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The Optimal Isolation and Characterization Conditions of Rabbit Dental Pulp-Derived Mesenchymal Stem Cells for Use in Regenerative Medicine

Salkin H[1,2,3]*, Gonen ZB[3,4], Ozcan S[3], Bahar D[3], Kutuk N[5] and Alkan A[5]

[1]Department of Pathology Laboratory Techniques, Beykent University, Turkey

[2]Department of Histology and Embryology, Erciyes University, Turkey

[3]Genome and Stem Cell Center, Erciyes University, Turkey

[4]Department of Oral and Maxillofacial Surgery, Erciyes University, Turkey

[5]Department of Oral and Maxillofacial Surgery, BezmiAlem University, Turkey

Research Article

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***Corresponding author:** Hasan Salkin, Department of Pathology Laboratory Techniques, Vocational School, Beykent University, Büyükçekmece/Istanbul, Turkey, Tel: +905301255808; Email: hasansalkin@beykent.edu.tr

Abstract

Background and Objectives: Currently, dental pulp stem cells have gained importance in stem cell research. Our aim was to isolate mesenchymal stem cells from the rabbit dental pulp and to characterize those using different methods.

Methods: Mesenchymal Stem Cells (MSCs) were obtained by enzymatic method from dental pulp of New Zealand Rabbits. MSCs that were derived from rabbit dental pulp (rabbit DPSCs) were characterized by using flow cytometry, immunofluorescence staining, quantitative Real Time PCR (qRT-PCR). For the characterization, CD29, CD44 and CD45 MSC markers were used. Adipogenic, osteogenic and chondrogenic differentiation tests were performed for multilineage differentiation. The clonogenic properties of the obtained rabbit DPSCs were demonstrated by the Colony forming units-fibroblast (CFU-F) Test. The results were analyzed statistically.

Results: Flow cytometry, immunofluorescence staining and qRT-PCR analysis showed that rabbit DPSCs were positive for CD29 and CD44 antibodies; and negative for CD45 antibody ($p<0.001$). Adipogenic, osteogenic and chondrogenic differentiation was demonstrated by adipo-red, alizarin red, alkaline phosphatase and safranin-O positive staining. In the CFU-F test, 127.7±13.98 colonies and 211.3±10.40 colonies were counted at 500 cells/well and 1000 cells/well, respectively ($p<0.001$).

Conclusion: rabbit DPSCs have been successfully isolated from rabbits; and it has been proven that the cells obtained are MSCs. It has been determined that there are high potency cells according to other stem cell sources. Rabbit is a model organism frequently used in *in vivo* studies. Hence, rabbit dental pulp stem cells can be used in both *in vitro* and *in vivo* clinical, surgical and tissue engineering studies.

Keywords: Dental pulp-derived stem cell; Rabbit; Isolation; Characterization; Differentiation

Abbreviations: MSCs: Mesenchymal Stem Cells; DPSC: Dental Pulp Stem Cells; FBS: Fetal Bovine Serum; RFU: Relative Fluorescence Units; CFU-F: Colony Forming Units-Fibroblast.

