured pancreas. The presence of canine cells in the rat pancreas was confirmed by PCR amplification of cRPS5, the canine-specific reference gene (Brinkhof B et al., 2006). MSCs have the capacity to migrate to the site of inflammation in response to diverse cytokines (Chamberlain

G et al., 2007; Kallmeyer K et al., 2015) and chemokines; indeed, functional CC chemokine receptor type (CCR)1, CCR7, CXC chemokine receptor type (CXCR)4, CXCR5, and CXCR6 are expressed by MSCs isolated from human adipose tissue (Baek SJ et al., 2011).

Inflammatory cytokines play an important role in the pathophysiology of SAP. TNF-α, IL-1β, and IL-6 are pro-inflammatory cytokines predominantly released predominantly by innate immune cells during SAP (Bhatia M et al., 2000; Zyromski N et al., 2003) that activate and induce the differentiation of T cells (Hsieh CS et al., 1993). T helper type 1 (Th1) and Th17 cells produce the pro-inflammatory cytokines IFN-γ, IL-12, IL-17, and IL-23, and their numbers are increased during SAP (Jung KH et al., 2011). MSCs derived from human tissues have the ability to suppress pro-inflammatory cytokine expression (Hua J et al., 2014; Jung KH et al., 2011; Jung KH et al., 2015; Meng HB et al., 2013; Yang B et al., 2013). In this study, migrated cATMSCs inhibited the expression of pro-inflammatory cytokines, whereas the levels of anti-inflammatory cytokines such as IL-4 and IL-10—which are mainly released by Th2 cells and can reduce SAP (Kusske AM et al., 1996; Van Leather JL et al., 1995), were upregulated in the injured pancreas. The anti-inflammatory effect of cATMSC infusion was supported by the observed decrease and increase in serum concentrations of IFN-γ and IL-10, respectively.

My results suggest that infused cATMSCs improve SAP in rats by inhibiting pro-

inflammatory cytokine and stimulating anti-inflammatory cytokine production. Previous in vitro studies have shown that canine MSCs suppress the proliferation of T cells that release inflammatory cytokines and mediators (Carrade DD et al., 2013; Kang JW et al., 2008). Furthermore, FoxP3+ regulatory T cells induced by co-culture with MSCs are known to induce apoptosis in innate immune cells and CD4+ T cells (English K et al., 2009; Pandiyan P et al., 2007). In the present study, cATMSCs suppressed the proliferation of co-cultured cPBMCs as well as rat splenocytes treated with ConA in a ratio-dependent manner. Recent studies have also shown that MSCs release soluble factors that have anti-inflammatory effects (Pitternger M et al., 2009). The TNF-inducible protein TSG-6, which is a major soluble factor related by MSCs, not only suppressed macrophage and T cells, but also stimulated the production of FoxP3+ regulatory T cells in damaged tissue (Francois M et al., 2012; Gonzalez-Rey E et al., 2009; Kota DJ et al., 2013). Similarly, CM containing soluble factors inhibited the proliferation of both cPBMCs and rat splenocytes in the present study. In addition, cAT-MSCs blocked the infiltration of CD3+ T cells and increased the FoxP3+ regulatory T cell population in the injured pancreas of SAP rats. Although the identify of the double factors and anti-inflammatory mechanism of cAT-MSCs require more detailed study, I speculate that cAT-MSCs inhibit inflammation by regulating T cells via paracrine mechanisms as well as cell-to cell contact based on previous studies and the results of the present study.

