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A pilot study of regenerative therapy by implanting synovium-derived mesenchymal stromal cells in equine osteochondral defect models

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Synovium-derived mesenchymal stromal cells (SM-MSCs) from seven Thoroughbreds with naturally occurring intra-articular fracture proliferated to over ten million cells by the second passage. Using three experimental Thoroughbreds, columnar osteochondral defects were made arthroscopically at the bilateral distal radius. Five million allogenic SM-MSCs were implanted into the right defect, and another five million were injected into the right radio-carpal joint (implantation site). No SM-MSCs were implanted into the left defect or the same joint (control site). At 3 and 6 weeks after surgery, ten million autologous SM-MSCs were injected into the right joints. Radiolucent volumes of defects calculated by analysis of postmortem CT images 9 weeks after surgery were decreased in implanted sites compared with control sites in all horses. The average scores for ICRS gross and histopathological grading scales in implanted sites were equal to or higher than those of the controls. These results suggest that allogenic implantation and subsequent autologous injection of SM-MSCs might not obstruct subchondral bone formation in defects.

Key words: *bone, cartilage, regeneration, stromal cell, synovium*

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Mesenchymal stromal cells (MSCs) derived from bone marrow (BM) [1, 13–15] or adipose tissue (AT) [2, 6] have been investigated for the regeneration of both hyaline cartilage and subchondral bone. Of the other candidates, synovium-derived MSCs (SM-MSCs) are of interest clinically because of their susceptibility to chondrogenic differentiation [4, 9]. SM-MSCs are similar to MSCs derived from synovial fluid (SF) in terms of abundant cartilage matrix production in culture plates following chondrogenic induction [5]. It has been hypothesized that these MSCs would be preferable candidates to treat articular cartilage defects. To apply the MSC therapy in racehorses, it should first be scientifically demonstrated that MSC therapy can promote recovery of fibrous or hyaline cartilage and subchondral bone. Therapeutic MSCs should be also supplied in quick response to the patient's demands. Previous studies using experimental animal models have suggested that autologous or allogenic implantation of SM-MSCs could be helpful to recover osteochondral defects with fibrocartilage or hyaline cartilage [8, 11, 12]. In general, autologous cell therapy requires a "two-step surgery" consisting of a first surgery to collect the autologous cells and a second surgery to implant the cultured cells. This might be unacceptable for actual racehorse practice owing to the repetitive invasion of general anesthesia and surgery. Therefore, the aim of this study was to investigate whether a "one-step surgery" procedure consisting of surgical implantation of allogenic SM-MSCs and postsurgical injection of autologous SM-MSCs is possible and helpful for tissue regeneration in experimentally induced osteochondral defects in horses.

Samples of synovium (SM) (Fig. 1a) were arthroscopically obtained from seven Thoroughbreds with intra-artic-

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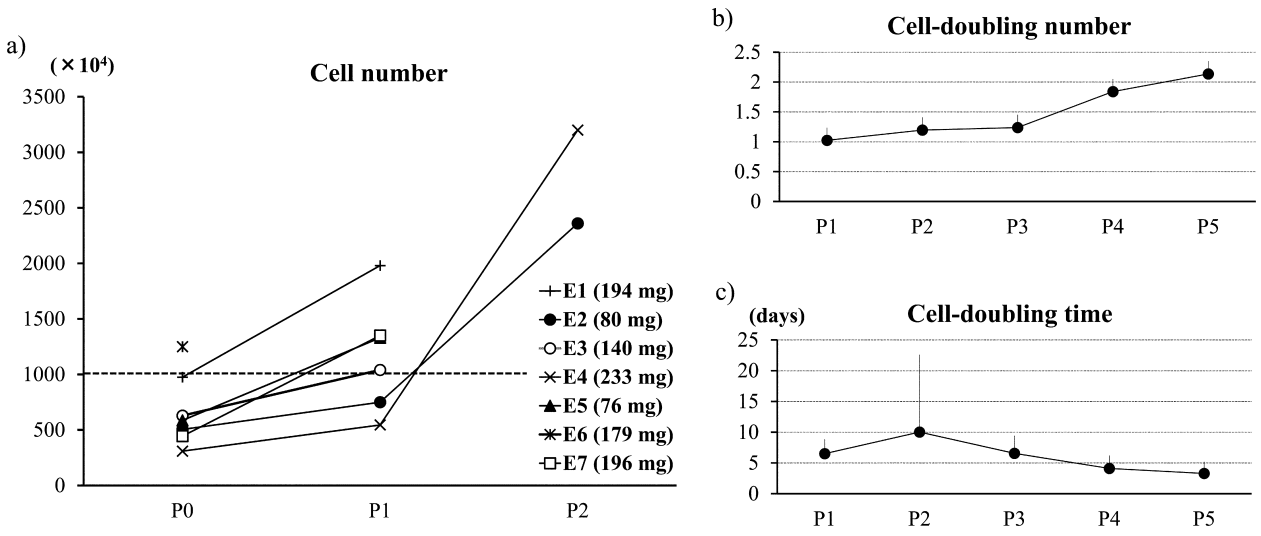