Introduction

MSCs can be isolated from various adult tissues such as bone marrow, synovium fluid, periosteum, adipose tissue, umbilical cord, and dental pulp [1]. MSCs have the ability to differentiate osteoblasts, chondrocytes and adipocytes under appropriate conditions, and these properties are called plasticity [2]. These cells are multipotent progenitor cells, and have the ability to differentiate into many cell types including adipocytes, osteoblasts and chondrocytes [3]. The stem cells in the dental pulp are predominantly characterized by MSCs. Dental pulp stem cells were first isolated in 2000 by Gronthos, et al. [4] from the third molar permanent teeth. In 2003, Miura, et al. [5] reported that they isolated stem cells from human milk teeth (SHED). The complex mechanism of odontogenesis and that of the dental germs are composed of two different embryonic layers, which are ectomesenchyme and ectoderm dental pulp-derived MSCs. Dental pulp stem cells are similar to primitive stem cells in umbilical cord gelatinous structure. Dental pulp-derived MSCs are a new research topic in the literature in terms of biological behavior and potential for differentiation [6]. Dental pulp tissue is thought to be derived from migrating neural crest cells during development; and multipotent/progenitor stem cells have been shown in various populations [7]. Other sources of dental stem cells besides pulp include apical papilla, periodontal ligament, and dental follicle [8-10]. Dental Pulp Stem Cells have a distinct advantage over other sources of dental stem cells due to their characteristics such as ease of access, their immature nature, and wide repertoire of differentiation. In 2010, Karaöz, et al. [11] examined the isolation and *in vitro* characterization of Dental Pulp Stem Cells from human natal teeth in detail, and compared the characteristics of bone marrow MSCs with human natal dental pulp stem cells. Dental Pulp Stem Cells (DPSC) were found to be very successful in terms of adipogenic, osteogenic, myogenic, chondrogenic and neurogenic differentiation. Differentiation of Dental Pulp Stem Cells is regulated by various regulators that regulate osteogenesis, TGF-β superfamily and various cytokines [12]. Dental Pulp Stem Cells have been isolated from various sources in the literature [13,14]; however, studies on the methods used in isolation and characterization of rabbit DPSCs, which is a model organism for orthopedics, maxillofacial surgery and regenerative medicine applications, have been limited. The aim of this study was to isolate mesenchymal stem cells from the rabbit dental pulp and to characterize them using different methods.

Materials and Methods

Animals

The teeth were obtained from 4 six-week-old New Zealand rabbits, each weighing 1.2 kg. The rabbits were obtained from Erciyes University Hakan CETINSAYA Experimental and Clinical Research Center (DEKAM). (Ethics Board Permission was obtained from Erciyes University, Animal Experiments Local Ethics Committee Presidency with decision number 13/91).

Isolation and Culture of Rabbit Dpscs

The lower and upper incisors of the rabbits were pulled out, and the gingival tissues were removed. The pulp tissue was removed from the tooth with the help of sterile tirnerf and taken to the culture dish. The pulpal tissue obtained was exposed to 5 mg / ml dispase (Stem Cell Technologies, #07913, USA) and 2.5 mg/ml collagenase type 1 (Serva, #1745401, Germany). The pulp tissue was thoroughly enzymatically dissolved for 30 min in a gently shaking incubator at 37°C until a single cell suspension was formed. The cells were centrifuged to remove collagenase and dispase. Cells were cultured in nucleoside Alpha MEM (Biochrom, #F0915, USA) supplemented with ascorbate-2-phosphate (Sigma, #A8960, Germany) containing 20% Fetal Bovine Serum (FBS) (Biological Industries, #04-001-1A, Israel Beit Haemek) and 100 IU/ml penicillin-100 µg streptomycin (Lonza, #17-602E, USA). Cells from one tooth were placed on two 25 cm^2 plastic tissue culture flasks and were incubated for 3 days in a 5% CO2 incubator containing 37°C humid atmospheres. On the third day, non-adherent cells were removed and fresh medium added to the growing cells. When the density of the adherent cells in the primary culture was 70-80%, they were passaged. For

passaging, the cells were washed with Ca^{2+} and Mg^{2+} free PBS (Phosphate Buffer Saline) (Lonza, #17-516Q, USA) and were incubated with 0.25% trypsin-EDTA (Lonza, #CC-5012, USA) solution for 3 min at 37°C. At least twice as much serum-containing growth medium was added to inhibit trypsin. The cells were then centrifuged at 350 x g for 5 min to allow pellet formation. The supernatant was discarded and the cells resuspended with the Growth medium (alpha MEM with nucleoside, 20%FBS, 1% 100 IU/ml penicillin-100 µg streptomycin) and counted on the Thoma slide. It was then seeded in $75cm^2$ tissue culture flasks at a density of $1x10^6$cells/flask. Growth medium (alpha MEM with nucleoside, 20%FBS, 1% 100 IU/ml penicillin-100 µg streptomycin) was changed every three days in the 10-14-day period and cells were passaged as confluent.

