CHAPTER IV
TRANSPLANTATION OF HUMAN WHARTON'S JELLY MESENCHYMAL STEM CELLS DERIVED-CHONDROCYTES IN GUINEA PIG MODEL WITH SPONTANEOUS OSTEOARTHRITIS

4.1 Plants and Phenotypic Similarities

Osteoarthritis (OA) is a degenerative joint disease commonly found in elderly people, and obese patients. Currently, OA treatments are determined based on their condition severity and medical professional's advice. The aim of this study was to differentiate human Wharton's jelly-derived mesenchymal stem cells (hWJ-MSCs) into chondrocytes for transplantation in OA-suffered guinea pigs. Early stage chondrocytes can be obtained by chondrogenic differentiation induction of MSCs for 14 days. For transplantation, Then, early OA-suffered guinea pigs were injected with hyaluronic acid (HA) containing either MSCs or 14-days old hWJ-MSCs-derived chondrocytes. Results showed that hWJ-MSCs-derived chondrocytes expressed specific markers of chondrocytes including Aggrecan, type II collagen, and type X collagen proteins, and *ß-catenin*, *Sox9*, *Runx2*, *Col2a1*, *Col10a1* and *ACAN* gene expression markers. Administration of HA plus hWJ-MSCs-derived chondrocytes (HA-CHON) produced better recovery rate of degenerative cartilages than HA plus MSCs or only HA. Histological assessments demonstrated no significant difference in Mankin's scores of recovered cartilages between HA-CHON-treated guinea pigs and normal articular cartilage guinea pigs. Transplantation of hWJ-MSCs-derived chondrocytes was more effective than undifferentiated hWJ-MSCs or hyaluronic acid for OA treatment in guinea pigs. This study provides a promising treatment used in early OA patients to promote the recovery and prevent the disease progression to severe osteoarthritis.

4.2 Introduction

Articular cartilage is a specialized type of connective tissue composed of cartilage cells and typically found in synovial joints. These cells produce extracellular matrix (ECM) and preserve the function of the tissue. Articular cartilage does not possess self-healing ability due to the absence of blood vessels, lymphatic vessels, and the nervous system (Sophia Fox et al., 2009). Arthritic cartilage degeneration can cause various symptoms, including growth abnormalities in children, injuries caused by stress from trauma, and age-related osteoarthritis (Song et al., 2004).

Current OA treatments are commonly determined based on disease severity, and the physician's recommendations including pharmacological and non-pharmacological therapies. The first-line pharmacologic treatment is acetaminophen to cure mild and intermittent symptoms, and then followed by non-steroidal anti-inflammatory drugs (NSAIDs) when acetaminophen is ineffective to alleviate pain. However, NSAIDs prescription should be considered due to their gastric ulcer complication, and cardiovascular risk (Yusuf, 2016). Combination of pharmacological and non-pharmacologic treatment, namely diet and weight loss, physical therapy and exercise, and nutritional supplements (glucosamine and chondroitin sulfate) is a common advice for OA treatment to reduce symptoms and improve functional performance of the joint. Surgery is an invasive procedure that should be conducted when the combined therapy is unsuccessful to produce desired outcomes (Yusuf, 2016). Additionally, there is a possibility of recurrence and complications after surgery in many patients. Consequently, a novel and more effective procedure for osteoarthritis is indispensable, like application of cartilage cells. However, extraction of these cells from a human requires invasive surgery, which is complicated and expensive (Ebihara et al., 2012; Wong et al., 2020). Research is currently underway to explore the potential use of mesenchymal stem cells (MSCs) in treating osteoarthritis. The stem cells possess the unique ability to stimulate the growth of cartilage cells and other types of cells. MSCs are utilized in treating various disorders and can be sourced from several different locations, including bone marrow, blood, adipose tissue, and dental pulp. They can be isolated and cultured with a high level of proliferation activity. Previous studies have identified Wharton's jelly, found in the

umbilical cord of humans, as a common source of MSCs. This tissue can be collected from pregnant women following childbirth, without complex collection process required (Troyer et al., 2008). As a result, MSCs isolated from the Wharton's jelly of human umbilical cords are a promising area of interest for future clinical trials.

The use of Dunkin Hartley guinea pigs as an animal model for studying spontaneous cartilage degeneration in the knee joint, which is similar to osteoarthritis in humans, has been well established (Tessier et al., 2003; Yan et al., 2014). Researchers reported that the knee joint of guinea pigs closely resembles that of humans affected by osteoarthritis (Fernandez et al., 1997; Kraus et al., 2010). Moreover, spontaneous cartilage degeneration in the Dunkin-Hartley guinea pigs are used for study (Bendele & Hulman, 1988). Previous studies demonstrated that injecting mesenchymal stem cells (MSCs) with hyaluronic acid (HA) into the articular cartilage of guinea pigs with osteoarthritis led to recovery (Sato et al., 2012). HA-based formulations are currently delivered into the joint to relieve pain and improve joint mobility of OA patients by partial restoration of the rheological properties of the synovial fluid (La Gatta et al., 2021).

In this study, we isolated MSCs from human Wharton's jelly of the umbilical cord and induced them into cartilage cells. We then transplanted the early chondrogenic differentiated MSCs into the guinea pigs which have osteoarthritis and monitored their progress to evaluate the effectiveness of the treatment. The results demonstrated promising outcomes in the experimental animals, suggesting that this treatment approach using early chondrogenic differentiated MSCs could be developed into a viable treatment option for patients with osteoarthritis. Furthermore, this method is simpler and less invasive than surgical treatments, making it a potentially safer option for patients. Therefore, the purposes of this study were to differentiate human Wharton’s jelly-derived mesenchymal stem cells (hWJ-MSCs) isolated from human umbilical cord tissues into chondrocytes, and characterize hWJ-MSCs-derived chondrocytes prior to transplantation in OA-suffered guinea pigs.

