Master's Thesis of Science in Agriculture

The Reparative Role of Fetal Horse Bone Marrow Cell-derived Extracellular Vesicles on Injured Chondrocytes

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The Reparative Role of Fetal Horse Bone Marrow Cell-derived Extracellular Vesicles on Injured Chondrocytes

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Abstract

Mesenchymal stem cells (MSCs) reside in diverse host tissues and organs, such as circulating blood, adult and fetal bone marrow (BM), spleen, amniotic fluid, cartilage, muscle tendons, placenta, adipose tissues, periosteum, synovial fluid, dermis, and dental pulp. MSCs have anti-inflammatory, anti-oxidative, anti-apoptotic, and proliferative roles in injured organs including osteoarthritic lesions. Extracellular vesicles (EVs) are nano-sized particles secreted by almost all cell types, and they mediate various biological processes via cell-to-cell communication. Recent studies consistently showed that the therapeutic function of MSCs could be mediated by EVs. Practically, MSC-derived EV provides several advantages for cell therapy, such as reduced risk of rejection, less oncogenic potential, ease of long-term storage, lower chance of thromboembolism, and readiness for immediate use.

In this study, I examined whether EVs produced from fetal bone marrow cells (BMCs) collected from Jeju horse promote the proliferation of equine chondrocytes. First, BMCs were isolated from a 170-day fetus and subsequently cultured, and then they were characterized in various methods including flow cytometry, differentiation analysis, and gene expression of immunomodulatory genes. I examined whether BMC-derived EVs have essential characteristics as EVs by various experiments, including nanoparticle tracking analyzer (NTA), dynamic light scattering (DLS), and immunoblotting. Functionally, BMC-derived EVs stimulated the proliferation of equine chondrocytes even without the supplementation of fetal bovine serum in the culture medium. I further showed that EVs could alleviate the apoptotic death

of chondrocytes that were injured by inflammatory cytokines. To Further, I observed that BMC-EVs upregulated the phosphorylation of Akt1, while reduced the phosphorylation of extracellular signal-regulated kinase (ERK)1/2 and p65 subunit of NFκB in inflammatory environment. Together, the present study showed that equine fetal BMC-EVs have potential to become an alternative, cell-free therapeutic for joint diseases in the horse.

Keywords: Apoptosis, BMC, Cell signaling, Extracellular vesicles, Osteoarthritis

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Contents

List of Tables

List of Figures

List of Abbreviations

ADAMTS – a disintegrin-like and metalloproteinase with thrombospondin motifs

Alp - Alkaline phosphatase

BMCs – Bone marrow cells

BSA – Bovine serum albumin

CCL5 – CC-chemokine ligand 5

DLS – Dynamic light scattering

DMEM - Dulbeccos's modified eagle medium

ERK1/2 - Extracellular signal-regulated kinase1/2

EVs – Extracellular vesicles

FBS - Fetal bovine serum

HSP70 – Heat shock protein70

GN - Gene name

IDO - Indoleamine 2, 3-dioxygenase

IL-10 – Interleukin-10

IL-13 – Intereukin-13

IL-17 – Interleukin-17

IL-1β - Interleukin-1β

IL-1R – Interleukin-1 receptor

IL-4 – Interleukin-4

IL-6 – Interleukin-6

iPSC – induced pluripotent stem cell

ISCT - International society for cellular therapy

LC mass spectrometry – Liquid chromatography-mass spectrometry

MCP1 – Monocyte chemoattractant protein 1

MIA - Monoiodoacetate

MMP – Matrix metalloproteinases

MSCs – Mesenchymal stem cells

MSK1 – Mitogen-and stress-activated kinase 1

NC – Negative control

NSAIDs – Non-steroidal anti-inflammatory drugs

NTA – Nanoparticle tracking analyzer

OA – Osteoarthritis

OS - Organism name

OX - Organism taxonomy

PC – Positive control

PE - Protein existence

SD – Standard deviation

SEM – Scanning electron microscopy

SV - Sequence version

TEM – Transmission electron microscopy

TGF-β1 - Transforming growth factor-β1

TNF-α - Tumor necrosis factor-α

TNFR – Tumor necrosis factor-α receptor

Ub - Ubiquiti

1.Introduction

According to the criteria of the International Society for Cellular Therapy (ISCT), human mesenchymal stem cells (MSCs) should exhibit plastic adherence. Second, they possess a specific set of cell surface markers, i.e., CD73, CD90, and CD105. Third, MSCs maintain the ability to differentiate in vitro into adipocytes, chondrocytes, and osteoblasts [1].

It was reported that osteoarthritis (OA) accounts for 60 percent of the total in the United States [2, 3]. In the horse industry, joint injury and disease is the most common cause of diminished exercise capability and disposal racing horse [4]. In order to solve these cases, it is necessary to extend their athletic life span through proper treatment.

1.1 Current method of treating osteoarthritis

Depending on the disease status, clinical protocols can be classified into a surgical method, using NSAIDs (Non-steroidal anti-inflammatory drugs), via physical therapy, opioids, or intra-articular injection of hyaluronic acid. Although NSAIDs have been commonly used for relieving inflammation due to their analgesic and anti-inflammatory effect, side effects such as the organ toxicity (e.g., liver and kidney) have also been critical. In particular, using NSAIDs for a long-term or repeated period of time cause gastrointestinal hemorrhage [5-7]. Thus, other alternatives, i.e., cellular therapies using autologous or allogenic origins, are now becoming recognized as a safe and

effective options. Also, the application of induced pluripotent stem cell (iPSC)-derived chondrocytes may be another choice depending on the regulation and safety guidelines [8]. Although several protocols are currently available for clinical purposes [9], cell-based therapy inherently possesses the risk of immune rejection and tumor formation in vivo [10, 11]. Accordingly, the application of extracellular vesicles, which can be obtained from desired cell types during culture, would be an ideal cell-free strategy that can solve the problems that can be raised upon implementing cell therapy [8, 12, 13].

1.2 Characteristics of EVs

EVs are a collective term for heterogeneous nano-sized lipid-bilayer membrane vesicles having a 30-2000nm diameter. Importantly, EVs play an essential role in intercellular communications due to a large variety of biologically active signaling molecules within EVs, including RNA species (messenger RNA and small RNA), proteins, enzymes, lipids, and DNA fragments [14]. So far, various characterization methods are available. Transmission electron microscopy (TEM) and Scanning electron microscopy (SEM) are usually used for verifying their cup- or round- shape [15-18]. TEM is more commonly used than SEM [19, 20]. The diameter size as well as their size distribution, can be measured by NTA [18, 21]. Finally, the presence of EV-specific markers (CD9. CD63, CD81, TSG101, and Alix) [17, 18] can be examined by immunoblotting or flow cytometry.

1.3 Preclinical studies of cell-derived EVs on osteoarthritis models

EVs contain a broad spectrum of biomolecules, including proteins, lipids, nucleic acids (DNAs, RNAs, small RNAs). Together with the notion that EVs are a natural player of cell-cell interaction in multicellular organisms, studies have focused on strengthening their specific function [22]. For example, miR-140-5p-overexpressing synovial MSC-derived exosomes led to an enhancement of chondrocyte proliferation and migration and prevented OA in a medial meniscus OA rat model [23]. Another study showed that human embryonic MSC-derived exosomes injected in the medial meniscus OA mouse model improved the synthesis of cartilage regeneration [24]. Another study compared the therapeutic efficacy between EVs from iPSC derived - mesenchymal stem-like cells and synovial membrane-derived MSC in Collagenase-induced mouse OA model, and showed that EVs from iPSC derived MSC was better in reducing OA progression [25].

Similarly, in a rat model of an osteochondral defect, EVs derived from human embryonic mesenchymal stems were able to reduce the disease progression [26]. Several mechanistic studies also showed that EVs from MSCs mediate cartilage repair by enhancing proliferation, attenuating apoptosis, modulating immune reactivity. For example, the treatment of MSC-derived exosomes led to an enhanced activity of Akt and ERK signaling in cultured chondrocytes in vitro, and increased infiltration of $CD163^+$ regenerative M2 macrophages over $CD86^+$ M1 macrophages was found in the osteochondral tissue in Surgical defect created on the model [13]. Also, EVs

from mouse BM-MSCs showed a therapeutic effect in collagenase induced arthritis model, as shown by Protection from osteoarthritis damage and a reduction of apoptotic cells injected in mouse chondrocyte, with a significant improvement cartilage generation. Finally, EV treatment was able to reduce osteophyte formation in a mice model of OA [27]. In an OA model created by making a rounded trephine groove osteochondral model in dogs (3mm diameter, 1mm depth), administration of mouse bone marrow MSC-derived EV led to a marked regeneration of cartilage and restoration of chondral tissue [28]. Also, it was shown that WNT5A expression was inhibited by miR-92a-3p delivery by exosomes, which led to an inhibition of cartilage degradation [29]. In a collagenase induced arthritis model in mice, EVs from mouse BM-MSCs inhibited T lymphocyte proliferation in a dose-dependent manner, and also decreased the percentages of CD4 and CD8 subsets. Also, fewer plasmablasts and more Breg-like cells in lymph nodes were found [30].

miRNAs are one of the major biological cargoes in EVs from parental cells, and it was shown that miR-100-5p was enriched in the exosomes derived from human Infrapatellar fat mesenchymal stem cells. Upon being injected intra-articular into OA mice induced by destabilization of the medial meniscus, the OA progression was dramatically attenuated, as shown by the reduction of articular damage and amelioration of gait abnormality. The molecular study also demonstrated that miR-100-5p inhibited the mTOR/ autophagy pathway [31]. Another study demonstrated that exosomes from miR-92a-3p-overexpressing BM-MSCs was able to promote the chondrocyte proliferation, and upregulated several matrix genes (Aggrecan, Col2A1, Sox9) and

decreased a subset of other matrix genes (Col2A10, Runx2, MMP13, Wnt5A).

Another study showed that EVs from human amniotic fluid stem cells has a therapeutic effect in MIA(Monoiodoacetate)-induced OA model in rats, as demonstrated by an enhanced pain tolerance and improved histological score. After three weeks of EV treatment, rat cartilage restoration with good surface regularity and with the characteristic of hyaline cartilage was shown. Moreover, markers of resolving macrophages (CD163, arginase 1, and TGF-β) were significantly increased after EV treatment [32].

Collectively, EVs from various stem cells alleviated the disease progression, as supported by results of tissue histology as well as inflammatory cytokine profiles in various preclinical OA models. We have provided a detailed list of studies that have attempted to use EVs from various parental cell types in OA animal models.

1.4 Role of the cytokines in osteoarthritis

So far, various soluble mediators have been reported to be involved in the progression of OA. Various pro-inflammatory (IL-1β, TNF-α, IL-6, IL-17) and anti-inflammatory cytokines are involved in OA pathogenesis (IL-4, IL-10, IL-13) [33, 34]. For example, an elevated level of IL-1β and TNF-α was found in OA synovial fluid, synovial membrane, and subchondral bone cartilage [35]. Mechanistically, these cytokines down-regulated the extracellular synthesis matrix (ECM) component by inhibiting anabolic activities of chondrocytes.