Flow Cytometry Analyses

After Passage 3 (P3), the rabbit DPSCs were removed from the flasks by trypsinization, and were washed twice with Ca^{2+} and Mg^{2+} containing PBS (Biological Industries, #02-020-1A, Israel Beit Haemek). To study the antibodies of CD29-FITC Conjugated (EMD Millipore Corp., #FCMAB269F, USA), primer antibody CD44 (Bio-Rad Lab., Inc., #MCA2504, USA) and primer antibody CD45 (Bio-Rad Lab., Inc., #MCA808GA, USA), cells were gathered in PBS containing Ca^{2+} and Mg^{2+} (Biological Industries, #02-020-1A, Israel Beit Haemek) at $1x10^6$ cells in three tubes. The cells were fixed with 4% paraformaldehyde (Merck #104005, USA) and incubated at room temperature for 10 min. After the incubation, the cells were washed twice with PBS containing Ca^{2+} and Mg^{2+}. Antibodies were added to the cells as the CD29-FITC Conjugated antibodies (EMD Millipore Corp., #FCMAB269F, USA), CD44 primer antibodies (Bio-Rad Lab., Inc., #MCA2504, USA) and CD45 primer antibodies (Bio-Rad Lab., Inc., #MCA808GA, USA) with 1:100 dilution, and were incubated at room temperature. The cells that were treated with CD29-FITC conjugated antibody (EMD Millipore Corp., #FCMAB269F, USA) were washed twice after the incubation with PBS containing Ca^{2+} and Mg^{2+} (Biological Industries, #02-020-1A, Israel Beit Haemek), and were then subjected to direct flow cytometry analysis. The cells that were treated with CD44 (Bio-Rad Lab., Inc., #MCA2504, USA) and CD45 (Bio-Rad Lab., Inc., #MCA808GA, USA) primer antibodies were centrifuged after the incubation, and Goat anti-Mouse secondary antibody (Abcam, #ab7064, UK) was added. The cells were then incubated with the FITC labeled secondary antibody in the dark for 30 min. At the end of the incubation, the cells were washed twice. Flow cytometry was performed using FACS Canto II (BD Biosciences, San Diego, USA).

Immunocytochemistry Analyses

The selected antibodies for flow cytometry were also selected from antibodies available for immunocytochemistry. For immunophenotyping cells, the samples were washed twice with PBS containing Ca^{2+} and Mg^{2+} (Biological Industries, #02-020-1A, Israel Beit Haemek), and were fixed with 4% paraformaldehyde (Merck #104005, USA) for 10 minutes. The cells were then incubated for 30 minutes at 37°C to block nonspecific binding of immunoglobulins with serum (Biological Industries, #04-001-1A, Israel Beit Haemek); and were washed twice with PBS containing Ca^{2+} and Mg^{2+} (Biological Industries, #02-020-1A, Israel Beit Haemek), and were incubated overnight at 4°C with primary antibodies: CD29-FITC Conjugated antibodies (EMD Millipore Corp., #FCMAB269F, USA), CD44 primer antibodies (Bio-Rad Lab., Inc., #MCA2504, USA) and CD45 primer antibodies (Bio-Rad Lab., Inc., #MCA808GA, USA). The cells were then washed 3 times with PBS containing Ca^{2+} and Mg^{2+} (Biological Industries, #02-020-1A, Israel Beit Haemek), and were incubated for 25 min in the dark with secondary antibody labeled with fluorescent dyes such as FITC (Goat anti-Mouse secondary antibody) (Abcam, #ab7064, UK). It was then washed 3 times with PBS and the nuclear regions of the cells were labeled with DAPI (Thermo Scientific #D1306, UK). After 24 hours, the cells were visualized on a fluorescence microscope (Nikon Eclipse Ti-E, Japan).