4.3 Materials and Methods

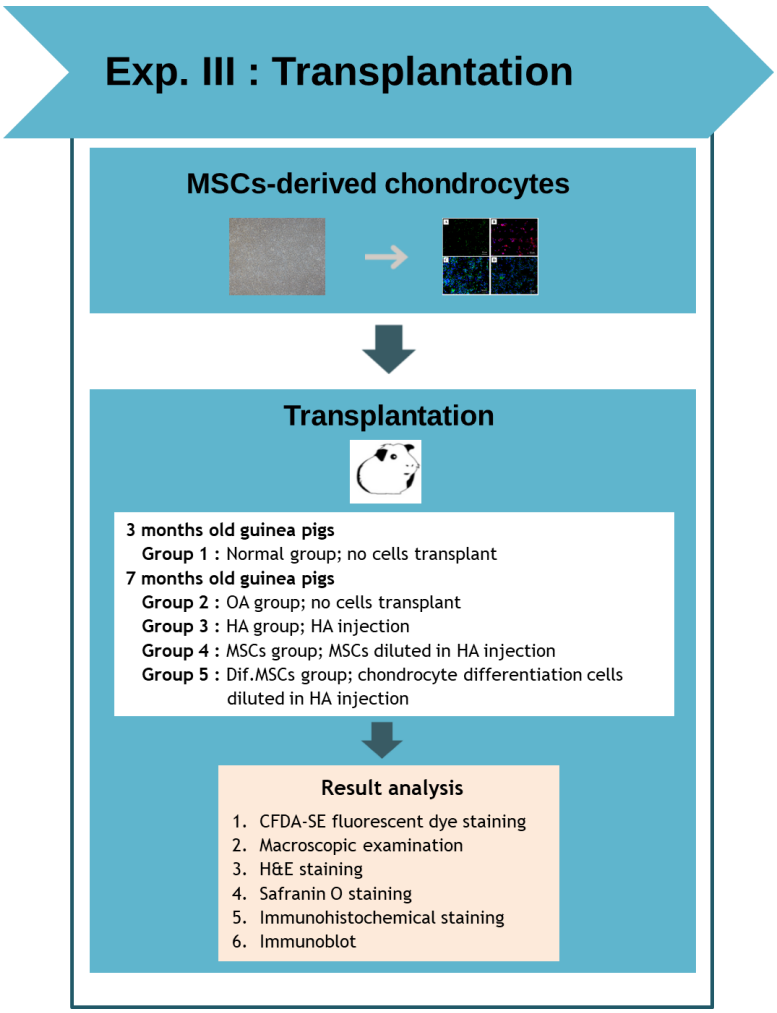
4.3.1 Ethics Statement

Ethical approval for this study was obtained from the Animal Ethics Committee of Suranaree University of Technology, Thailand (Approval Number: U1-03131-2559).

4.3.2 Reagents

All chemical compounds were purchased from Sigma-Aldrich Corporation (St. Louis, Missouri, USA), antibodies were obtained from Thermo Fisher Scientific (Waltham, MA, USA). The cell culture media and cell culture ware was obtained from Gibco (Paisley, UK) and SPL Life Science (Gyeonggi-do, South Korea), respectively. Unless stated otherwise.

4.3.3 Experimental design



4.3.4 Experimental animals

Guinea pigs of 3 months age were identified as normal articular cartilage guinea pigs, while guinea pigs of 7 months were identified as having minor osteoarthritis (OA). The study randomly assigned guinea pigs to five groups (N= 10/group) using a Completely Randomized Design (CRD) depicted in Figure 4.1: (1) Normal group consisting of guinea pigs of 3 months with no cells transplant (2) OA group consisting of OA guinea pigs of 7 months with no cells transplant, (3) HA group consisting of OA guinea pigs of 7 months given Hyaluronic acid (HA) injection (4) HA+MSCs group consisting of OA guinea pigs of 7 months given Hyaluronic acid (HA) injection with hWJ-MSCs, and (5) HA+dif.MSCs group consisting of OA guinea pigs of 7 months given Hyaluronic acid (HA) injection with chondrogenic differentiated cells. The guinea pigs were marked by notching on the pinna of the ear for identification among treatments. Carboxyfluorescein diacetate succinimidyl ester (CFDA-SE; Thermo Fischer Scientific) was used to label cells before transplantation (Yoon et al., 2013).

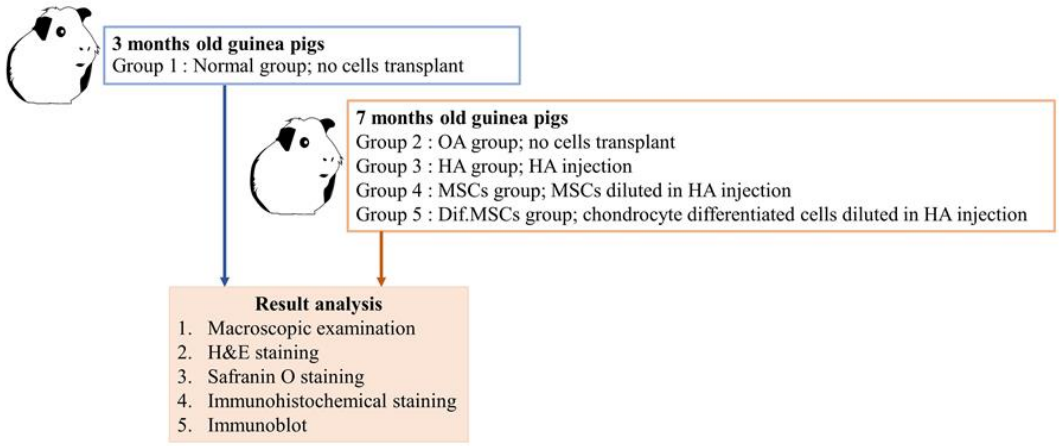


Figure 4.1 Experimental design of cell transplantation.