Another study showed that IL-1β reduces the expression of type II collagen, which is a major ECM component constituting the cartilaginous tissues in several animal species [36, 37]. Also, the expression of Aggrecan, which is one of the major components of the cartilage, was found to be decreased by IL-1β treatment in chondrocytes and cartilage [38]. Indirectly, IL-1β and TNF-α stimulate chondrocyte to produce a proteolytic enzyme such as matrix metalloproteinases (MMPs), including MMP-1 (interstitial collagenase), MMP-3 (stromelysin 1), MMP-13 (collagenase 3) [39-41]. In addition, a disintegrin-like and metalloproteinase with thrombospondin motifs (ADAMTS) is also one of the major players in cartilage degradation in OA. The expression of ADAMTS-4 is induced by IL-1β and TNF-α, while the expression of ADAMTS-5 was not affected by IL-1β and TNF-α [42]. However, subsequent studies have shown that IL-1β is relevant to ADAMTS-5. IL-1β treatment rabbit nucleus pulposus in the ADAMTS-5 mRNA level increase [43]. Additionally, miR30a has an important role in controlling ADAMTS-5 caused by IL-1β [44].

Fetal MSCs have more advantages than adult MSCs [45-49], and few studies have been conducted on attempting fetal MSC-derived EVs in osteoarthritis. Therefore, in this study, I investigated whether equine fetal MSC derived EVs have therapeutic effects on the equine chondrocytes injured by being treated with inflammatory cytokines.

2. Materials and Method

2.1 Isolation and culture of bone marrow cells (BMCs)

Bone marrow cells were isolated from the humerus and femur of the fetal horse (140 days). This study was approved by Institutional Animal Care and Use Committees of Seoul National University (SNU-171103-2) was conducted in accordance with approved guidelines. After both ends of the bones were cut open, the BMCs were harvested through flushing with a 50 mL DMEM (Dulbecco's modified eagle medium, Thermo Fisher Scientific, Waltham, MA, USA) with 10% FBS (Fetal bovine serum, Altas Biologicals, Fort Collins, CO, USA) with 1% Antibiotics-Antimycotics (Genedirex, Taoyuan, Taiwan) in a 16-gauge syringe. Harvested BMCs were cultured in MesenPRO^{TM} RS (Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 0.1% Mycozap (Lonza, Basel, Switzerland). Cells were replenished with fresh culture medium every four days. When the cell growth reached 90% confluence, BMCs were split into 1:4 by being treated with 0.05% Trypsin-EDTA (Genedirex, Taoyuan, Taiwan).

2.2 Culture of equine chondrocytes

Equine chondrocyte primary cells (Cellider biotech, Zaragoza, Spain) were cultured in DMEM (Thermo Fisher Scientific, Waltham, MA, USA) with 10% FBS with 1% Antibiotics-Antimycotics (Genedirex, Taoyuan, Taiwan) under 5% CO2 condition at 37℃. When the cell growth reached 90% confluence,

equine chondrocytes were split into 1:3 by being treated with 0.05% Trypsin-EDTA (Genedirex, Taoyuan, Taiwan).

2.3 Flow cytometry

BMCs were trypsinized and washed twice before resuspension in PBS containing 2% FBS. Cells were adjusted to 1×10^6 in 100 μL of cell suspension. For cell surface labeling, cell suspensions were incubated at 4℃ for 45min or 1h with 1 μL of antibodies (1:100) against MSC surface markers such as mouse anti-human CD29 (clone TS2/16, BioLegend, San Diego, CA, USA), mouse anti-CD34 (clone 4H11, Invitrogen, Carlsbad, CA, USA), mouse anti-CD90 (clone MRC OX-7, Abcam, Cambrige, UK), mouse anti-human CD105 (clone SN6, Bio-rad, Hercules, CA, USA), rat anti-CD44 (clone IM7, Carlsbad, CA, Invitrogen). For the unconjugated primary markers, the secondary antibody goat anti-mouse IgG H&L Dylight 488 (Abcam, Cambrige, UK) and goat anti-rat IgG Alexa Fluor 488 (BioLegend, San Diego, CA, USA) were used. Cell surface marker analysis was performed using a BD FACS CantoTM II Cytometer and FACS DIVA software (Ver 6.1.3, BD Bioscience, Franklin Lakes, NJ, USA).

2.4 In vitro differentiation of BMCs

BMCs (passages between 4-5) were plated in triplicate in 4-well plate (SPL, KOR) for chondrogenic a pellet cultured incubated at 37℃ and 5% CO_2. After one day, the culture medium was removed, and the differentiation medium StemPro chondrogenesis (Thermo Fisher Scientific, Waltham, MA, USA) was add changed every three days. Osteogenic and Adipogenic differentiation were incubated at 37℃ and 5% CO_2. After reaching 80% confluence, the culture medium was removed, and the differentiation medium StemPro osteogenesis and StemPro adipogenesis (Thermo Fisher Scientific, Waltham, MA, USA) were added to the cultures. The medium was changed every three days, and their differentiation potential was examined after 2 weeks of differentiation. Chondrogenic differentiation was examined by staining with Alcian Blue staining kit (Lifeline Cell Technology, Frederick, MD, USA) to identify sulfated proteoglycans deposits. Osteogenic differentiation was examined by staining with 2% Alizarin Red staining kit (Lifeline Cell Technology, Frederick, MD, USA) to identify calcium deposits. Adipogenic differentiation was examined by staining with Oil Red O staining (Sigma-Aldrich, St. Louis, MO, USA) to identify lipid droplets.

2.5 qRT-PCR

BMCs were harvested in Trizol (Invitrogen, Carlsbad, CA, USA), and the concentration of total RNA was measured using DeNovix DS-11 (DeNovix, Wilmington, DE, USA). RNA was reverse transcribed with cDNA Synthesis Kit (Philekorea, Daejeon-si, Korea), and qPCR was performed using the AccuPower 2X GreenStar qPCR Master Mix (Bioneer, Daejeon-si, Korea) in StepOne^{TM} Real-Time PCR System (Applied Biosystems, Waltham, MA, USA). Genes for trilineage differentiation were *Acan, Collagen type II, Sox9, Alp, Runx2, Osteonectin*. Genes for the immunomodulatory study were *TGF-β1, IDO, IL-6, IL-10*. After the expression of each gene was normalized against *Gapdh*, the relative expression among each treatment group were compared by the $2^{-\Delta\Delta Ct}$ method [50].

Table 1. The sequences of primers used for confirming differentiation potential of BMCs

Target genes	Sequences	Product size (bp)	Annealing Tm. (℃)
Gapdh	F: 5'-TCCCTGCTTCTACTGGTGCT-3' R: 5'-CGTATTTGGCAGCTTTCTCC-3'	147	60
Acan	F: 5'-CAGTCACACCTGAGCAGCAT-3' R: 5'-TTCGATGGTCCTGTCATTCA-3'	102	59
Collagen type II	F: 5'-CAACAACCAGATCGAGAGCA-3' R: 5'-GTCTCCGCTCTTCCATTCAG-3'	106	57
Sox9	F: 5'-AGAAGGAGAGCGAGGAGGAC-3' R: 5'-ACGTGAGGCTTGTTCTTGCT-3'	133	59
Alp	F: 5'-GATGGCCTGAACCTCATCGA-3' R: 5'-AGTTCGGTCCGGTTCCAGAT-3'	83	60
Runx2	F: 5'-CTCCAACCCACGAATGCACTA-3' R: 5'-CGGACATACCGAGGGACATG-3'	80	59
Osteonectin	F: 5'-GGTGTGTGAGCTGGATGAGA-3' R: 5'-TGCACACCTTCTCAAACTCG-3'	95	58

Table 2. The sequences of primers used for investigating immune modulatory function of BMCs

Target genes	Sequences	Product size (bp)	Annealing Tm. (°C)
Gapdh	F: 5'-TCCCTGCTTCTACTGGTGCT-3' R: 5'-CGTATTTGGCAGCTTTCTCC-3'	147	60
TGF-β1	F: 5'-AGGCTCAAGTTAAGCGTGGA-3' R: 5'-CAGCCGGTTACTGAGGTAGC-3'	84	59
IDO	F: 5'-CATTGTGATTCCTGCACACC-3' R: 5'-ACATCAGTGCCTCCAGTTCC-3'	93	59
IL-6	F: 5'-CACCACTGGTCTTTCGGAGT-3' R: 5'-TCAGGGGTGGTTACTTCTGG-3'	156	59
IL-10	F: 5'-CAAGCCTTGTCGGAGATGAT-3' R: 5'-CTCACTCGGAGGGTCTTCAG-3'	125	58

2.6 Measuring the viability of BMCs

Analysis of cell viability was measured by using the CellTiter-Glo 3D cell viability assay kit (Promega, Madison, WI, USA). The CellTiter-Glo 3D was used due to its high consistency based on the amount the cellular ATP, which represents the metabolically active cells. CellTiter-Glo 3D cell viability assay reagent directly to cells cultured in serum-supplemented medium. The amount of luminescence by cellular ATP was measured by Cytation (BioTek, Winooski, VT, USA).

2.7 Collection of EVs

EV-depleted FBS was prepared by ultracentrifuge at 40,000 × g for 8h at 4℃. Upon reaching 80% confluency, the culture media from BMCs were replaced with fresh medium supplemented with 2% EV-depleted FBS and the cells were subsequently cultured for an additional 48h. After incubation, the BMC culture medium was harvested, centrifuged 2,000 × g for 20min at 4℃, and the supernatants were filtered through 0.2 μm pore filters to remove the particle larger than 200 nm. And then, the supernatants were concentrated using Vivaspin 20 (100,000 MWCO) (Sartorius, Gottingen, Germany). Next, EVs were isolated by using Exo2D™ Kit (Exosome plus, Suwon-si, Korea). The mixture was mixed by rocking for 30min at 4℃, followed by centrifugation at 3,000 × g for 30min at 4℃. EV pellet was then resuspended in EV-free PBS that had been filtered through a 20 micrometer-pose sized syringe filter. The protein concentration was measured by the Pierce™ BCA Protein Assay kit

(Thermo Fisher, Waltham, MA, USA). The aliquots stored at –80℃.

2.8 Coomassie staining

EV lysate and purified BSA were boiled at 95-100℃ for 5 minutes. Denatured lysates were then loaded and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Gels were washed twice in dH_2O and then stained in Coomassie brilliant blue (Biosesang, Seongnam-si, Korea) for 4h followed by being washed in a destaining solution (8.1: 1.2: 0.7 = dH_2O: methanol: glacial acetic acid).

2.9 DLS assessment

To determine the size and zeta potential of EVs, EVs were diluted in PBS and dispensed into a cuvette. The size of EVs was measured and analyzed using Zetasizer Nano ZS (Malvern Panalytical, Malvern, UK) and Zetasizer software (Ver 7.13, Malvern Panalytical, Malvern, UK), respectively.

2.10 NTA assessment

Nanoparticle analysis was conducted to determine the particle size and concentration of EVs using NanoSight 300 (Malvern Panalytical, Malvern, UK). EVs were suspended EV-free D.W (Distilled Water).

2.11 LC-MS/MS for peptides analysis and identification

I was able to obtain the results by requesting MS/MS analysis to Yonsei Proteome Research Center in Seoul, and when sending the sample, EVs were lysate using RIPA buffer to extract EV protein. Nano LC-MS/MS analysis was performed with an Easy n-LC (Thermo Fisher Scientific, Waltham, MA, USA) and an LTQ Orbitrap XL mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA) equipped with a nano-electrospray source. Samples were separated on a C18 nanopore column (150 mm × 0.1 mm, 3 μm pore size; Agilent, Santa Clara, CA, USA). The mobile phase A for LC separation was 0.1% formic acid, 3% acetonitrile in deionized water, and the mobile phase B was 0.1% formic acid in acetonitrile. The chromatography gradient was designed for a linear increase from 0% B to 32% B in 23 min, 32% B to 60% B in 3 min, 95% B in 3 min, and 0% B in 6 min. The flow rate was maintained at 1500 mL/min.

Mass spectra were acquired using data-dependent acquisition with a full mass scan (350–1800 m/z) followed by 10 MS/MS scans. For MS1 full scans, the orbitrap resolution was 15,000, and the AGC was 2×10^5. For MS/MS in the LTQ, the AGC was 1×10^4.