RNA Isolation and Quantitative Real Time PCR

Once the $1x10^6$ MSC in passage 4 was washed with Ca^{2+} and Mg^{2+} free PBS (Phosphate Buffer Saline) (Lonza, #17-516Q, USA), Total RNA Isolation Reagent (Trizol) (Roche, #11667157001, Germany) was added and incubated for 5 min at room temperature. Chloroform (Merck #107024, USA) was then added to the cells and centrifuged at 12.000 *g* for 15 min. After the centrifugation, the RNA collected in the aqueous phase was pipetted into another tube, and was incubated at room temperature with isopropanol (Sigma, #278475, USA). After the incubation, it was centrifuged at 12.000g for 10 min, and 70% ethanol (Merck, #100983, USA) was added to the pellet that was formed after the centrifugation and vortexed thoroughly. It was centrifuged at 7500g for 5 min. The resulting supernatant was discarded, the pellet was dried and nuclease-free water (Roche, #03315843001, Germany) was added and the RNA concentration was measured at 260 nm with a

NanoDrop spectrophotometer (Thermo Scientific, #ND1000, UK). Roche High Fidelity cDNA Synthesis Kit (Roche, #05081963001, Germany) was used for cDNA synthesis. CD29, CD44 and CD45 gene primers were designed by Roche Company (Roche, Germany) and expressions were normalized to housekeeping gene GAPDH, and the MSC surface markers were evaluated at the gene expression level. The information about the genes is given in Table 1.

Name of Gene	5'- sequence-3'	NCBI Gene ID (Oryctolagus cuniculus)
CD29 (ITGB1-integrin subunit beta 1) CD44-CD44 molecule	*F: AGAATGTCACCAACCGTAGCA*	100008898
	R: CACAAAGGAGCCAAACCCA	
	F: GGATGGCACCCGCTACA	100301546
	R: GGAGACCCACTGCTCACG	
CD45 (PTPRC-protein tyrosine phosphatase receptor type C)	*F: TACTCTGCCTCCCGTTG*	100358807
	R: GCTGAGTGTCTGCGTGTC	
GAPDH (glyceraldehyde-3-phosphate dehydrogenase)	*F: TTTGTGATGGGCGTGAAC*	*100009074*
	R: CCCTCCACAATGCCGAAGT	

Table 1: The information and sequences of the gene primers studied.

Colony Forming Units-Fibroblast (CFU-F) Assay

CFU-F assay was performed to demonstrate the clonogenic properties of the obtained rabbit DPSCs. The cells were seeded in 9.6 cm^2 petri dishes at 500 cells/well and 1000 cells/well, and were cultured in growth medium (nucleoside Alpha MEM (Biochrom, #F0915, USA) supplemented with ascorbate-2-phosphate (Sigma, #A8960, Germany) containing 20% Fetal Bovine Serum (FBS) (Biological Industries, #04-001-1A, Israel Beit Haemek) and 100 IU/ml penicillin-100 µg streptomycin (Lonza, #17-602E, USA)). for 2 weeks. At the end of two weeks, the cells were stained with crystal violet (Sigma, #C0775, USA), the colonies were displayed and the colony counts were determined. The staining process was briefly carried out as follows. Media was removed from petri dishes and 0.05% crystal violet was added. Petri dishes were incubated for 20 min at room temperature. Petri dishes were washed in tap water. The stained colonies were counted.

Adipogenic Differentiation

The cells in P3 were seeded in 6-well plates (30.000cell/cm2). Upon reaching 90-100% confluency, the growth medium (nucleoside Alpha MEM (Biochrom, #F0915, USA) supplemented with ascorbate-2-phosphate (Sigma, #A8960, Germany) containing 20% Fetal Bovine Serum (FBS) (Biological Industries, #04-001-1A, Israel Beit Haemek) and 100 IU/ml penicillin-100 µg streptomycin (Lonza, #17-602E, USA)) of the cells was replaced with adipogenic differentiation medium. For this, hMSC mesenchymal stem cell adipogenic induction medium containing insulin, L-glutamine, dexhametasone, indomethacin, IBMX (3-isobuty-l-methyl-xanthine), GA-1000 (Lonza MD, #PT-3102B, USA) and Adipogenic continuing medium containing insulin, L-glutamine, GA-1000 (Lonza MD, #PT-3102A, USA) were used. Firstly, the cells were cultured with induction medium for 3 days, and on the next 3 days, they were cultured with adipogenic continuing media. This cycle was repeated 3 times for 21 days and stimulated for 21 days by replacing with fresh medium every three days. The resulting lipid droplets and triglycerides in the cells were stained with AdipoRed™ (Lonza, #PT-7009, USA) according to the product protocol. Immediately prior to the assay, each plate were rinsed with PBS, and were added AdipoRed™, using the volume 140µl in 5ml PBS. The plate was incubated for 10 to 15 minutes and, was read using relative fluorescence units (RFU) in a microplate reader (Promega, USA).