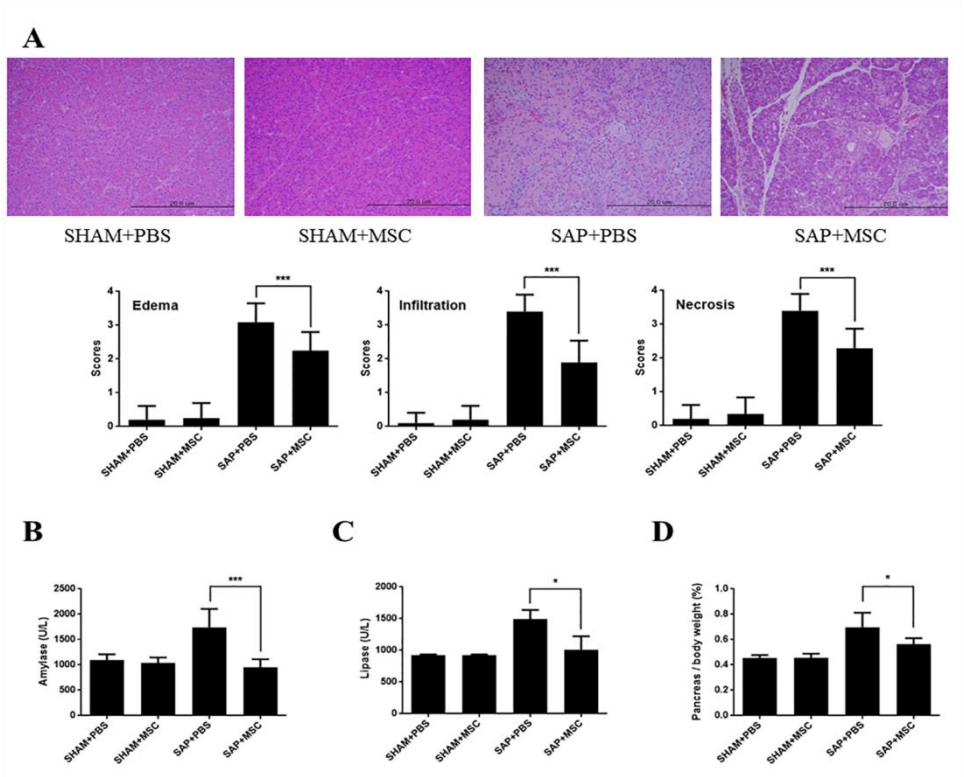

Figure 2. Therapeutic effects of cAT-MSCs in rats with severe acute pancreatitis (SAP). (A) Histopathological analysis. (B) Serum amylase activities (U/L). (C) Lipase activists (U/L). (D) Pancreas to body weight ratio. Data are shown as the means +/- standard deviation (SD). *$p < 0.05$ ***$p < 0.001$. Scale bar = 20 µm (A).