2.12 Database searching

The PEAKS Studio 10.0 (Bioinformatics Inc, Arlington, VA, USA) was used to identify peptide sequences present in a protein sequence database. Database search criteria were OS, Organism Name; OX, Organism Taxonomy; GN, Gene Name; PE, Protein Existence; SV, Sequence Version. Only peptides resulting from trypsin digests were considered.

2.13 CCK-8 assay

Analysis of cell viability/proliferation, cell viability/proliferation was measured by Cell Counting Kit-8 (Dojindo Laboratories, Kumamoto, Japan). Cells were seeded into 96-well plate (SPL Life Sciences, Pocheon-si, Korea) at a density of 5.0×10^3 cells per well with 100 μL medium and incubated at 37℃. At the designated time point, 10 μL CCK-8 solutions were added. After further incubation for 3 hours at 37℃, the amount of formazan generated by cellular dehydrogenase activity was measured (450 nm) by a microplate reader (TECAN, Mannedorf, Switzerland).

2.14 Apoptosis assay

Cytokine-treated equine chondrocyte apoptosis was measured with the Annexin V-FITC Apoptosis Detection Kit I (BD Bioscience, Franklin, NJ, USA). Cells were trypsinized and washed twice with cold PBS and then resuspended 1×10^6 cells in 500 μL of binding buffer. 5 μL of Annexin V-

FITC and 5 μL of PI (Propidium Iodide, I mg/mL) were added into the cell resuspended for 30min at room temperature in the dark. The cells were analyzed BD FACS Canto™ II Cytometer and FACS DIVA software (Ver 6.1.3, BD Bioscience, Franklin, NJ, USA).

2.15 Immunoblotting

EV and cellular protein total protein concentration evaluated by Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA). EV and cell lysates were loaded into each well and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The protein bands were transferred to nitrocellulose membranes after which were blocked in 5% skim milk or 5% BSA. Primary antibodies (dilution 1:1000) against CD 63 (Abcam, Cambrige, UK), HSP 70, phospho-Akt, Akt, phospho-Erk, Erk (Santa Cruz, Dallas, TX, USA), phospho-NFκB (Cell signaling, Danvers, MA, USA), NFκB, or β-Actin (Abcam, Cambrige, UK) were incubated with the membrane at 4°C overnight. After the membrane was washed three times with TBST (9: 1: 0.01 = dH_2O: 10X TBS: 10% Tween20), membranes were incubated with horseradish peroxidase-linked anti-mouse or anti-rabbit secondary antibody for 1h at room temperature. After being washed three times with TBST for 10min, the reactivity was examined by an enhanced chemiluminescence kit (Thermo Fisher Scientific, Waltham, MA, USA). The image of the membrane was taken using UV or white light on a Davinci-K Gel Imaging System (Davinch-K, Seoul-si, Korea). Bands were quantified by Image J (Version 1.50, National Institutes of Health, Bethesda, MD, USA).

2.16 Immunocytochemistry

To identify whether the EVs can incorporate into equine chondrocytes, cells were cultured for 2 days at a density of 1.0×10^4 cells per well in 8-well coated cover slides. EVs from JJ BMCs were labeled with PKH26® red fluorescent cell linker kit (Sigma-Aldrich, St. Louis, MO, USA). Labeled EVs (10, 50, 100 μg/mL) were co-cultured with equine chondrocytes for 24 hours. The nuclei were stained with 4',6-diandino-2-phenylindole (DAPI) (Sigma-Aldrich, St. Louis, MO, USA), and filamentous-actin were stained with phalloidin (Abcam, Cambrige, UK). Images were obtained using the Cytation 5 (Biotek, Winooski, VT, USA).

2.17 Statistical analysis

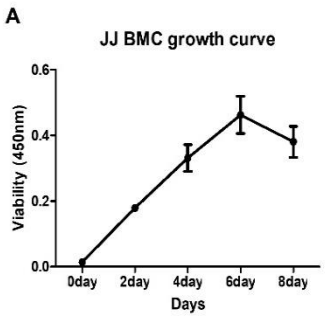
Differences between two groups were analyzed by Student's *t*-test using GraphPad Prism software (Ver. 5.0 GraphPad Software, San Diego, CA, USA). P values less than 0.05 were considered as significantly different.

3. Results

3.1 Characterization of BMCs

First, to see if the BMCs are growing well, the growth curve was measured. 3.0×10^3 cells were seeded in 96-well plates, and the culture medium was renewed every four days. The surface protein of BMCS for flow cytometry was selected based on the ISCT guidelines in human MSCs [51]. Flow cytometric analysis of BMCs at passage 8-10 showed that they were positive for several conventional MSCs surface markers such as CD44, CD90, and CD105, although the reactivity was not exclusive positive (84. 7% for CD29, 22.5% for CD90, and 64.5% for CD105), except CD44 (94.3%). A hematopoietic stem cell marker CD34 was negative. Then, BMCs were subjected to differentiation to chondrogenic, osteogenic, and adipogenic lineages for 15 days, and then stained for analyzing of the deposition of proteoglycan (Alcian blue), calcium (Alizarin Red), and neutral lipid (Oil Red O). As result, BMCs were readily differentiated to chondrogenic and osteogenic cells, however, their potential to differentiate into adipocytes was reduced (Figure 1B). Next, the expression of critical mRNAs specific for chondrogenic or osteogenic lineages was analyzed. As shown in Figure 1C and 1D, quantitative real-time PCR analysis revealed that the expression of chondrogenic (Acan, Collagen type II and Sox9) and osteogenic (Alkaline phosphatase, Runx2, and osteonectin) genes were significantly increased at day 15 and 21, compared with non-differentiated BMCs (Figure 1C, 1D). These data are in line with previous studies that demonstrated the expression

of these genes during the differentiation of human-induced pluripotent stem cells [52, 53]. Subsequently, their immunoregulatory role under inflammatory stimuli was investigated. As shown in Figure 1E and 1F, BMCs were stimulated for 24 or 48 h with TNF-α or IL-1β. After treatment with TNF-α and IL-1β for 24 h, the expression of TGF-β1, IDO, and IL-6 was significantly upregulated. The expression of IL-10 was reduced by TNF-α at 24 h, although IL-1β treatment induced a substantial increase (Figure 1E). When BMCs were treated with these cytokines for 48 h, the expression of TGF-β1, IDO, IL-6, and IL-10 was up-regulated by being treated with both TNF-α or IL-1β (Figure 1F). These results can identify that the BMC has the characterization of the MSC.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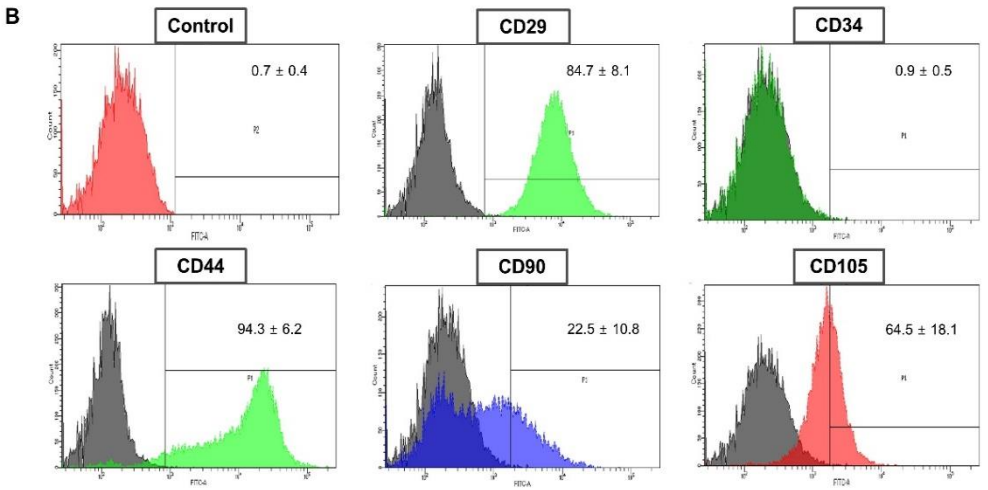
0.4
0.2
0.0
0day
2day
4day
6day
8day
Days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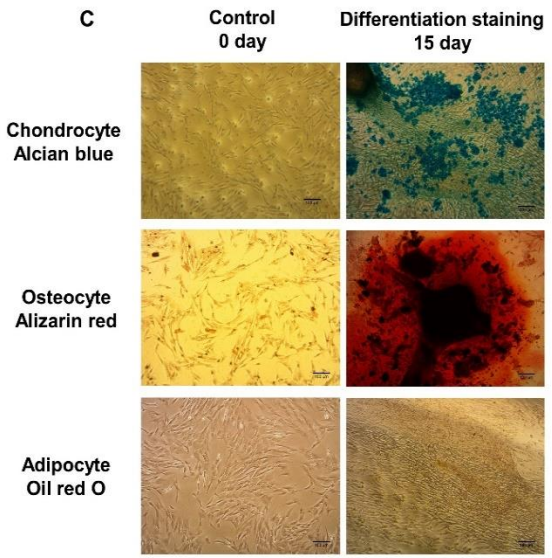
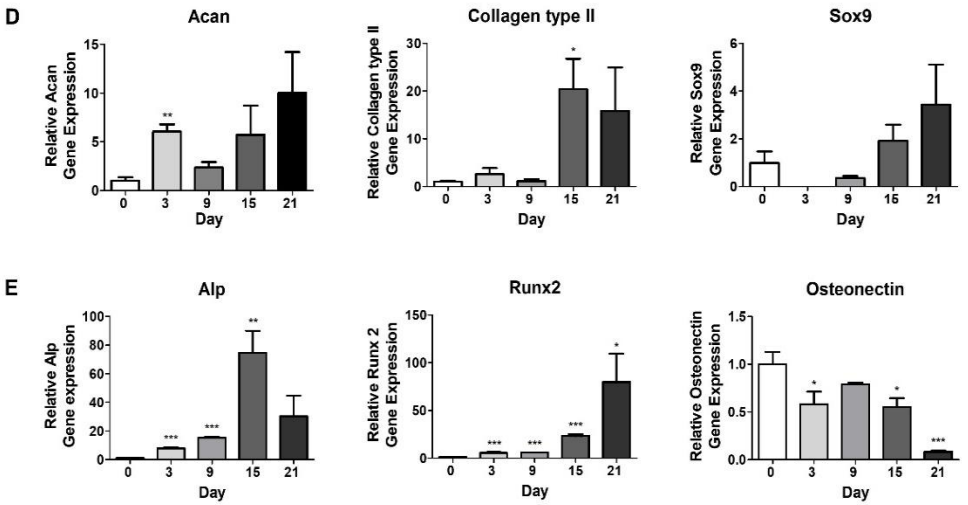
Control
0.7 ± 0.4
CD29
84.7 ± 8.1
CD34
0.9 ± 0.5
CD44
94.3 ± 6.2
CD90
22.5 ± 10.8
CD105
64.5 ± 18.1


C
Control
0 day
Differentiation staining
15 day
Chondrocyte
Alcian blue
Osteocyte
Alizarin red
Adipocyte
Oil red O


D
Acan
Relative Acan
Gene Expression
Collagen type II
Relative Collagen type II
Gene Expression
Sox9
Relative Sox9
Gene Expression
Day
E
Alp
Relative Alp
Gene expression
Runx2
Relative Runx 2
Gene Expression
Osteonectin
Relative Osteonectin
Gene Expression