4.3.5 Preparation of chondrocytes derived from hWJ-MSCs

At day 14th of chondrogenic induction, chondrocytes derived from hWJ-MSCs at passage 5 were collected for transplantation. The hWJ-MSCs-derived chondrocytes were detached and separated by a 30 minute digestion process using

0.2% collagenase type II followed by 0.25% Trypsin. Before injection, these cells were labeled with CFDA-SE.

4.3.6 Cell transplantation

In group 4 and 5, each guinea pigs received a 100 µl injection containing a total of 1×10^6 cells (hWJ-MSCs in group 4 and chondrocytes derived from hWJ-MSCs in group 5) that were labeled with CFDA-SE and suspended in HA (Hyruan®III; LG Chem, Seoul, South Korea). The injection was administered into the medial compartment of the knees of the guinea pigs (Sato et al., 2012).

4.3.7 Macroscopic examination

Five weeks following cell transplantation, the guinea pigs were euthanized using carbon dioxide fumigation. The proximal tibiae of each guinea pigs were then opened, and their cartilage repair in the degenerative knee was examined (Sato et al., 2012). This study used five proximal tibia samples to perform the macroscopic examination. The tibiae's distal heads were first stained with India ink for a minute. The cartilage surfaces were then washed with PBS(-) before being examined and scored following a criteria in Table 4.1.

Table 4.1 Scoring criteria for osteoarthritis symptoms according to cartilage damage examined by India ink staining.

Score	cartilage surface
0	normal, perfectly smooth surface, no black areas
1	small area of rough surface, only stick black on small area < 10%
2	medium area of rough surface, stick black on small area 10 - 30%
3	large area of rough surface, stick black on wide and dark area >30%
4	Cartilage loss areas are deep but not damaged to the bone
5	Cartilage loss areas are deep and damaged to the bone

4.3.8 Histology and Immunohistochemistry

Five proximal tibia samples were fixed in 10% buffered formaldehyde at 4°C for 72 hours. To decalcify the tissues, they were soaked in 5% nitric acid for seven days, and then embedded in optimal cutting temperature (OCT) compound. Tissue slices with a thickness of 15 mm were sectioned (Kawamoto and Shimizu,

2000). The study conducted histology, immunohistochemistry, and fluoroscopic analysis on guinea pigs tissue series. The tissue slides were stained using hematoxylin and eosin (H&E) and safranin O before being examined and scored following the Mankin et al.'s (Mankin et al., 1971) and Armstrong et al.'s (Armstrong et al., 1994) methodology. In the case of immunohistochemistry, the tissue slides were incubated using primary polyclonal antibodies (rabbits against type II collagen) with a 1:200 dilution (COSMO BIO, Tokyo, Japan) for an hour at room temperature. After washing with PBS, the tissue slides were incubated in HRP anti-mouse IgG secondary antibody for 30 minutes at room temperature. The tissue slides were visualized using VECTA STAIN ABC Reagent (Vectastain Elite Kit; Vector Laboratories). To conduct fluoroscopic analysis, CFDA-SE labeled cells were studied in a tibial frontal section using a fluorescence microscope with 492 nm and 517 nm settings (Sato et al., 2012).

4.3.9 Immunoblot analysis

Proteins were extracted from tissues and separated via SDS-PAGE using a 15% resolving gel. After electrotransfer to PVDF membranes (Immun-Blot® PVDF Mem-brane), the membranes were blocked using a solution of 5% skim milk in TBST (Tris-buffered saline with 0.1% Tween 20) for 1 hour at room temperature. To detect type I collagen, type II collagen, and matrix metalloproteinase-13 (MMP13 dilution 1:20)), the membranes were incubated with primary antibody solutions (1% BSA in TBS Tris-buffered saline) overnight at 4°C. After being washed with TBST, secondary antibodies (goat anti-rabbit or goat anti-mouse) conjugated with horseradish peroxidase (HRP; Abcam, Cambridge, U.K) were applied for 1 hour at room temperature at a dilution of 1:2000 in 5% skim milk in TBST. The chemiluminescent substrate was applied using an ECL substrate kit (Ultra-high sensitivity, Abcam, Cambridge, UK) following the manufacturer’s suggestions. The protein bands were then imaged using Image Quant™ LAS 500 (GE Healthcare Life Sciences, Massachusetts, USA), with β-actin used as a were not quantified.

4.3.10 Statistical Analysis

Statistical analysis was conducted on three to five samples, and the data were presented as the mean ± standard deviation (S.D.). To compare differences between the control and treated groups, a one-way analysis of variance (ANOVA) was employed, followed by Tukey-Kramer Honest Significant Difference (HSD) Post hoc

test. Results with a *p*-value less than 0.05 were regarded as significant, whereas those with a *p*-value less than 0.01 were deemed highly significant.

4.4 Results

4.4.1 Chondrocyte transplantation results

Dunkin Hartley guinea pigs were used as animal models. Guinea pigs were divided into five groups: 1) 3-month-old guinea pigs with normal knee joints (normal), 2) 7-month-old guinea pigs with no treatment (spontaneous osteoarthritis; OA), 3) 7-month-old guinea pigs with HA injections, 4) 7-month-old guinea pigs with HA+MSCs injections, 5) 7-month-old guinea pigs with HA+ differentiated chondrogenic MSCs. The differentiated chondrogenic MSCs were stained with CFDA-SE fluorescent dye before transplantation. After staining with CFDA-SE, the stained cells survived and grew normally when cultured (Figure 4.2). Intra-articular injection was performed at the knee joints of the guinea pigs. After transplantation, all the guinea pigs were healthy, and there were no observable any abnormalities at the knee joints after the injection.