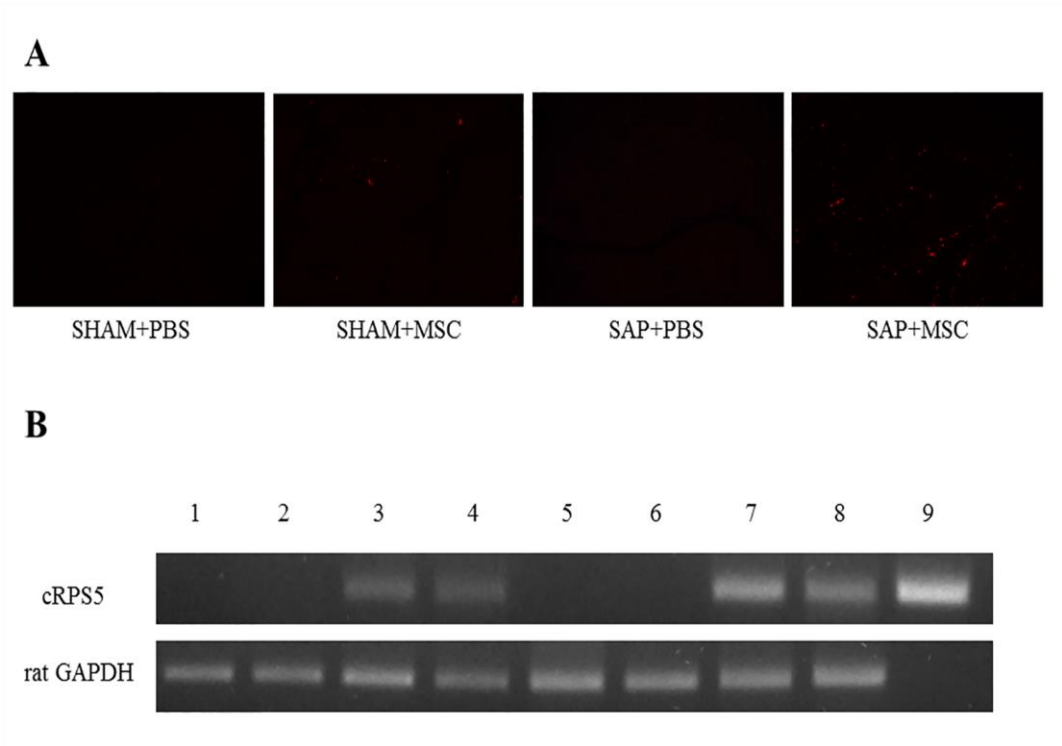

Figure 3. Tracking of infused cAT-MSCs. (A) Pancreatic tissue sections following systemic administration of CM-Dil-labeled cAT-MSCs in rats with or without SAP. (B) PCR amplification of cRPS5 in pancreatic tissue. Lane 1 and 2, SHAM + PBS; Lanes 3 and 4, SHAM + MSC; Lane 5 and 6, SAP + PBS; Lane 7 and 8; SAP + MSC; Lane, canine DNA. 200 x (A).