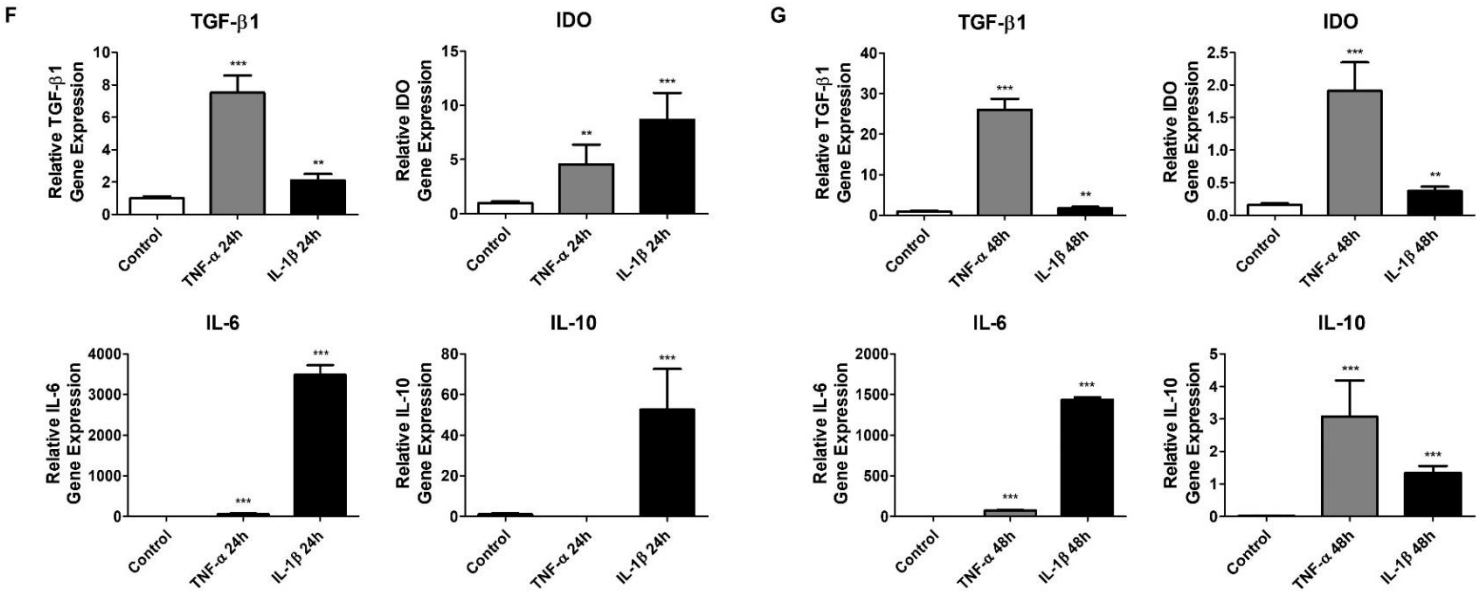

F
TGF-β1
Relative TGF-β1 Gene Expression
Control
TNF-α 24h
IL-1β 24h
IDO
Relative IDO Gene Expression
IL-6
Relative IL-6 Gene Expression
IL-10
Relative IL-10 Gene Expression
G
TNF-α 48h
IL-1β 48h

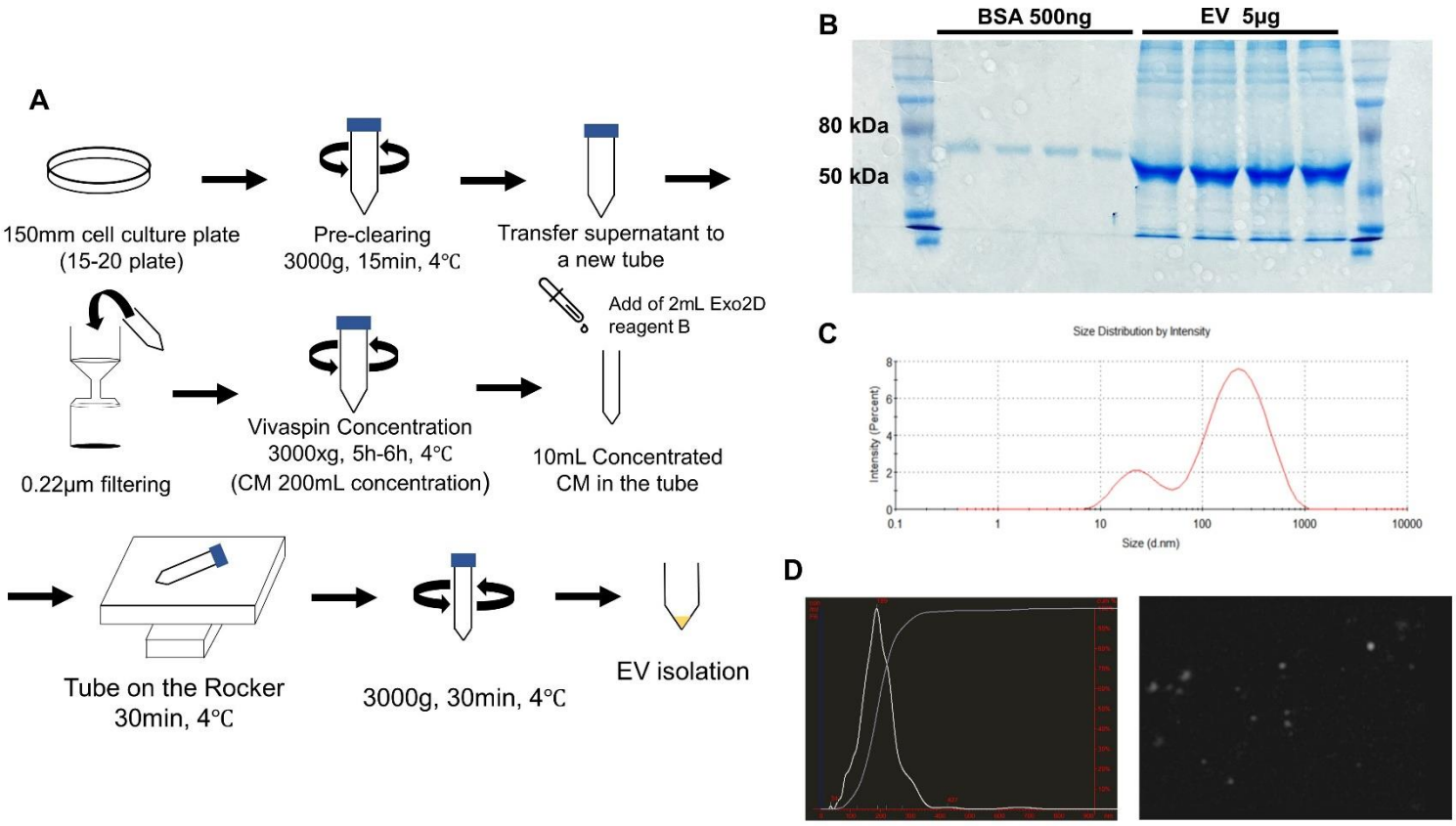
Figure 1. Characterization of BMCs. (A) Growth curve of BMC (passages between 8-10) in Mesenpro^{TM} RS. (B) Flow cytometry analysis of BMCs. Panels were selected based on previous literature describing several positive and negative markers for MSCs in other mammals [1]. (C) Microscopic images of differentiated cells from BMCs. Cells that were grown and passaged 3 times were subjected to differentiation into cells, including chondrocytes, osteocytes, and adipocytes for fifteen days. Alcian blue, Alizarin red, and Oil red O staining was used. Scale bars are 100μm. (D, E) The expression of chondrocyte-specific genes (D) or osteogenic genes (E) were analyzed on days 0, 3, 9, 15, and 21 of differentiation. (F, G) Expression analyses of immunomodulatory genes after being stimulated by inflammatory cytokines. Alp, Alkaline phosphatase; Transforming growth factor β1, TGF-β1, Transforming growth factor β1; IDO, Indoleamine 2, 3-dioxygenase; IL-6, Interleukin 6; IL-10, Interleukin 10. All data are expressed as mean ± standard deviation (SD) from three replications. *$p < 0.05$, **$p < 0.01$, ***$p < 0.005$. Scale bars are 100 μm.

3.2 Characterization of BMC-derived EVs

EVs were isolated from the supernatant collected for 48 hours during the culture of BMCs. A brief process for isolating EVs was shown in Figure 2A. As an initial attempt to characterize the EVs, the lysates of EVs were subjected to SDS-PAGE and then stained with Coomassie blue. As shown in Figure 2B, EVs were stained with Coomassie blue, indicating that EVs had various proteins of different sizes. Also, the EVs were free of bovine serum albumin (BSA), as compared with the BSA protein that I have used as a reference protein. To identify the most dominant proteins within these EVs, the lysates were subjected to LC mass spectrometry (liquid chromatography-mass spectrometry) analysis.

Interestingly, several subtypes of Collagen proteins, e.g., collagen type I α, inter α trypsin inhibitor, collagen type V α, collagen type XII α, and fibronectin were identified as the most enriched proteins found from equine protein database. To assess whether they meet their biophysical traits as EVs, their size was analyzed by DLS. Two peaks were prominently found, a population (14-17%) with relatively small size (36-38 nm), and the other (83-86%) with large size (200-300 nm) (Figure 2C). Additionally, NTA (Nanoparticle Tracking Analyzer) was conducted. The mean is 200 nm, and the deviation is 73 nm. And the highest graph is 189 nm. It has been well accepted that EVs can induce physiological changes to recipient (target) cells by various EV-to-cell interaction mechanisms including endocytosis. To examine whether BMC-derived EVs can be uptake by equine chondrocytes,

PKH26-labeled EVs were incubated with equine chondrocytes for 24 hours, and their localization was analyzed by immunofluorescence after cells were counterstained with F-actin (green) and DAPI (blue). As shown in Figure 2E, the PKH26-labeled spots were detected in the cytoplasm of equine chondrocytes in a dose-dependent manner, indicating that EVs were uptake into the recipient cells (equine chondrocytes) (Figure 2E). Western blot analysis revealed that EVs were positive with tetraspanin CD63 and heat shock protein HSP70 (Figure 2F), which are well-known markers for EVs derived from MSCs of various animal species [54]. Collectively, these results suggest that BMC-derived EVs have a general characteristic of EVs, and they can be internalized into equine chondrocytes.