Fig. 1. Growth curves (a) of SM-MSCs derived from IAF joints of 7 Thoroughbreds (E1 to E7). Values in parentheses indicate weights (mg/joint) of synovial samples. Cell-doubling numbers (b) and cell-doubling times (c) from passages 0 to 5 (P0–P5) of the cells (data are presented as mean+SD). Cell-doubling number=ln (Nf/Ni)/ ln (2). Nf, final number of cells; Ni, initial number of cells. Cell-doubling time=cell culture time/cell-doubling number.

ular fracture (IAF) (5 males and 2 females, 2 to 5 years of age) for analysis of MSC proliferation, cell surface antigens, and multipotency. One hundred milligrams of SM was also obtained from another case of IAF (3 year-old, male Thoroughbred) to expand MSCs for allogenic implantation. SM was digested in 20 m*l* of phosphate-buffered saline (PBS) containing 0.1% collagenase (Collagenase Type I, Worthington Biochemical, Lakewood, NJ, U.S.A.) at 37°C for 90 min before filtering through a 70-μm-pore nylon membrane (Cell Strainer, BD, Franklin Lakes, NJ, U.S.A.) and centrifuging at 160 × g for 5 min at room temperature. After decanting the supernatant, the pellet was resuspended and plated onto a 150-cm^2 culture dish (Tissue Culture Dish Φ150, TPP, Trasadingen, Switzerland) in complete culture medium (CCM): Dulbecco's Modified Eagle's Medium (Life Technologies, Carlsbad, CA, U.S.A.) containing 10% fetal bovine serum (FBS, Thermo Fisher Scientific, Waltham, MA, U.S.A.) and 1% antibiotic-antifungal preparation (100 U/m*l* Penicillin G, 100 μg/m*l* streptomycin, 0.25 μg/m*l* amphotericin B; antibiotic-antimycotic, Life Technologies). CCM was only added on day 3 and then not changed for 6 days (Passage 0, P0). Following incubation at 37°C with 5% CO_2 for 6 days, cells that adhered to the bottom of the flask were washed with PBS and harvested with 0.05% Trypsin and 0.2 mM EDTA (Trypsin-EDTA, Life Technologies). After centrifugation, the supernatant was removed, and cells were replated at a density of 1.0 × 10^6 cells in 150-cm^2 dishes to be cultured for 6 days. The medium was changed every 3 days for 6 days (P1). This serial process of passage was repeated until the number of cells reached the required quantity. Numbers of cells were determined at every passage with a cell counter (TC10, BioRad, Hercules, CA, U.S.A.) to determine the proliferation rates of cells, which were calculated as the cell-doubling number, cell-doubling time, and daily duplication rate. Immunological surface markers and multipotency of cells were analyzed at P5. Ten thousand cells were resuspended in 500 μ*l* of staining buffer (SB; PBS containing 1% FBS) and incubated for 30 min at 4°C with 20 μg/m*l* of antibodies (mouse immunoglobulin G) against CD11a/18 (gifted), CD34 (BD), CD44 (AbD Serotec, Kidlington, U.K.), CD45 (BD), CD90 (BD), CD105 (AbD Serotec), MHC class I (gifted), and MHC class II (gifted). Mean fluorescence intensity (MFI) of cells was evaluated by flow cytometry, as previously reported [5, 7]. Total RNA from the cultured cells was isolated and converted to cDNA by RT. The PCR primers and the expected sizes of products for the multipotency marker (sex determining region Y-box 2, Sox2) and the induction marker genes were presented in a previous study [5].