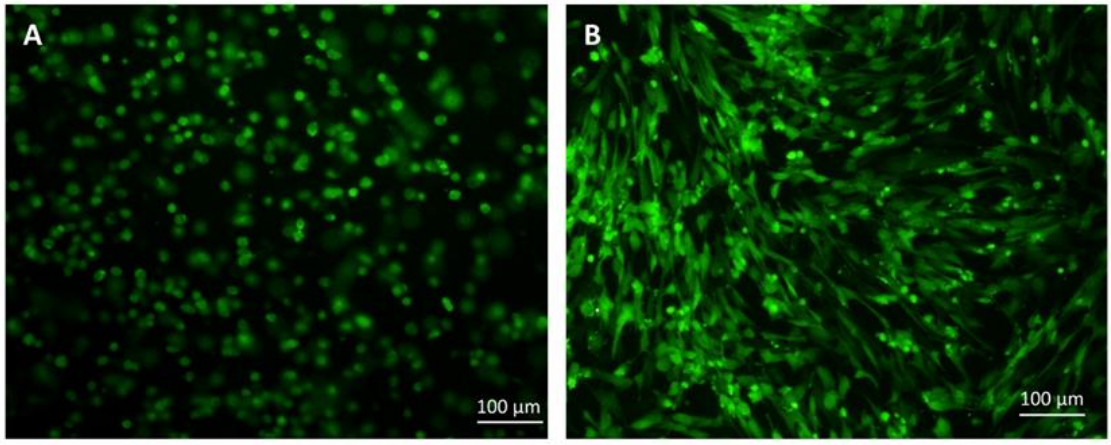


Figure 4.2 The cells stained with CFDA-SE fluorescent dye: (A) cells suspended in HA, (B) cells cultured for 7 days. Scale bar = 100 µM.

4.4.2 Macroscopic examination results

Five samples of the proximal tibia were obtained from each guinea pig group and dyed with India ink as shown in Figure 4.3. The rough cartilage surface clearly showed black color of India ink particles. In group 1, there were no blacked

out areas on the cartilage surfaces. Only the medial sides of the tibial cartilages in groups 2-5 of 7-month-old guinea pigs were blacked out. The cartilage of the group 2 (not- transplanted), the blacked areas were wider than group 3, 4 and 5. Degenerative symptom scores of each group are displayed in Figure 4.3 F. The degenerative scores of the 7-month old guinea pigs in groups 2 – 5 were significantly higher than group 1 (the 3-month old guinea pigs; $P< 0.01$).

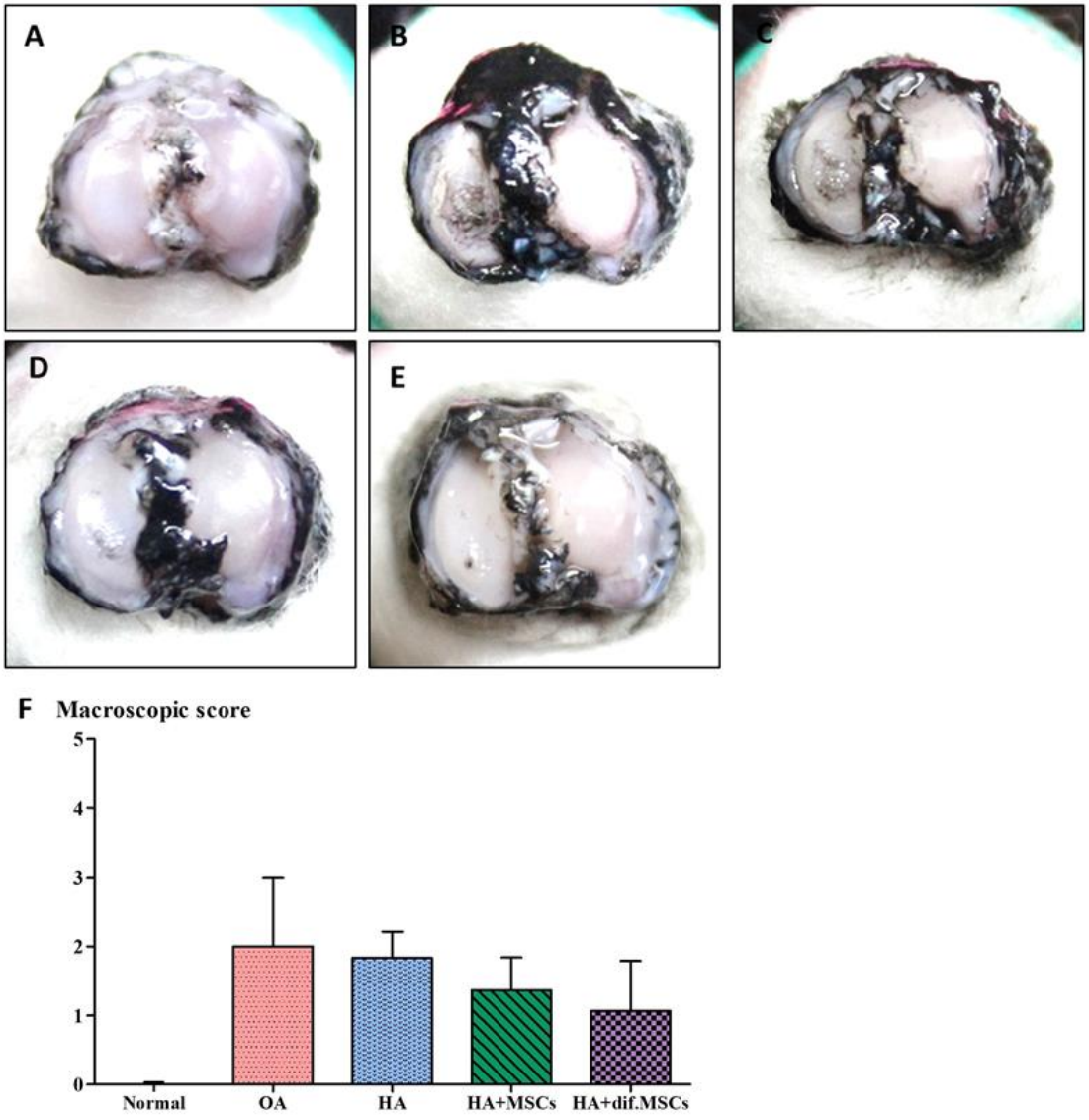


Figure 4.3 The osteoarthritis scores of each group were examined by (A-E) India ink staining and (F) macroscopic score.