A
150mm cell culture plate
(15-20 plate)
Pre-clearing
3000g, 15min, 4℃
Transfer supernatant to
a new tube
Add of 2mL Exo2D
reagent B
0.22μm filtering
Vivaspin Concentration
3000xg, 5h-6h, 4℃
(CM 200mL concentration)
10mL Concentrated
CM in the tube
Tube on the Rocker
30min, 4℃
3000g, 30min, 4℃
EV isolation
B
BSA 500ng
EV 5μg
80 kDa
50 kDa
C
Size Distribution by Intensity
Intensity (Percent)
Size (d.nm)
D

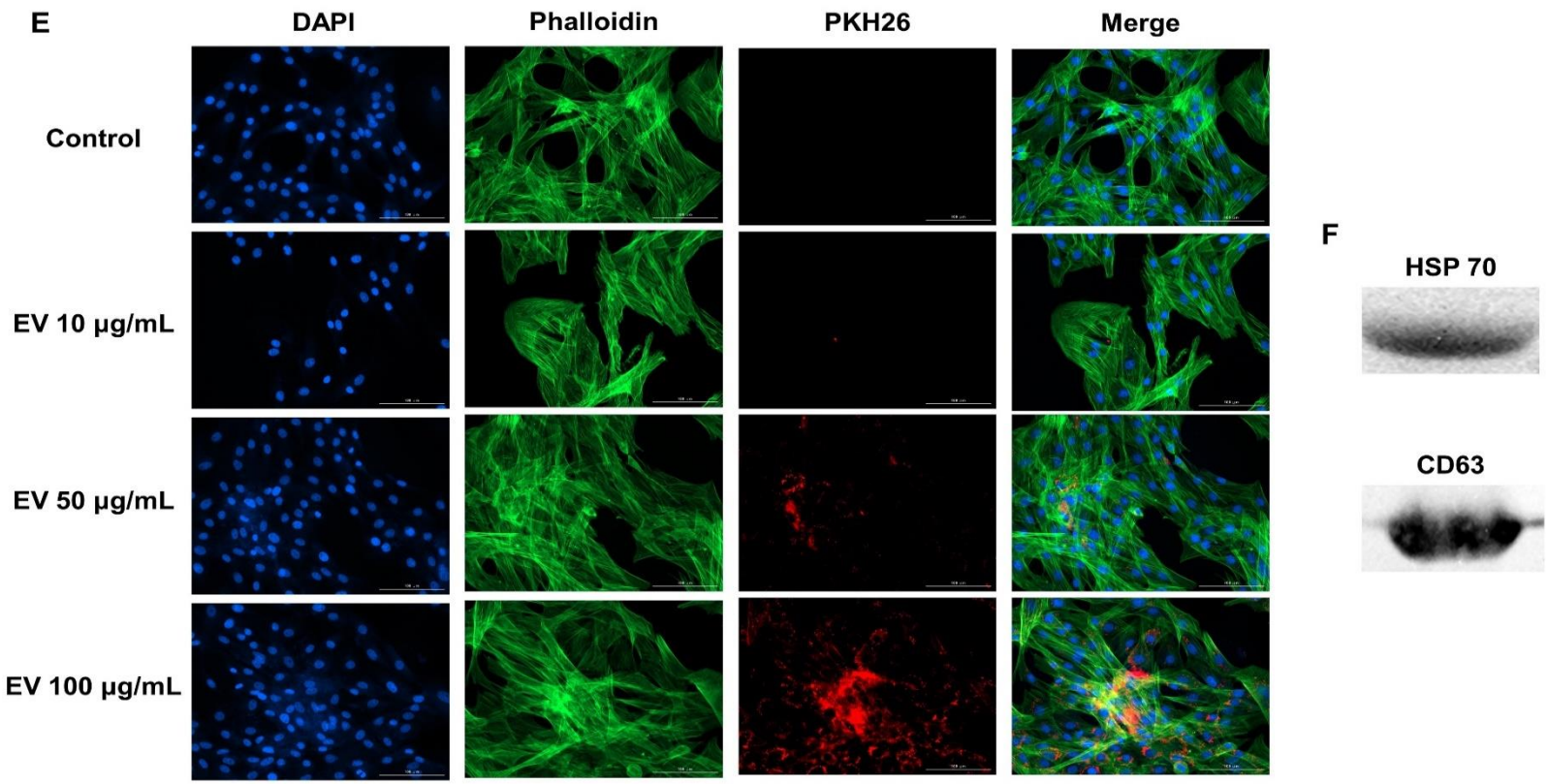

E
DAPI
Phalloidin
PKH26
Merge
Control
EV 10 μg/mL
EV 50 μg/mL
EV 100 μg/mL
F
HSP 70
CD63

Figure 2. Characterization of BMC-derived EVs. (A) Schematic workflow extracellular vesicle (EV) isolation. (B) Coomassie blue staining of EV content. BSA (Bovine serum albumin) was loaded as a reference control. (C) Zetasizer analysis of BMC-derived EVs. The mean diameter was 137.5 nm for BMC-derived EVs. (D) NTA analysis of BMC-derived EVs. The mean diameter was 200 nm. (E) Visualization of the uptake EV into equine chondrocyte. BMC-derived EVs (10, 50, or 100 μg/mL) were stained with PKH26 (red) and then incubated with equine chondrocyte for 24h. Before analysis, cells were counterstained with Phalloidin 488 (green) and DAPI (blue). (F) Immunoblot analysis for HSP70 and CD63 in EVs. Scale bar are 100 μm.

Table 3. Mass spectrometry results of highly expressed proteins in BMC-derived EVs

Abundant proteins in JJ BMC-EVs
Collagen type I alpha 1 chain OS=Equus caballus OX=9796 GN=COL1A1 PE=4 SV=1
Inter-alpha-trypsin inhibitor heavy chain 2 OS=Equus caballus OX=9796 GN=ITIH2 PE=4 SV=1
Collagen type I alpha 2 chain OS=Equus caballus OX=9796 GN=COL1A2 PE=4 SV=2
Collagen type V alpha 2 chain OS=Equus caballus OX=9796 GN=COL5A2 PE=4 SV=1
Collagen type XII alpha 1 chain OS=Equus caballus OX=9796 GN=COL12A1 PE=4 SV=1
Fibronectin OS=Equus caballus OX=9796 GN=FN1 PE=4 SV=2

OS, Organism name; OX, Organism taxonomy; GN, Gene name; PE, Protein existence; SV, Sequence version.

3.3 Assessment of the role of BMCs and BMC-EVs in the viability of equine chondrocytes

For functional analysis, I investigated whether BMCs or BMC-EVs can promote the proliferation of equine chondrocytes. Since the trophic role of FBS can often mask the effect of MSCs, the chondrocytes were starved for 12 or 24 hours, and then co-cultured with BMCs for 24 or 48 hours. As result, the trophic effect of MSCs on the growth of chondrocytes was more prominent after 24 hours of starvation (Figure 3B) compared with those starved for 12 hours (Figure 3A). Next, equine chondrocytes were treated with various concentration of EVs (10, 25, 50 and 100 μg/mL), and the increase of cells was analyzed by CCK-8 after 24, 48, and 72 hours of treatment. As shown in Figure 3C, BMC-EVs were able to stimulate the proliferation of chondrocytes in a time- and dose-dependent manner. Of note, the viability of equine chondrocytes at 72 hours of treatment was reduced when treated with 100 μg/mL compared with those treated with 50 μg/mL.

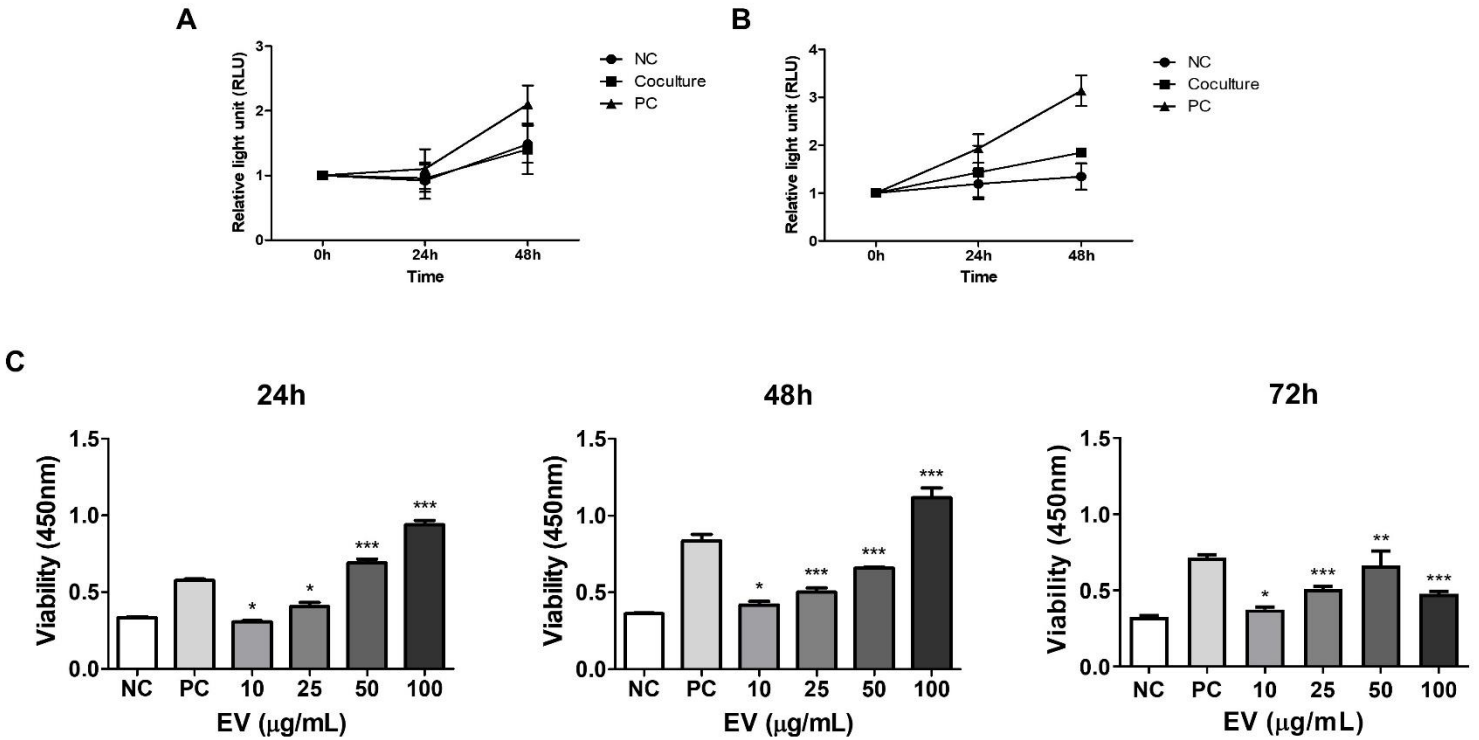

A
Relative light unit (RLU)
0
1
2
3
0h
24h
48h
Time
NC
Coculture
PC
B
Relative light unit (RLU)
0
1
2
3
4
0h
24h
48h
Time
NC
Coculture
PC
C
24h
48h
72h
Viability (450nm)
0.0
0.5
1.0
1.5
NC
PC
10
25
50
100
EV (μg/mL)
*
**
***

Figure 3. Assessment of the role of BMCs and BMC-derived EVs in equine chondrocyte. (A, B) The effect of co-culturing chondrocytes with BMCs. (C) The effect of BMC -derived EVs on the proliferation of chondrocytes. NC (negative control) and PC (positive control) indicate chondrocytes cultured without and with FBS, respectively. FBS, Fetal bovine serum. All data are expressed mean ± standard deviation (SD) from three replications. *$p < 0.05$, **$p < 0.01$, ***$p < 0.005$.

3.4 Establishing an optimal concentration of cytokine for inducing chondrocyte injury

Since no data was available on the effect of inflammatory stimuli on the equine chondrocytes that were obtained commercially, a pilot experiment was conducted on the relationship between the viability and various concentration of TNF-α or IL-1β. Thus, the viability of equine chondrocytes was analyzed following treatment with various concentrations of cytokines (0.1, 1, 10, 100, and 200 μg/mL) for up to 72h. The results of the cell viability CCK-8 assay showed that these cytokines significantly reduced the viability of equine chondrocyte in a time- and dose-dependent manner (Figure 4).