Three experimental Thoroughbreds (3- and 5-year-old males, 4-year-old female) with no joint diseases were placed under general anesthesia by isoflurane inhalation following induction with 2 mg/kg of ketamine HCl (Ketalar, Daiichi Sankyo Propharma, Tokyo, Japan) and premedication with 5 μg/kg of medetomidine HCl (Domitor, Zenoaq, Koriyama, Japan) and 10 μg/kg of butorphanol tartrate (Vetorphale, Meiji Seika, Tokyo, Japan). Columnar osteochondral defects (6-mm diameter and 5-mm depth) were made arthroscopi-

Table 1. Percentages (%) of positive cells with specific molecular markers by flow cytometry

Molecular markers	CD44	CD90	MHC-I	CD11a/CD18	CD105	MHC-II	CD34	CD45
Positive cells (%)	94.5	94.4	93.7	80.4	64.4	83.6	0.2	0.1

cally at the medial part of the bilateral distal radius with a drill bit (6-mm diameter), and approximately 120 mg of synovium was collected for isolation of autologous MSCs. Of the suspension of 1.0×10^7 allogenic SM-MSCs in 1 m*l* of saline prepared prior to the arthroscopic surgery, 5.0×10^6 allogenic SM-MSCs were implanted into the osteochondral defect at the right distal radius with the joint space inflated by air (which remained stationary for 10 min [10]), while the remaining 5.0×10^6 were injected into the right radio-carpal joint space just after the skin suture (implantation site). The left osteochondral defect and radio-carpal joint were not treated with SM-MSCs (control site). All horses were postoperatively allowed to move freely in their stables. Flunixinmeglumine (a single daily dose of 1 mg/kg for 4 days after surgery, Banamine, DS Pharma Animal Health, Osaka, Japan) and Kanamycin (a single daily dose of 5 g for 3 days after surgery, Kanamy Inj.250, Fujita Pharmaceutical, Tokyo, Japan) were used. At 3 and 6 weeks after the surgery, 1.0×10^7 autologous SM-MSCs suspended in 1 m*l* of saline were injected into the right radio-carpal joint. Nine weeks after the surgery, experimental horses were arthroscopically evaluated for osteochondral defects and then were euthanized by, as generally recommended, administration of an overdose of general anesthetic and depolarizing muscle relaxant. Both forelimbs were also examined for osteochondral defects with a CT scanner (Toshiba, Tokyo, Japan). Multiplanar reconstruction allowed 2-D images of bone defects to be created from the original axial plane in either the coronal, sagittal, or oblique plane. A series of sagittal images with 0.5-mm slice thicknesses were used to quantify the radiolucent volume (RV, mm^3) of defects, which was calculated as an integrated value of radiolucent areas of each 2-D sectional image. Macroscopic evaluation of osteochondral defects was scored in accordance with the International Cartilage Repair Society (ICRS) gross grading scale. Finally, both distal radiuses were fixed in 10% neutral-buffered formalin for 2 weeks and then longitudinally sectioned parallel to the sagittal plane. Blocks of tissues were embedded in paraffin following decalcification with formic acid for one month. Serial sections (5-μm thick) were placed on glass slides and evaluated with safranin O staining. Histopathological findings were also scored with the ICRS histological grading scale. The above procedures were approved by the Animal Care and Use Committee of Japan Racing Association (approval number 2015-3; date of approval April 15th, 2015).