4.4.3 Histology results

CFDA-SE fluorescence-stained cells in the knee sample of guinea pigs were examined using cryosection method and observed under a fluorescent microscope. Human nuclei were also stained with red fluorescent antibody. Green fluorescent cells of CFDA-SE with red staining were observed at the human nuclei. This confirmed that the cells attached to the cartilage surface were human cells that were transplanted into the guinea pig's knee joint (Figure 4.4). The results also pointed out that hWJ-MSCs-derived chondrocytes could adhere to the cartilage surfaces together with recovery of the damaged cartilage.

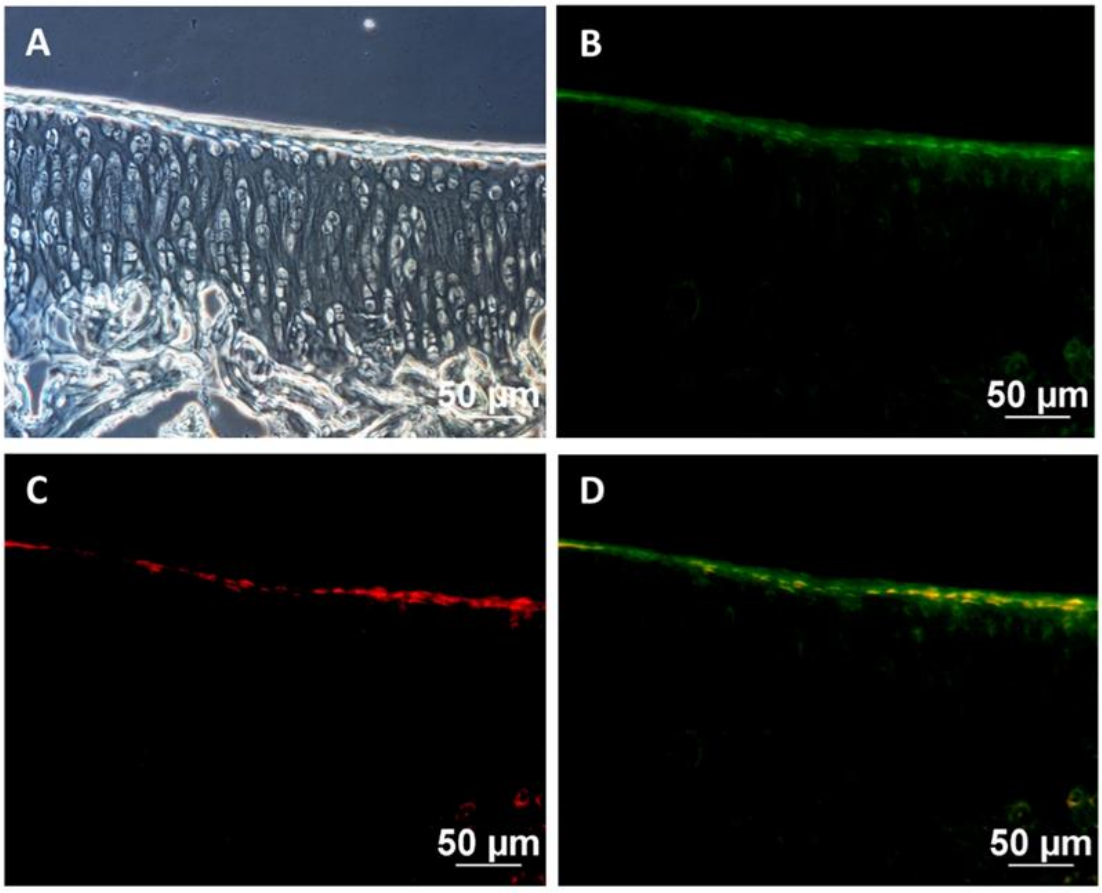


Figure 4.4 Cell tracking after transplantation: (A) bright field images, (B) CFDA-SE-stained transplanted cells (green), (C) human nuclei (red), (D) merged images. Scale bar = 50 um.

The knee joints of guinea pig were cryosectioned and examined histologically using H&E and Safranin O staining techniques (Figure 4.5-4.6), respectively. A smooth cartilage surface, a uniform cartilage surface layer, and the extracellular matrix were present in group 1. However, the cartilage of 7-month-old guinea pigs in groups 2-5 showed the cartilage imperfection at the medial side of the tibia indicated by un-smooth thickness of the cartilage surface layer, the rupture of the cartilage surface (the arrows in Figure 4.5-4.6, extracellular matrix loss, and the formation of a cavity within the cartilage tissue. Group 2 had the most severe cartilage damage among all groups. Cartilage damage was reduced in group 3 (HA injection) and group 4 (HA injection with MSCs). On average, MSCs caused the least cartilage damage across all samples. In group 5 (HA injection with chondrogenic differentiated cells), the lowest cartilage damage was noticeable, compared to other groups. Cartilage damage scores based on the Mankin criterion procedure are illustrated in Figure 4.7. The knee joints of guinea pig in groups 1, 2, 3, 4 and 5 showed cartilage damage scores as 1.6 ± 0.5, 6.6 ± 1.8, 6.4 ± 2.1, 4.8 ± 1.5, and 3.0 ± 1.9, respectively. The values in groups 2, 3, and 4 were significantly higher than group 1 ($P < 0.01$), but there was no difference between group 5 and group 1.