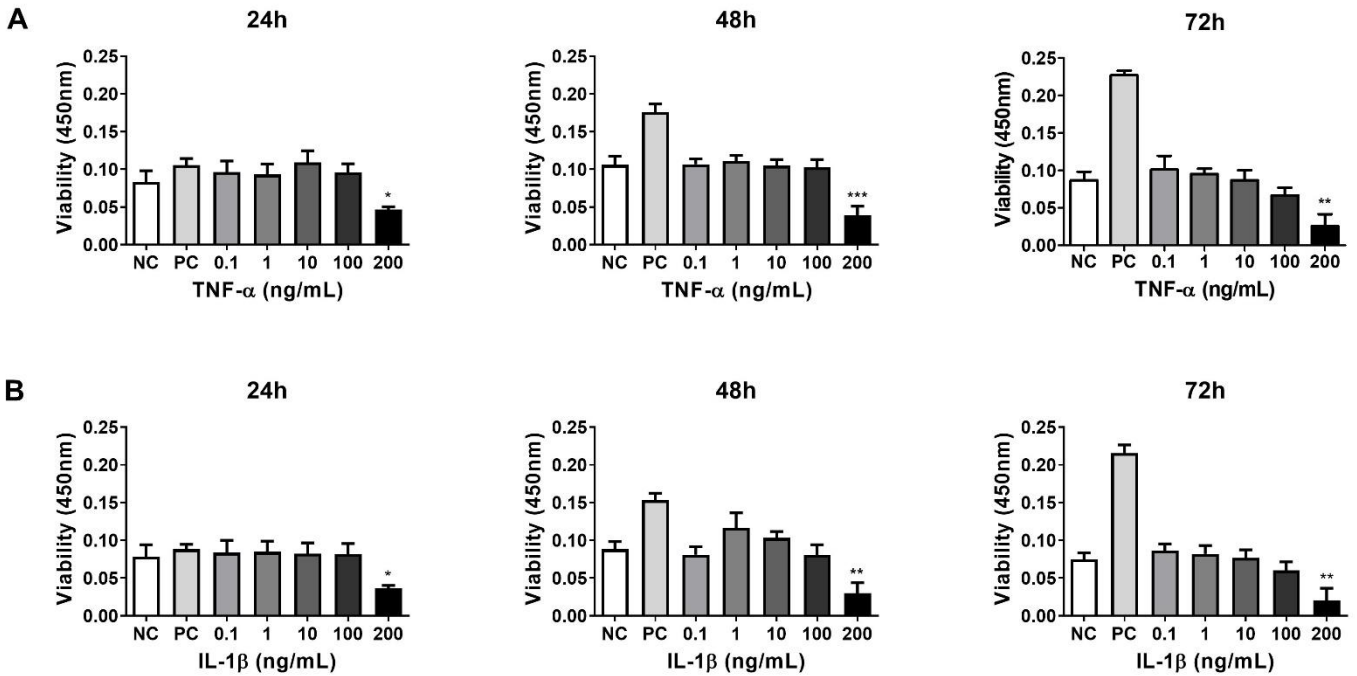
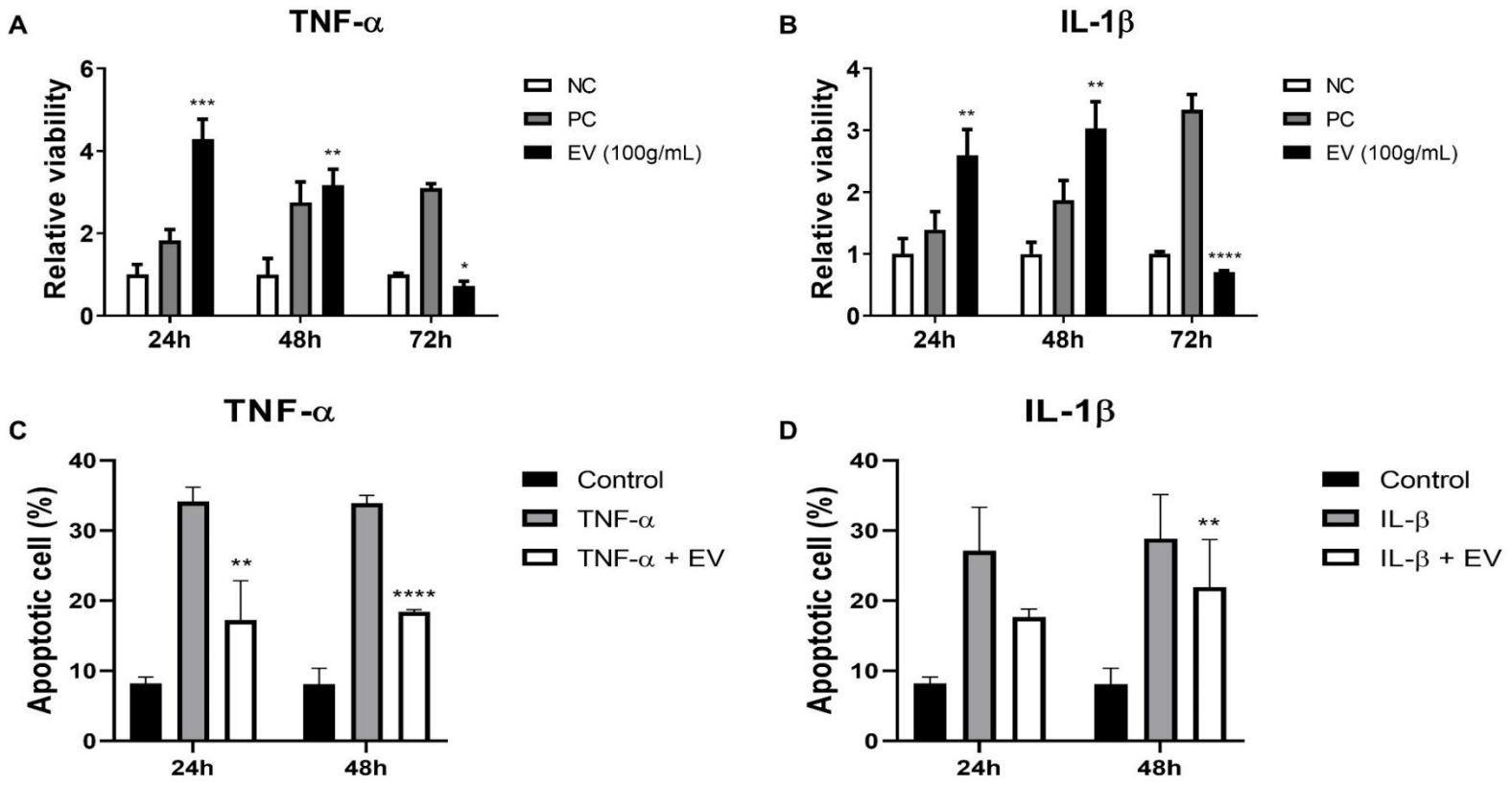

A
24h
48h
72h
Viability (450nm)
0.25
0.20
0.15
0.10
0.05
0.00
NC PC 0.1 1 10 100 200
TNF-α (ng/mL)
*
***
**
B
IL-1β (ng/mL)

Figure 4. The effect of BMC-derived EVs on the viability Inflammatory model in equine chondrocyte viability. Viability of equine chondrocytes incubated for 24, 48, and 72 h with different cytokines with TNF-α and IL-1β. NC and PC equine chondrocytes incubated in culture medium without cytokine TNF-α, IL-1β. (A) Relative viability of chondrocytes after treated with TNF-α for 24, 48 and 72 h. (B) Relative viability of chondrocytes after treated with IL-1β in equine chondrocyte for 24, 48 and 72 h. NC (negative control) and PC (positive control) indicate chondrocytes cultured without and with FBS, respectively. TNF-α, Tumor necrosis factor-α; IL-1β, Interleukin-1β; FBS, fetal bovine serum. All data are expressed as mean ± standard deviation (SD) from three replications. *$p < 0.05$, **$p < 0.01$, ***$p < 0.005$.

3.5 The effect of the BMC-derived EVs on the survival of injured chondrocytes

This study was conducted to investigate whether BMC-EVs can exert their trophic function on chondrocytes that are under inflammatory injury. EV treatment led to enhanced viability that was even higher than the positive control. Such effect was not found at 72 hours (Figures 5A and 5B). To further examine their role in enhancing the survival of injured chondrocytes, the apoptotic profile of chondrocytes was analyzed. As showed by Annexin V/PI staining, cytokine treatment led to a significant increase in the rate of apoptosis. However, the apoptotic cell death was significantly reduced in cells co-treated with EVs (Figure 5C-E).


A
TNF-α
Relative viability
6
4
2
0
***
**
*
24h
48h
72h
NC
PC
EV (100g/mL)
B
IL-1β
Relative viability
4
3
2
1
0
**
**
****
24h
48h
72h
NC
PC
EV (100g/mL)
C
TNF-α
Apoptotic cell (%)
40
30
20
10
0
**
****
24h
48h
Control
TNF-α
TNF-α + EV
D
IL-1β
Apoptotic cell (%)
40
30
20
10
0
**
24h
48h
Control
IL-β
IL-β + EV