Osteogenic Differentiation

P3 cells were seeded on 6-well plates coated with 0.1% gelatin (3000 cell/cm2) in growth medium (nucleoside Alpha MEM (Biochrom, #F0915, USA) supplemented with ascorbate-2-phosphate (Sigma, #A8960, Germany) containing 20% Fetal Bovine Serum (FBS) (Biological Industries, #04-001-1A, Israel Beit Haemek) and 100 IU/ml penicillin-100 µg streptomycin (Lonza, #17-602E, USA)). After 24 hours, the growth media of the cells were replaced with hMSC Mesenchymal Stem Cell Osteogenic differentiation medium containing dexamethasone,

ascorbate, L-glutamine, penicillin/streptomycin, β-glycerophosphate (Lonza, #PT-3002, USA) and stimulated for two weeks by replacing with fresh osteogenic differentiation medium every three days. The calcium deposits in the differentiated cells were stained with Alizarin Red (Sigma, #A5533, USA) and alkaline phosphatase (System Biosciences, #AP100B-1, US).

Chondrogenic Differentiation

The cells were transferred to chondrogenic differentiation in vitro. A hMSC Chondrogenic Differentiation Basal Media and Chondrogenic Singlequots Kit (Lonza, #PT-3003, USA) containing ITS premix, proline, sodium pyruvate, dexamethasone, L-glutamine, GA-1000 and ascorbate was used for this purpose. They were also supplemented with 10 ng/ml TGF-B3 (Lonza, #PT-4124, USA) on this medium. To induce chondrogenic differentiation, $2.5x10^5$ cells were taken in 15 ml polypropylene tubes, and were centrifuged at 150 xg to form 5 min pellets. The pellet was cultured with the complete chondrogenic medium (Chondrogenic Differentiation Basal Media containing ITS premix, proline, sodium pyruvate, dexamethasone, L-glutamine, GA-1000 and TGF-B3) for 27 days and the medium was changed twice a week. At the end of the 27th day, the pellets were embedded in paraffine block and 5 µm sections were taken. The differentiation was shown on a fluorescence microscope (Nikon Eclipse Ti-E, Japan) with the safranin-O (Sigma, #S8884, USA) dye.

Statistical Analyses

Statistical analyses were performed using GraphPad Prism version 6.00 for windows (GraphPad Software, San Diego California, and the USA). A two-tail, unpaired Student's ***t***-test and One-Way ANOVA was applied for analyzing the difference between average responses of CD29, CD44 and CD45 expressions and CFU-F and multiline age differentiations. Multiple comparisons were performed using Tukey's Multiple Comparisons Test. Three levels of significance were considered: * P<0.05, ** P<0.01, and *** P<0,001.

Results

Isolation and Culture of Rabbit DPSCs

After isolation of rabbit dental pulp tissue, rabbit DPSCs that were adhered to the culture flasks showed a predominantly fibroblast-like morphology during early days of incubation (Figure 1A). On the 4-5th days of the incubation, the cells began multiplying, and were observed to develop into small colonies called Colony-Forming Units (Figure 1B). Approximately two weeks later, the first passage was performed when the cells in the primary culture reached 80% confluence. Subsequent passages indicated that the cells continued to multiply at a greater rate (Figure 1C-D). Some of the cells were frozen after each passage for later stages, and were found to retain morphology and differentiation abilities when reconstituted.