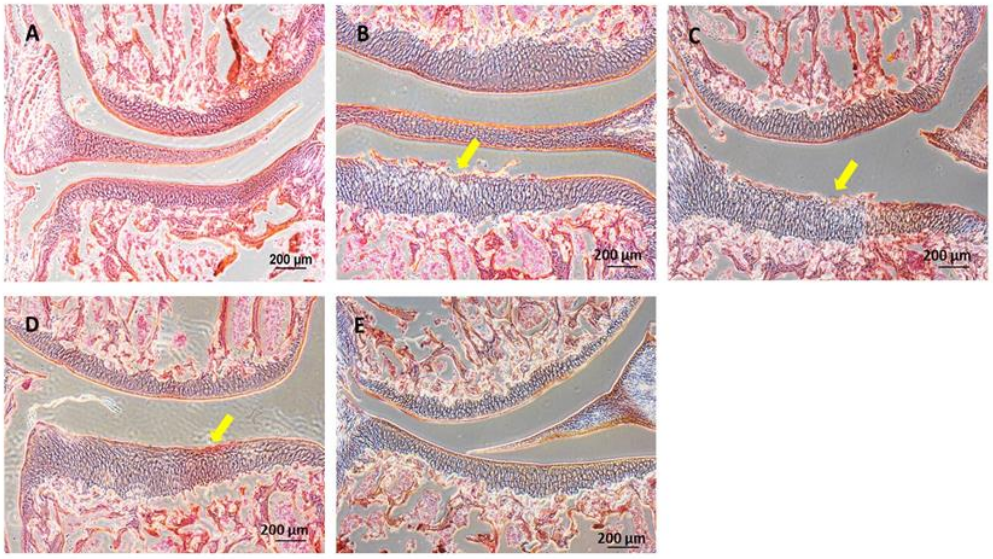


Figure 4.5 Histological examination by H&E staining: (A) group 1; 3 months old, (B) group 2; 7 months old, (C) group 3; 7 months old with HA injection, (D) group 4; 7 months old with HA + MSCs injection, (E) group 5, 7 months old with HA + chondrocyte differentiated cells injection. Scale bar = 200 um.

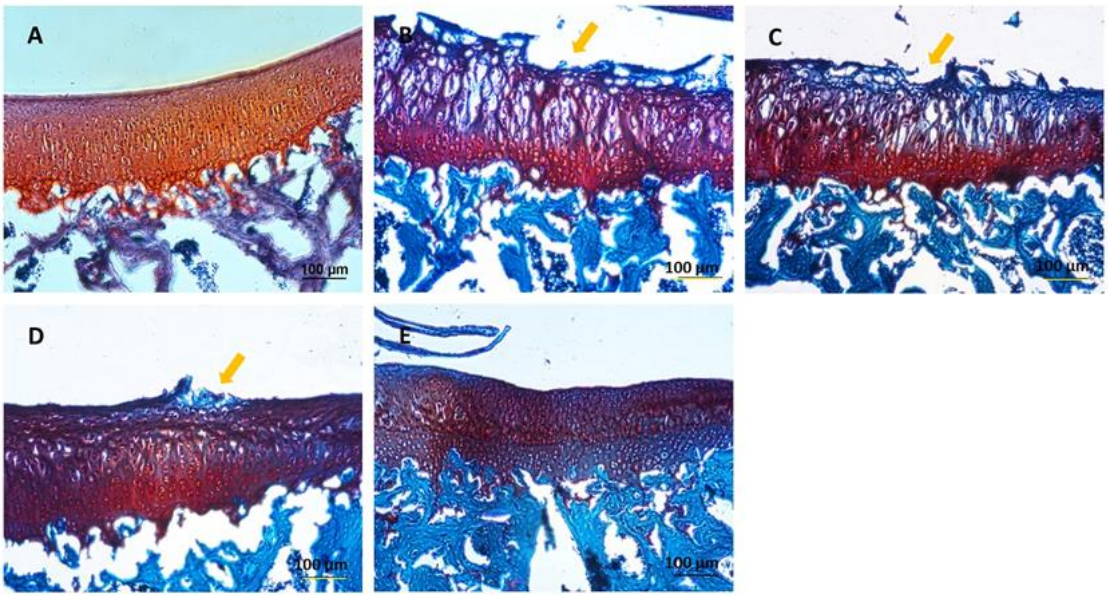


Figure 4.6 Histological examination by Safranin O staining: (A) group 1; 3 months old, (B) group 2; 7 months old, (C) group 3; 7 months old with HA injection, (D) group 4; 7 months old with HA + MSCs injection, (E) group 5, 7 months old with HA + chondrocyte differentiated cells injection. Scale bar = 100 µm.

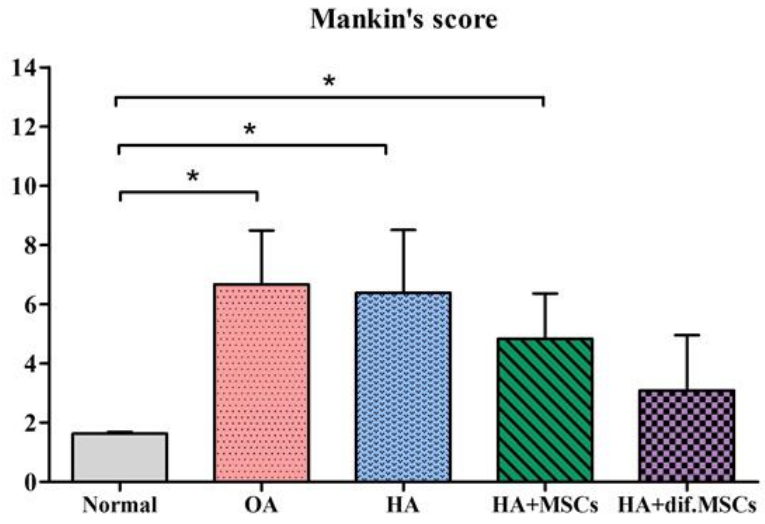


Figure 4.7 Cartilage damage scores based on the Mankin criteria (* $P < 0.01$).