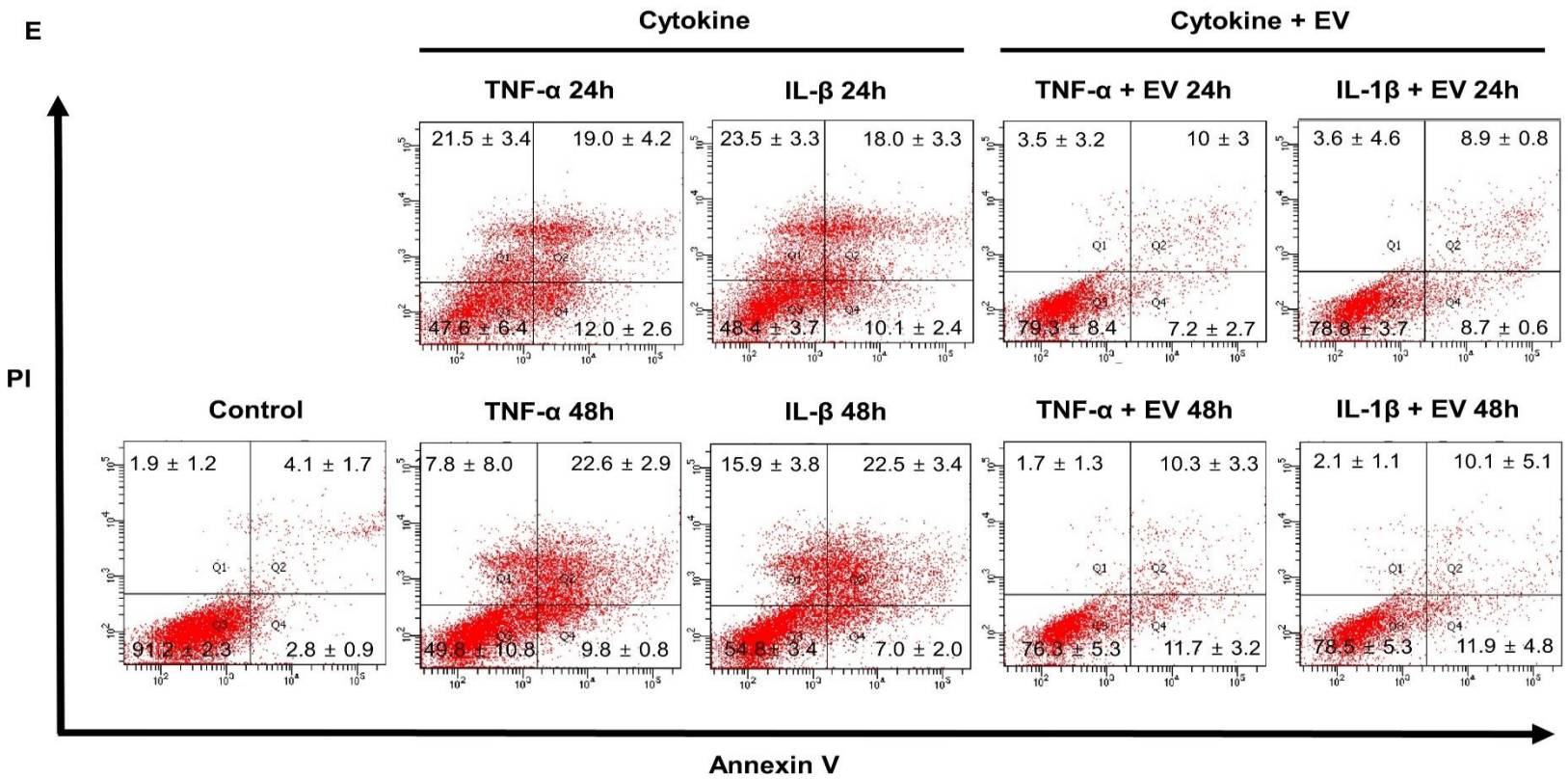

E
Cytokine
Cytokine + EV
TNF-α 24h
IL-β 24h
TNF-α + EV 24h
IL-1β + EV 24h
21.5 ± 3.4
19.0 ± 4.2
47.6 ± 6.4
12.0 ± 2.6
23.5 ± 3.3
18.0 ± 3.3
48.4 ± 3.7
10.1 ± 2.4
3.5 ± 3.2
10 ± 3
79.3 ± 8.4
7.2 ± 2.7
3.6 ± 4.6
8.9 ± 0.8
78.8 ± 3.7
8.7 ± 0.6
PI
Control
TNF-α 48h
IL-β 48h
TNF-α + EV 48h
IL-1β + EV 48h
1.9 ± 1.2
4.1 ± 1.7
91.2 ± 2.3
2.8 ± 0.9
7.8 ± 8.0
22.6 ± 2.9
49.8 ± 10.8
9.8 ± 0.8
15.9 ± 3.8
22.5 ± 3.4
54.8 ± 3.4
7.0 ± 2.0
1.7 ± 1.3
10.3 ± 3.3
76.3 ± 5.3
11.7 ± 3.2
2.1 ± 1.1
10.1 ± 5.1
78.5 ± 5.3
11.9 ± 4.8
Annexin V

Figure 5. The effect of BMC-derived EVs on the survival of chondrocytes. (A, B) The survivability of BMC-EVs on chondrocytes injured by TNF-α (A) or IL-1β (B) was examined at 24, 48, or 72 h. NC and PC are equine chondrocytes cultured without or with FBS, respectively. (C, D) Comparison of the percentages of TNF-α- or IL-1β-induced apoptotic chondrocytes co-treated with or without BMC-EVs. (E) Representative image of the flow cytometry data on the apoptotic chondrocytes treated with or without BMC-EVs under inflammatory injury. Cells were stained with Annexin V-FITC/PI to determine the apoptotic cell death. All data are expressed mean ± SD from three replications. *$p < 0.05$, **$p < 0.01$, ***$p < 0.005$, and ****$p < 0.001$ against negative control (A, B) or cells treated solely with TNF-α (C) or IL-1β (D).

3.6 Immunoblotting

To determine the mechanism of the BMC-derived EVs on the survival of chondrocytes, the expression of Akt and ERK, which is known to be essential for cell proliferation [55], was examined. Also, the expression of NFκB was also examined, because and NFκB is known to play a critical role in inflammatory responses via changing the transcription of several genes [56-58]. After the equine chondrocytes were simultaneously treated with cytokine and BMC-derived EVs, immunoblot analysis was conducted for the detection of phosphorylated and total Akt, ERK, and NFκB. As result, I found that Akt was activated in equine chondrocytes following BMC-derived EVs treatment as compared with that had not been treated with EVs. ERK was increased when cytokines were treated, while reduced when co-treated with BMC-derived EVs. Finally, reduced activity of NFκB was found after treated with BMC-derived EVs.

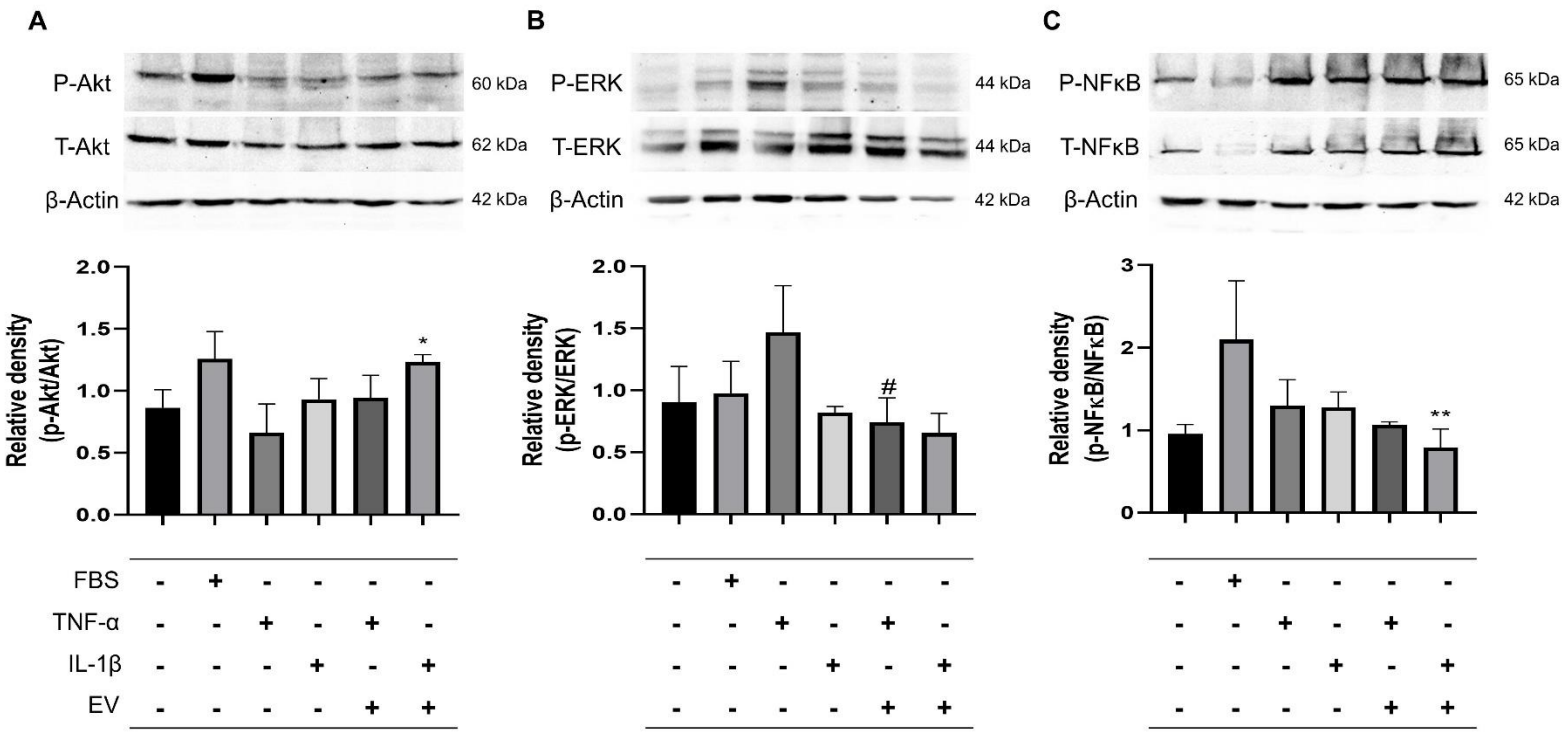

A
P-Akt
60 kDa
T-Akt
62 kDa
β-Actin
42 kDa
Relative density (p-Akt/Akt)
2.0
1.5
1.0
0.5
0.0
*
B
P-ERK
44 kDa
T-ERK
44 kDa
β-Actin
42 kDa
Relative density (p-ERK/ERK)
2.0
1.5
1.0
0.5
0.0
#
C
P-NFκB
65 kDa
T-NFκB
65 kDa
β-Actin
42 kDa
Relative density (p-NFκB/NFκB)
3
2
1
0
**
FBS
TNF-α
IL-1β
EV

Figure 6. Immunoblotting. Immunoblot analysis of the signaling pathway molecules in chondrocytes. (A) Chondrocytes were stimulated with TNF-α or IL-1β for 24 h in the presence or absence of BMC-EV, after which the cell extracts were analyzed by immunoblotting for Akt1, ERK1/2, and NFκB. Beta-actin was used as the loading control. (B) Densitometric analysis of the relative level of phosphorylated Akt1, ERK1/2, and NFκB. The value was normalized against that observed for negative control (NC). ERK, Extracellular signal-regulated kinase; FBS, Fetal bovine serum; TNF-α, Tumor necrosis factor-α; IL-1β, Interleukin-1β; EV, Extracellular vesicle. All data are expressed as mean ± SD from three replicates. *P=0.0401 against cells treated with IL-1β in the absence of BMC-EV. #P = 0.0414 against cells treated with TNF-α in the absence of BMC-EV. **P=0.0093 against cells treated with IL-1β in the absence of BMC-EV.

4. Discussion

Data from human clinical trials in cellular therapy or cell-based products for arthritic diseases and studies of OA are encouraging. However, the feasibility of utilizing these biological sources remains mostly unknown.

OA is one of the most prevalent joint diseases and a major public health problem. It is characterized by progressive articular cartilage destruction and synovitis [59, 60]. Current therapies attempt to relieve the symptoms, but they cannot stop or reverse the ongoing cartilage degeneration [61]. The ideal treatment aiming for an optimal OA joint repair should promote regenerative properties of chondrocytes and fight the destructive effects of inflammation.

During the last several decades, the reparative role of MSCs has been evidenced by various preclinical studies. Previously reported that MSC therapeutic effective in repair or regeneration of cartilage in an animal model [62, 63]. However, several limitations such as difficulties in long-term storage and maintenance, while sustaining its viability, should be overcome to make cell therapy feasible [64]. Regarding the mechanism of therapeutic function of MSCs in cartilage repair, the unique ability of MSCs to differentiate into several mesenchymal lineages, i.e., chondrocytes, accounts for the replacement of the dead or injured chondrocytes [65]. Additionally, it has been recently found that the secretome of MSCs also plays a vital role in the repair of injured chondrocytes [66, 67]. Since then, EVs including exosomes or were found to be efficacious against in cartilage regeneration. In particular, MSC exosomes or EVs therapeutic have been effective in cartilage regeneration in a

rodent model of OAs [13, 23-27, 29-32]. Despite such success and potential uses of MSC-derived EVs for cartilage repair, no attempt was made on the use of MSC-derived EVs in equine.

MSCs can be derived from various tissues including bone marrow, spleen, amniotic fluid, cartilage, muscle tendons, adipose tissues, periosteum, synovial fluid, thymus, trabecular bone, dermis, dental pulp, and lung in adult. In a developmental point of view, MSCs from fetal tissues may also provide a useful tool for cell therapy since they are known to express more pluripotent genes such as iPSC markers [45]. Compared to adult MSCs, fetal MSCs had an increased ability to proliferation more than adult MSCs [45-47] and increased the anti-inflammatory capacity, fitness, and homing ability [68].

Recently, many studies have been conducted on the characteristics of fetal MSCs. It was reported that fetal MSCs do not elicit pluripotency-associated issues that may be raised when using ESCs, and further, the therapeutic effect can be higher than adult MSCs [45, 47, 48]. The number of MSCs in newborns consists of 1:1,000 bone marrow cells, and only 1:2.0 x 10^6 at age 80 [69]. So fetal MSCs are found at a higher frequency in tissues than in adult MSCs and are readily available in fetal and extra fetal tissues. Tissues can be obtained from the fetal liver, umbilical cord, umbilical cord blood, placenta, and amniotic fluid [45, 70, 71]. Fetal MSCs are more effective in avoiding immune recognition than adult MSCs [72-74]. Fetal MSCs are known to be less immune than adult MSCs. Fetal MSCs have a lower surface of HLA Class I than adult MSCs [47, 72, 73], and fetal MSCs do not express HLA class

II on the surface or within the cell. Fetal MSCs are more suitable for clinical treatment given increased fitness. As MSC aging progresses, it has been reported that differentiation shifts from osteogenic differentiation to adipogenic differentiation and that fetal MSCs has better osteogenic potential than those from adults [75]. Indeed, a comparative study showed that many osteogenic genes were more abundantly expressed fetal than adult MSCs [76].