The cells adhering to the bottom of culture flasks were spindle shaped and highly proliferative reaching to over 1.0×10^7 cells by P2 (Fig. 1a). The cell-doubling numbers (means) were 1.02, 1.20, 1.24, 1.84, and 2.14 at P1, P2, P3, P4, and P5, respectively (Fig. 1b). The cell-doubling times (means) were 6.51, 10.02, 6.56, 4.11, and 3.29 days at P1, P2, P3, P4, and P5, respectively (Fig. 1c). According to the results of flow cytometry (Table 1), the cells presented evidently positive MFI shifts with antibodies against CD44 and CD90, as well as MHC class I. In contrast, positive signals for CD11a/18, CD105, and MHC class II were weak, and no signal was detected for CD34 or CD45. Genetic expression of Sox2 in the cells was confirmed by RT-PCR (Fig. 2a). Following osteogenic induction, the cells positively expressed Runx2, ALP, and OC (Fig. 2b). Following chondrogenic culture in plates, the cells expressed Agg, Col-II, and Sox9 (Fig. 2c). During tenogenic differentiation, the cells expressed Scx and TenC (Fig. 2d).

RV values were decreased in implanted sites compared with controls in all horses (Table 2). The average score for the ICRS gross grading scales in the implantation sites were equal to or higher than in controls (Table 2). In horse No. 1, the osteochondral defect was completely filled with yellowish thick fibrous tissue in the implantation site (Fig. 3a), but small thin fibrous tissue was located over the bleeding bone in the control site (Fig. 3d). In horse No. 2, subchondral bone was more widely covered with thin fibrous tissue in the implantation site (Fig. 3b) than in the control site (Fig. 3e). In horse No. 3, subchondral bone was widely covered with thin fibrous tissue in both sites (Fig. 3c and 3f), but the defect was extended in the control site (Fig. 3f). The average score for the ICRS histological grading scale in the implantation sites were also equal to or higher than in the controls (Table 2). Histopathology in horse No. 1 showed that the subchondral defect was decreased in the implantation site (Fig. 3g), in contrast to the extended bone loss observed in the control site (Fig. 3j). In horse No. 2, subchondral bone loss was also reduced in the implantation site (Fig. 3h) compared with the control site (Fig. 3k). However, this definitive difference was not seen in horse No. 3 (Fig. 3i and 3l). The recovery of hyaline cartilage in the joint surface was not satisfactory.

In this study, equine mesenchymal stromal cells isolated from synovium taken from either injured or clinically sound joints showed a consistent pattern of CD marker expression, as previously reported for SF-MSCs [5]. The