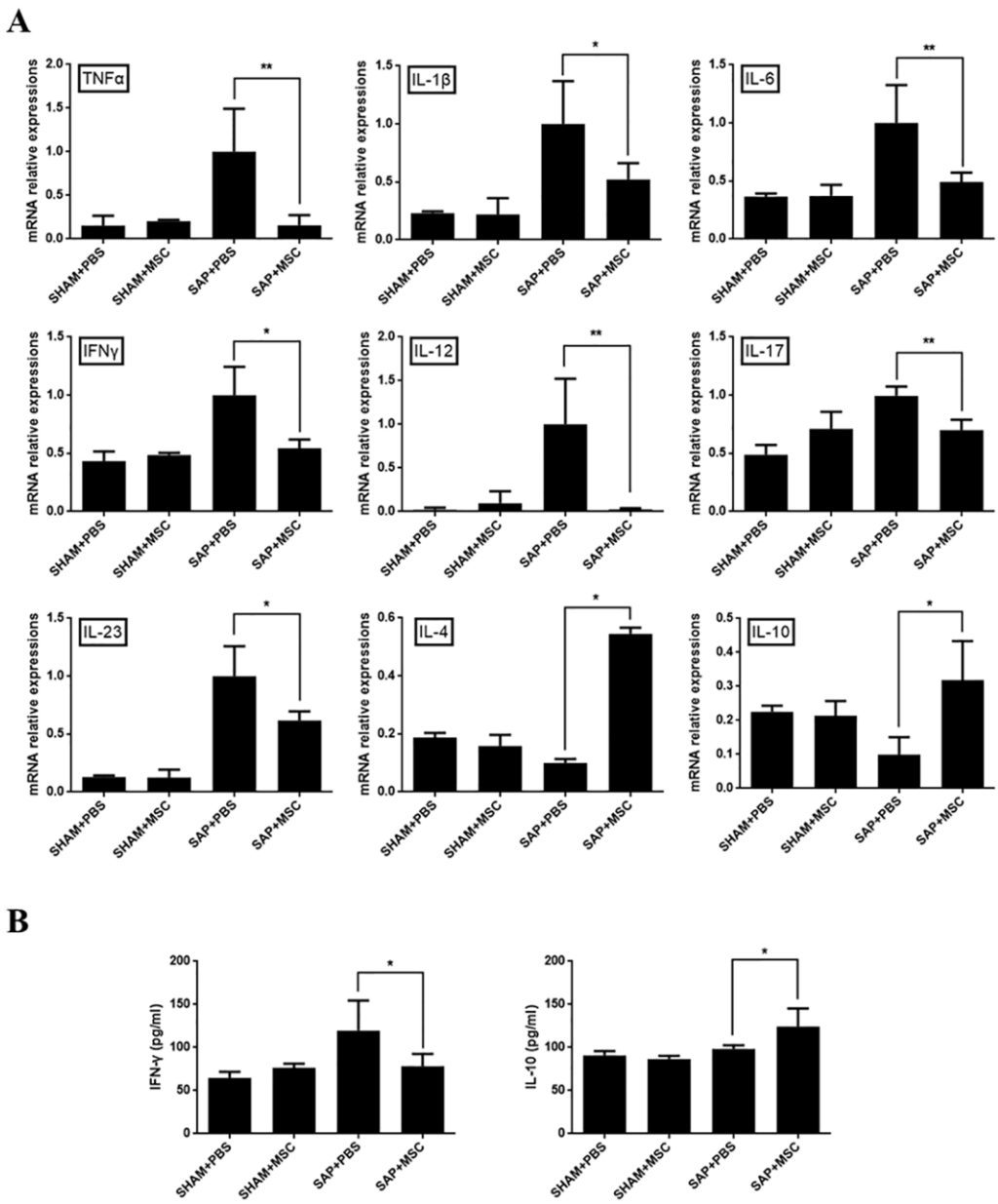

Figure 4. Effects of cAT-MSCs on inflammatory cytokines levels. (A) mRNA expression of pro- and anti-inflammatory cytokines in pancreas tissue. (B) serum level of IFN-α and IL-10. Data are shown as the means +/- SD. *$p < 005$, **$p < 0.01$.