IL-1β and TNF-α are involved in producing other inflammatory cytokines in osteoarthritis. IL-1β and TNF-α induce the generation of inflammatory cytokines such as IL-6 [43] and IL-8 [77], monocyte chemoattractant protein 1 (MCP1) [78] and CC-chemokine ligand 5 (CCL5) [79], all of which are well-reported players in sustaining tissue inflammation. IL-6 exists at a low concentration level in normal chondrocyte. However, its concentration in sera and chondrocytes is increased in osteoarthritic conditions, after which it causes the increases in IL-1β and TGF-β, which in turn they promoted the production of IL-6 [80, 81]. Studies also demonstrated that IL-6 stimulates the expression of MMP-1 and MMP-13 in bovine and humans (cell type) [82, 83], and IL-6 reduced the expression of type II collagen (cell type) [84]. Other studies showed that the expression of IL-17 is upregulated by IL-1β, TNF-α, and IL-6, after which IL-17 upregulated NO and MMPs production [85]. Also, IL-17 led to a reduced expression of proteoglycan [86].

In the present study, I explored whether EVs derived from BMCs can exert reparative function in injured chondrocytes. I hypothesized that MSC EVs might enhance the viability of chondrocytes. I tested this hypothesis by

assessing the effect of MSC EVs on proliferation and apoptosis of equine chondrocytes in cell culture. To this end, I isolated and cultured MSCs from the bone marrow tissue. In addition, experiments using EVs from bone marrow MSCs were conducted. BMC-derived EVs had better effects on the increased viability of equine chondrocytes, and the growth kinetics were better to those of cell-cultured in serum-supplemented medium. The Annexin V assay involved in apoptotic cell decreased following EV treatment (Figure 5). Furthermore, Akt phosphorylation was increased BMC-derived EVs treated in equine chondrocytes. However, the NFκB phosphorylation was decreased BMC-derived EVs treated in equine chondrocytes. Based on these findings, I suggest that BMC-derived EVs have a function in enhancing the growth and survival of equine chondrocytes.

Mostly, cell proliferation and survival are mediated by receptor-mediated signal transduction via phosphorylation of Akt and ERK including chondrocytes [13]. The activation of Akt and/or ERK prosurvival signaling by EVs has been reported to be an essential pathway in EV mediated tissue repair and regeneration, and have been demonstrated in wound healing [87, 88], bone repair [89], and myocardial regeneration following ischemia injury [90]. However, inflammation caused by TNF-α and IL-1β upregulated ERK (Figure 6) [91-93]. IKK-mediated p65 phosphorylation occurs in the cytoplasm and/or in the nucleus. TNF-α and IL-1β trigger Mitogen- and stress-activated protein kinase1-mediated phosphorylation of p65 a Ser276 in the nucleus via an ERK-dependent pathway (Figure 7) [57, 92]. EVs suppress the phosphorylation of NFκB (Figure 6) and prevent its translocation to the nucleus [94].

Based on the flow cytometry analysis, the expression profile of surface antigens was heterogeneous. CD90 is thymocyte antigen, glycophosphatidylinositol (GPI) anchored cell surface protein [95], whose main functions are cell adhesion and migration [96], apoptosis [97], T-cell activation [98], fibrosis [99, 100], and tumor suppressor [101-103]. CD105 is membrane glycoprotein located on the cell surface and part of the TGF-β receptor [104-106], which plays various roles such as cell migration, development of the cardiovascular system, or vascular remodeling [107-111]. Perhaps there is a connection with T-cell-related immunity, and I expect that fetal cells had a lower expression of CD90 and CD105 than adult MSCs. Further research is needed to identify the nature of their expression profile of conventional surface markers of MSCs in equine BMCs.

An extracellular matrix such as proteoglycan and collagen is required for proper cartilage regeneration [112-115]. In line with these findings, various subtypes of collagen proteins were detected in the BMC-derived EVs after proteomic analysis (Table 3). This suggests that collagen proteins in BMC-derived EVs may exert the function of EVs on the survival of chondrocytes.

When BMC-derived EVs were treated during the culture of chondrocytes in a serum-free medium, an increase of viability was found (Figure 3). Unexpectedly, at 100 μg/mL, their viability was decreased compared with those treated with 50 μg/mL. Similarly, the viability of chondrocytes was also decreased in inflamed chondrocytes upon treated with

the same concentration of EVs 100 μg/mL at 72 h (Figure 5). Such may be due to the unknown components (e.g., lipids) that may exist in the lipid bilayer structure of EV. Indeed, the membrane of EV has phospholipid, sphingolipid, and cholesterol [116].

Conclusions

The present study demonstrated that BMCs have the characteristics of MSCs. BMC-derived EVs potently enhanced the viability and stimulated the proliferation of injured chondrocytes. Also, BMC-derived EVs were competent in repairing equine chondrocytes that are undergoing inflammatory stress and that they downregulated the activity of NFκB in injured chondrocytes. BMC-derived EVs also enhanced the activation of Akt signaling while reducing ERK1/2 activity. In conclusion, BMC-derived EVs had potential that can become a novel strategy for the prevention or treatment of OA in horses.

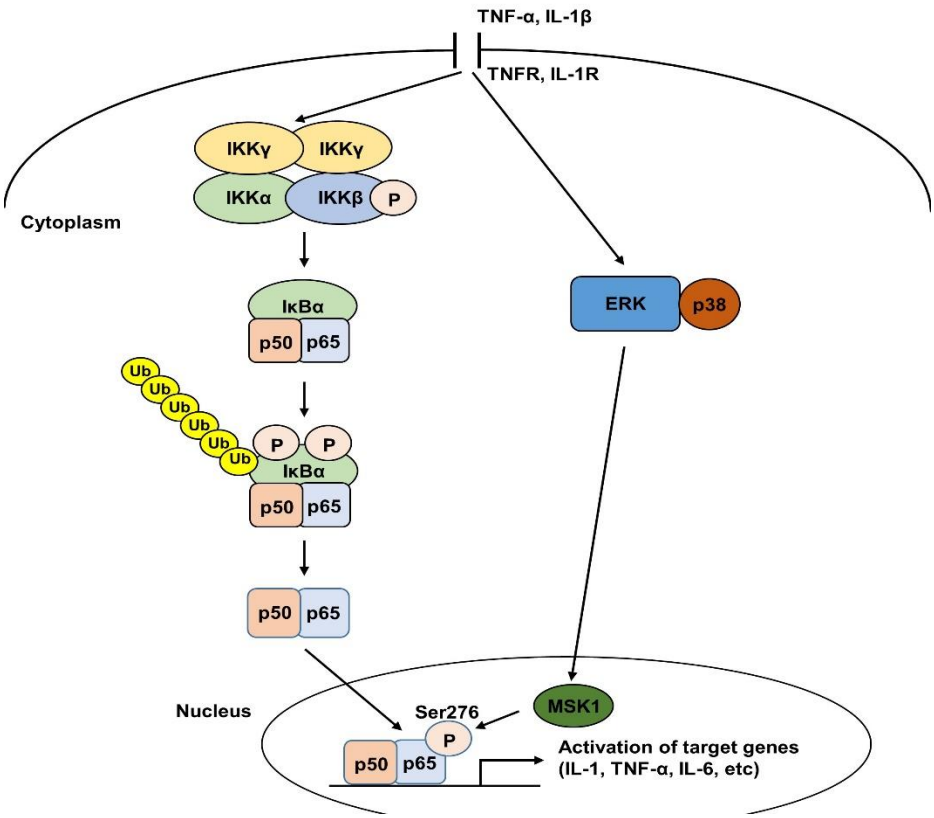


Figure 7. The schematic illustration of the NFκB pathway. The schematic illustration of the NFκB pathway. TLR ligands and cytokines such as TNF uses a wide variety of signaling adaptors to engage and activate the IKKβ subunit of the IKK complex. Activated IKKβ leads to the phosphorylation of IκBα, which results in its ubiquitination and subsequent proteasome-induced degradation. NFκB is then phosphorylated, and enters the nucleus and controls the transcription of genes that are involved in inflammation, survival, and division. ERK and p38 activate MSK1, which phosphorylates nuclear p65 at Ser276. TNF-α: Tumor necrosis factor-α; IL-1β: Interleukin-1β; TNFR: Tumor necrosis factor receptor; IL-1R: Interleukin-1 receptor; ERK: Extracellular signal-regulated kinase; MSK1: Mitogen-and stress-activated kinase 1; Ub: ubiquitin.

국문 초록

MSC는 비교적 분리와 배양 과정이 간단하며, 내재적으로 세포밖소포체와 성장인자를 분비하여 염증조절, 항섬유화, 조직 재생 능력을 지닌다. MSC는 다른 세포와 구분되는 몇 가지 특성을 보유하며, 대표적으로 표면 마커(CD73, CD90, CD105)의 발현과 중간엽조직으로의 분화(지방세포, 연골세포, 골세포)로 분화할 수 있는 능력을 들 수 있다.

세포밖소포체(EV)는 거의 모든 세포에서 분비되는 나노 크기의 입자로 세포간의 통신을 통해 다양한 생물학적 과정을 조절하며, 세포 밖 소포체는 MSC를 세포간 신호로 조절한다. 더욱이 MSC에서 파생되는 세포 밖 소포체는 거부 위험이 적고, 암 발생가능성이 낮고, 장기 저장 용이성, 혈전증 감소 그리고 쉽게 즉시 사용할 수 있다는 장점이 있다.

본 연구는 제주마 태아로부터 채취한 골수세포가 활발히 증식하는지 여부와 궁극적으로 이로부터 생산된 세포밖 소포체가 손상된 말 연골세포의 증식과 회복을 촉진하는지에 관한 것이다. 결과를 요약하면 첫째, 골수 세포는 140일령의 말 태아에서 추출한 후에 배양을 하였고, 그 후에 유세포 분석, 분화능 분석 그리고 면역조절 유전자 발현을 통하여 세포의 특성을 파악하였다. 그 다음에 골수세포 유래 세포 밖 소포체가 세포 밖 소포체의 특성을

가지고 있는 알아보기 위하여 NTA, Coomassie staining, 동적광산란 (DLS) 과 Immunoblot을 진행하였다. 골수세포 유래 세포 밖 소포체가 말 연골세포가 무혈청 배양조건에서도 증식을 자극한다는 것을 확인하였으며, 염증성 자극에 의한 연골세포 사멸을 억제하는 것을 확인하였다. 세포밖 소포체의 작용기전을 알아보고자 세포 밖 소포체를 연골세포에 처리하였다. 그후에 Immunoblot를 통해 Akt-1/2/3의 인산화를 증가시키는 것을 확인하였다. 이와는 대조적으로 세포 외 신호 조절 키나아제 (ERK)-1/2와 NFκB의 하위 단위인 p65의 활성은 세포밖 소포체에 의해 감소했다는 것을 알게 되었다.

종합적으로, 본 연구는 태아 골수세포에서 유래된 세포 밖 소포체가 연골세포의 성장을 증가시킬 수 있고, ERK-1/2와 NFκB signaling을 감소시킬 수 있으며, PI3/Akt경로를 통해 연골세포의 생존을 촉진할 수 있다는 것을 입증했다.

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