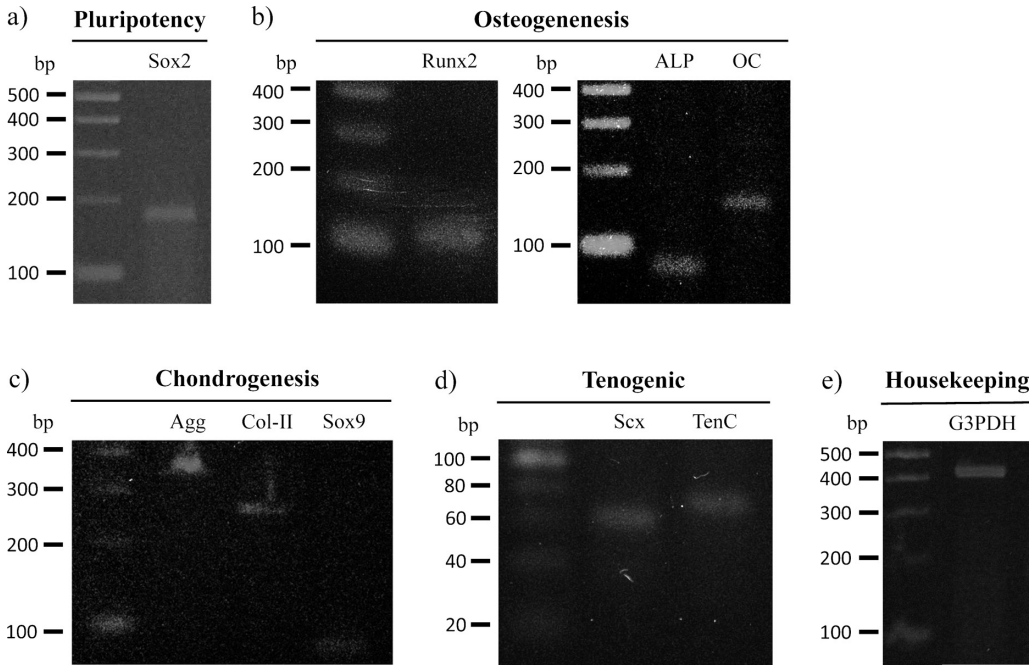

Fig. 2. Gene expression of SM-MSCs. Sox2, sex determining region Y-box 2; Runx2, runt-related transcription factor 2; ALP, alkaline phosphatase; OC, osteocalcin; Agg, aggrecan; Col2, type II collagen; Sox9, sex determining region Y-box 9; Scx, scleraxis; TenC, tenascin C; G3PDH, glyceraldehyde-3-phosphate dehydrogenase.

Table 2. Radiolucent volume (RV) values and ICRS grading scale scores of osteochondral defects at 9 weeks after the surgery

	Horse No. 1		Horse No. 2		Horse No. 3	
	RF	LF	RF	LF	RF	LF
Radiolucent volume (mm^3)	34.9	68.7	51.5	69.3	78.3	104.2
ICRS gross grading scale scores: average (0–4)	1.75	0.75	0.5	0.5	1.0	0.75
ICRS histological grading scale scores: average (0–3)	1.0	0.67	0.5	0.33	1.0	1.0

RF, right forelimb is the implantation site; LF, left forelimb is the control site.

synovium-derived cells also expressed the pluripotent marker Sox2, proliferated to over ten million cells before the second passage, and differentiated into chondrogenic, osteogenic, and tenogenic cells in response to specific periods of culture with special nutrient conditions. These results suggest that synovium-derived stromal cells are well-qualified SM-MSCs. As chondrogenic differentiation induction in plate culture generated a gelatinous sheet that was intensely stained with Alcian blue (data not shown), these MSCs could be highly susceptible to cartilage differentiation and useful for cell therapies aimed at cartilage regeneration.

This study was a trial run of a SM-MSC therapy for post-surgical osteochondral defects in racehorses. It has been well established that autologous cell implantation is more suitable than allogenic methods in terms of avoiding immunological rejection, in particular because these cells are positive for MHC class I and II antigens. Accordingly, in this study, autologous synovium was collected at the time of arthroscopic surgery to create the defects, and then SM-MSCs were isolated. Of note, the autologous SM-MSCs expanded to over 1×10^7 cell between passages 0 and 2 (within 3 weeks of culture) from approximately 120 mg of synovium, and 1×10^7 autologous SM-MSCs were injected at both 3 and 6 weeks after the surgery. The number of MSCs used was assumed to be sufficient to control the progression of postsurgical OA, as it was previously reported that 8–9 $\times 10^6$ BM-MSCs were effective to repair osteochondral defects in human knee OA [3]. Although the number of experiments was too limited to indicate the significant therapeutic effects in this study, intra-articular injection of autologous SM-MSCs within 3 weeks after arthroscopic