4.4.4 Immunohistochemistry results

collagen (Figure 4.8). The area with a high type II collagen expression will be darkened. The cartilage of the group 1 showed high type II collagen expression, dark colored covering over the cartilage. Even though the cartilage of group 2-5 showed unequal expression, there was no difference between among groups.

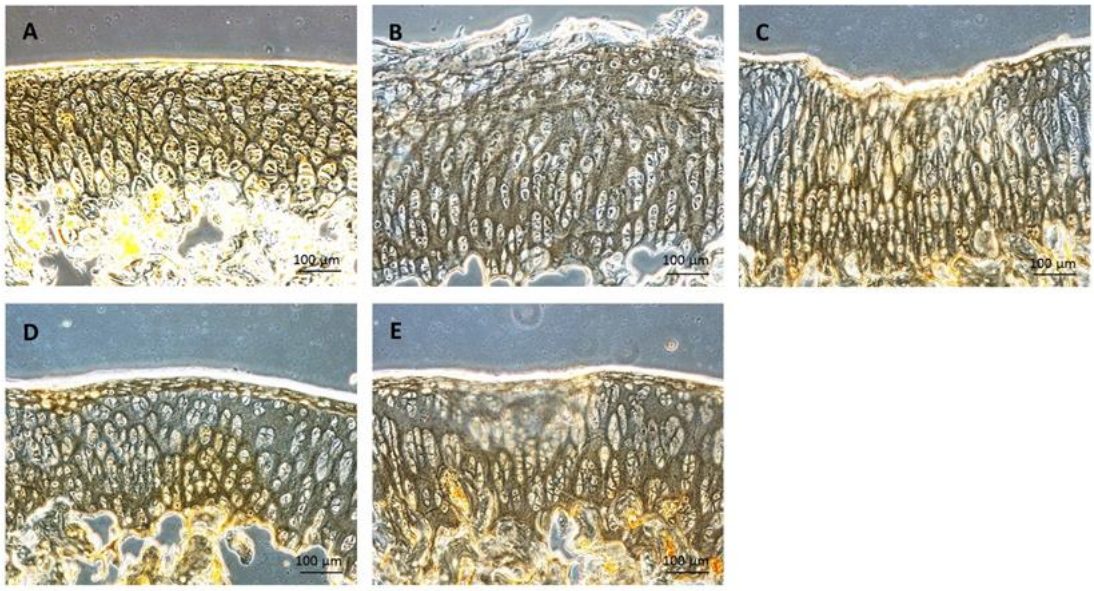


Figure 4.8 Immunohistochemistry for type II collagen: (A) group 1; 3 months old, (B) group 2; 7 months old, (C) group 3; 7 months old with HA injection, (D) group 4; 7 months old with HA + MSCs injection, (E) group 5, 7 months old with HA + chondrocyte differentiated cells injection. Scale bar = 100 um.

4.4.5 Immunoblot results

Total proteins from guinea pig tibia cartilages were extracted and analyzed for type II collagen, type I collagen, MMP13 and β-actin proteins. The results were compared with human knee cartilage. Protein bands isolated by gel electrophoresis and immunoblot are shown in Figure 4.9. Intensity changes of type II collagen, type I collagen and MMP13 versus β-actin protein, acts as an internal control are present in Figure 4.10.

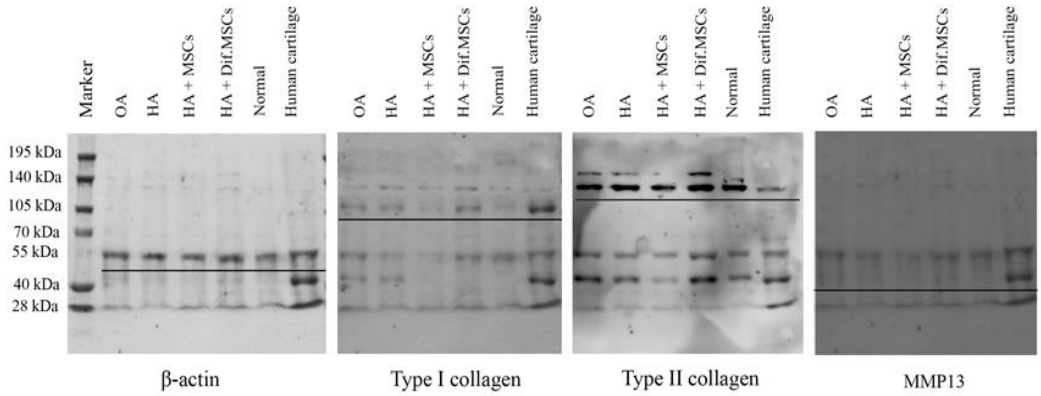


Figure 4.9 Immunoblot analysis after protein bands were isolated by gel electrophoresis.

Intensity changes of Col-II/ β-actin in the cartilage of group 1 were significantly higher than groups 2-3 ($P< 0.05$), but there was no difference among group 1 and group 4 and group 5. The results of the intensity changes of Col-I/ β-actin, on the other hand, revealed no difference in cartilage in all groups. For the results of human cartilage with severe osteoarthritis, the intensity changes of Col-II/ β-actin in cartilage were lower than all groups of guinea pigs. However, the intensity changes of Col-I/ β-actin in human cartilage were greater than in all other groups. MMP13/ β-actin intensity changes were similarly low in all guinea pig groups and lower than in human osteoarthritis cartilage. The highest Col-II/Col-I protein expression was seen in group 1. Col-II/Col-I protein expression in human osteoarthritis cartilage was very low and lower than those in guinea pigs in all experimental groups.