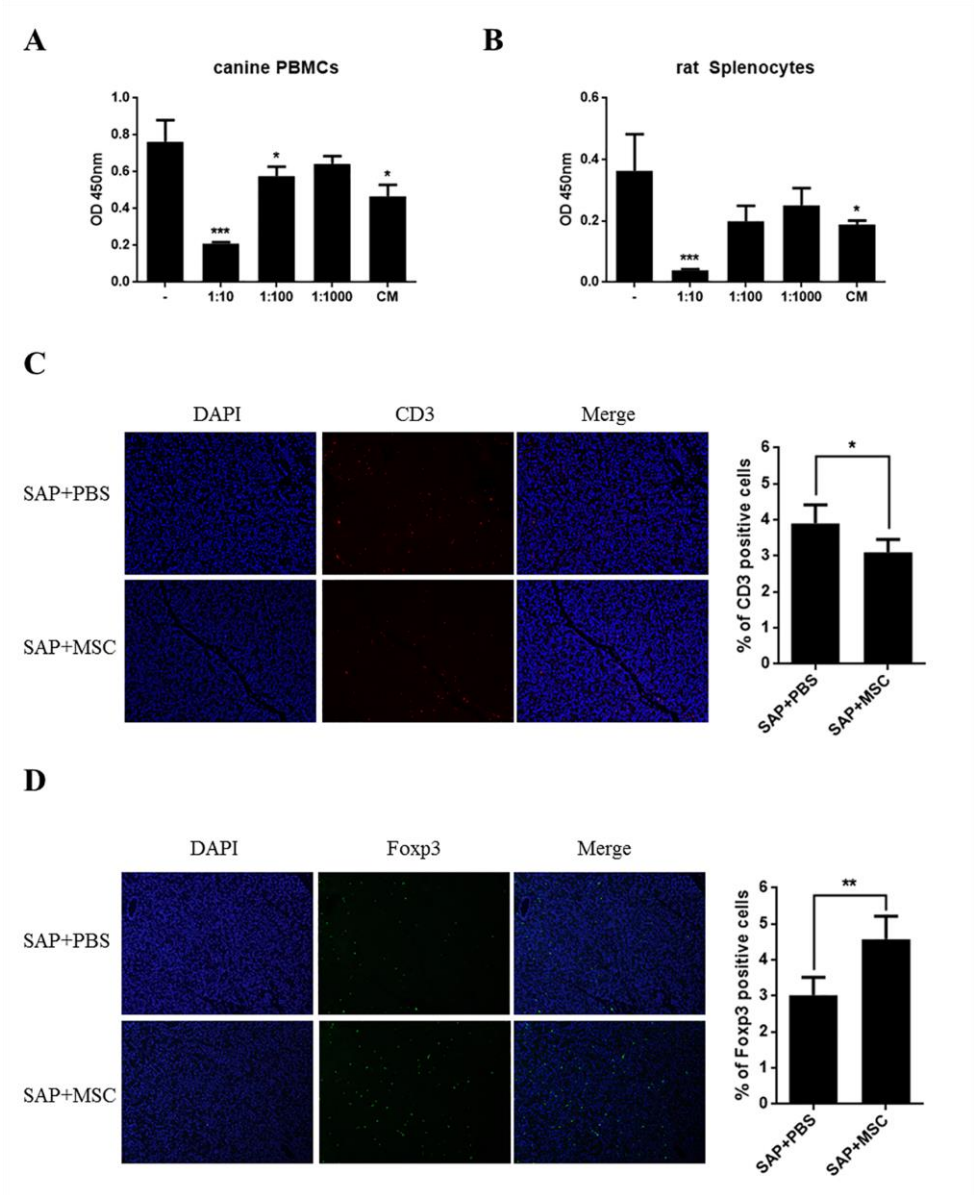

Figure 5. T cell regulation by cAT-MSCs. cAT-MSCs suppressed the proliferation of cPBMCs (A) and rat splenocytes (B) stimulated with ConA. Detection of CD3+ T cells (C) and FoxP3+ regulatory T cells (D) in pancreatic tissue. Percentages of CD3+ or FoxP3+ cells are shown as the means +/- SD *$p < 0.01$, ***$p < 0.001$. 200x (C and D).

General conclusion

The purpose of this study was to investigate the anti-inflammatory potential of canine AT-MSCs in rats with SAP. The results presented herein demonstrate that cAT-MSCs can improve pancreatic injury and regulate inflammatory cytokines by inducing FoxP3+ regulatory T cells and suppressing T cell proliferation in rats with SAP. Therefore, I suggest that cAT-MSCs are an attractive candidate for cell-based clinical therapy in SAP dogs.

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국문초록

급성췌장염랫드모델에서

개의 지방유래 중간엽 줄기세포의 항염증효과

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중증 급성 췌장염은 개들의 전신 합병증과 높은 사망률과 관련이 있다. 간엽 줄기 세포 는 몇 가지 염증 모델에서 치료 가능성에 대한 연구가 있었다. 본 연구에서는 3% sodium taurocholate solution 을 췌관으로 역행 주사하여 유도한 중증 급성 췌장염의 랫드 모델에서 개의 지방유래 중간엽 줄기세포

(cATMSC)의 효과를 조사 하였다. dioctadecyl-3,3,3-tetramethylindo-carocyanine perchlorate (1 x 107 cells / kg)로 표지 된 cATMSC 를 랫트에 전신 투여하고 조직 병리학적, 정량적 실시간 PCR 및 면역 세포 화학적 분석을 위해 3 일 후 췌장 조직을 채취 하였다. 대조군에 비해 중증 급성 췌장염을 유발한 랫트의 췌장에서 cATMSCs 가 더 많이 검출되었다. cATMSC 주입은 췌장 부종, 염증 세포 침윤 및 선세포 괴사를 감소 시켰고 전 염증성 사이토 카인 TNF-α, Interleukin(IL) -1β, -6, -12, -17 및 -23 의 췌장 발현을 감소시켰다. 또한 Interferon-γ 를 억제하는 반면, 중증 급성 췌장염 랫드에서 항 염증성 사이토 카인 IL-4 및 IL-10 의 발현을 촉진했다. 더욱이, cATMSCs 는 손상된 췌장에서 분화 3-positive T cell 의 클러스터 수를 감소 시켰고, Forkhead box P3-positve T cell 의 수를 증가시켰다. 이러한 결과는 cATMSC 가 개에서의 중증 급성 췌장염 치료를위한 세포 기반 치료 전략으로 효과적 일 수 있음을 나타낸다.

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