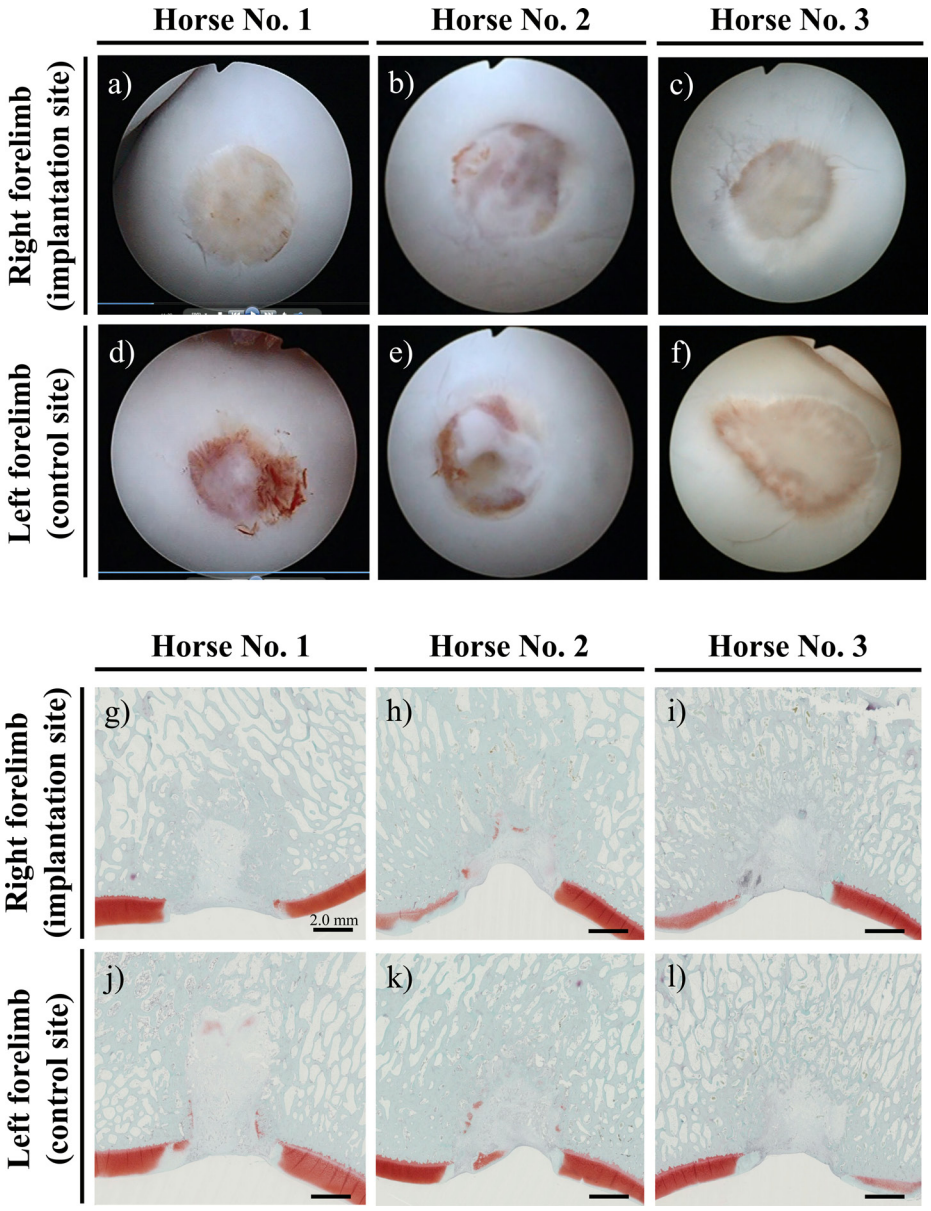

Fig. 3. Arthroscopic findings of osteochondral defects and histopathology of osteochondral defects by safranin O staining.

ablation of osteochondral fragments should be feasible and acceptable for clinical use in racehorses.

Nevertheless, the need to culture SM-MSCs for a few weeks to increase their numbers remains a limiting factor of autologous implantation, and therefore allogenic implantation was also tested in this study. Allogenic implantation of SM-MSCs has been reported to promote the recovery of osteochondral defects with fibrocartilage in an experimental animal model [12]. In this study, the right defects in which allogenic SM-MSCs were directly implanted showed no clinical and pathological adverse signs compared with the controls. Nine weeks after the surgery, the RV values of all horses were decreased in the implantation sites as compared with the control sites. This result suggests that allogenic implantation and subsequent autologous injection of SM-MSCs might not obstruct subchondral bone formation in osteochondral defects. However, the following limitations are undeniable: 1) more horses are required to demonstrate a statistical difference in RVs, and 2) time-dependent changes should be presented in terms of postsurgical RVs, using antemortem inspection with CT scans. Cartilage recovery was evaluated by the ICRS gross grading scale, in which coverage of defects in the implantation sites (scores of 4, 1, and 2 for Horse Nos. 1, 2, and 3, respectively) was equal

to or higher than in the control sites (scores of 3, 1, and 1 for Horse Nos. 1, 2, and 3, respectively). Based on the histopathology at the center of cylindrical defects, defects were mainly covered with fibrous tissue and fibrocartilage in both sites. In conclusion, allogenic implantation and subsequent autologous injection of SM-MSCs into osteochondral defects of thoroughbreds might be helpful for subchondral bone formation but does not promote the regeneration of hyaline cartilage.

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