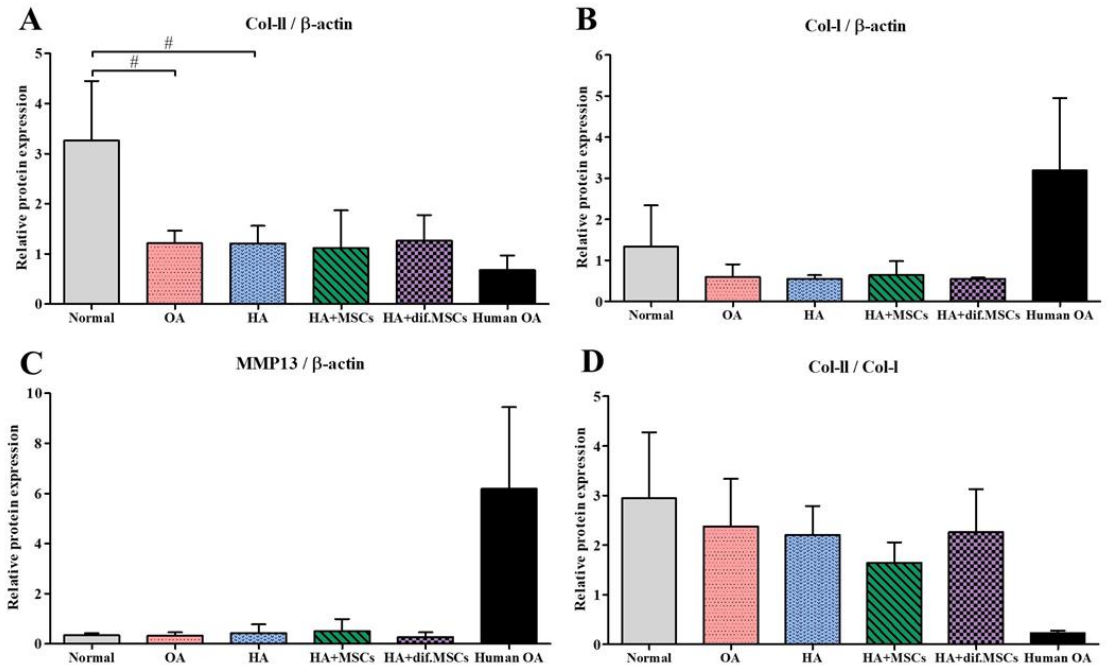


Figure 4.10 (A) Intensity changes of type II collagen, (B) type I collagen and (C) MMP13 proteins in guinea pig cartilage and human cartilage with osteoarthritis, compared with β-actin protein used as an internal control (#$P< 0.05$), and (D) intensity changes of type II collagen/type I collagen in guinea pig cartilage and human cartilage with osteoarthritis.

4.5 Discussion

The current study compared the effects of cell transplantation for knee osteoarthritis between MSCs and MSCs-derived chondrocytes. It was observed that induction of cartilage cells led to the production of hypertrophic chondrocytes, a common type of cartilage cell found in osteoarthritis patients in the long term (Armiento et al., 2019; Ding et al., 2012). In this work, MSCs were induced into cartilage cells in the early stage of chondrogenic differentiation for 14 days. At day 14, the gene expression of Sox9 and β-Catenin increased, while Col10a1 and Runx2, both of which are associated with hypertrophic chondrocyte aging in cartilage, did not express (Armiento et al., 2019; Ding et al., 2012). In this study, Dunkin Hartley guinea pigs were used, as they are prone to develop osteoarthritis with age. The normal articular cartilage of three-month-old guinea pigs was compared to that of seven-month-old guinea pigs with early-stage osteoarthritis (Kraus et al., 2010). The

knees of the guinea pigs were injected with either cell-free HA (Hyruan®III), or with either MSCs or chondrogenic differentiated cells. In the study, it was observed that seven-month-old guinea pigs that received only one injection of cell-free HA did not show any significant difference compared to the not-injected group. HA-based formulations, viscosupplements, are intended to recover the rheological properties of the synovial fluid, resulting in improvement of pain and joint mobility (Salgado et al., 2021). Chemically modified HA improved mechanical performance during high-frequency solicitation and showed a prolonged viscosupplementation effect, compared to the unmodified, linear HA-based product (La Gatta et al., 2021). However, the guinea pigs that received injections of HA containing either MSCs or early cartilage-differentiated cells showed a reduction in osteoarthritis. The injected cells adhered to the cartilage surface, thereby repairing the damaged cartilage, and making it smoother, similar to normal cartilage. Histological analysis showed that the cartilage tissue in the injected cells had a smoother surface compared to the non-injected group. It was concluded that using HA containing early chondrogenic differentiated cells from MSCs could be an effective method for restoring articular cartilage and could be more effective than using MSCs alone for treating osteoarthritis. Inflammatory responses made of xenogeneic/allogenic materials in case of cells or organ transplantation are notable problems. Allogeneic chondrocytes seeded on xenogeneic scaffolds did not suppress graft inflammation, but induced variable inflammatory responses involving mast cells and macrophages (Klabukov et al., 2023). Although allogenic MSCs were shown to support cartilage regeneration and decrease the symptoms of OA (de Windt et al., 2017), the inflammatory responses from xenogeneic MSCs and MSCs-derived chondrocytes remained poor studied and should be further investigated.

4.6 Conclusions

For transplantation, MSCs-derived early stage chondrocytes were intra-articular injected into the knee joints of 7-month old guinea pigs which has symptom of early spontaneous osteoarthritis. After transplantation for 5 weeks, the joints were collected. The results from the injection of MSCs-derived early stage chondrocytes were compared with those from the injection of undifferentiated MSCs and the

injection of Hyaluronic acid. The results revealed that the transplanted cells were integrated into the guinea pig cartilage surfaces and restored the degenerated cartilages. The injection of MSCs-derived early stage chondrocytes recovered the degenerated cartilages better than the injection of undifferentiated MSCs and the injection of Hyaluronic acid. The tissues of the recovered cartilages after the injection of MSCs-derived early stage chondrocytes were resembled to the cartilages of 3-month old guinea pigs, which has no symptom of osteoarthritis, with no significant difference in the Mankin's scores from the histological assessment.

4.7 References

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