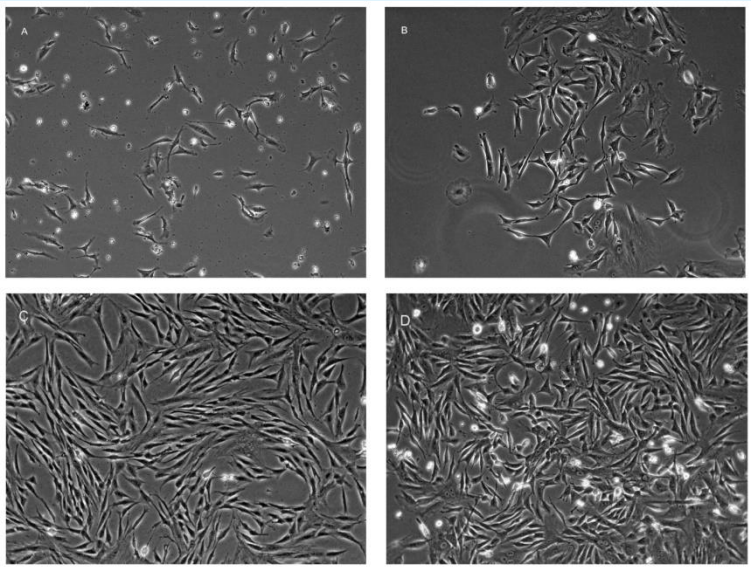

Figure 1: (A): Isolation of rbDPSCs. Fibroblast-like morphology-adherent cells, (B): the first passage cells, which proliferate in the form of CFU-F, (C): the second passage cells, (D) third passage cells. Microscope magnification is 10x.

Immunophenotypic Analysis (Flow Cytometry, Immunocytochemistry and qRT-PCR)

Analysis of surface markers by flow cytometry showed that rabbit DPSCs were 94.4+1.47% (n=3, p=0.0299) CD29 positive, 98.03+0.95% (n=3, p<0.001) CD44 positive and 3.4+1.01% (n=3, p<0.001) negative for CD45 (Figure 2). Positivity of the CD markers detected by flow cytometry was also confirmed by immunofluorescence staining. As a result of observation under fluorescent microscope after staining, the cells were found to express CD29 and CD44 violently, but were negative for CD45 (Figure 3). The CD29, CD44 and CD45 markers we used to characterize rbDPSCs were also studied by gene expression in the cells. Three repetitions and at least two readings were made for each marker. In our findings, rbDPSCs were strongly expressed in CD29 and CD44, and these markers were normalized to housekeeping gene GAPDH. CD45 expression was found to be zero (negative) (Figure 4). The findings were found to be statistically significant (n=3, p=0.0003).

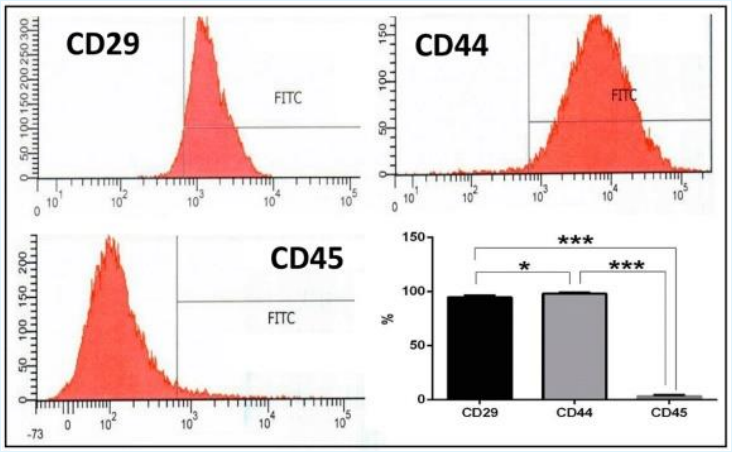

Figure 2: Identification and immunophenotyping of rbDPSCs expressing CD29, CD44 and CD45 markers by flow cytometry. CD29 and CD44 were strongly expressed, and CD45 was negative.

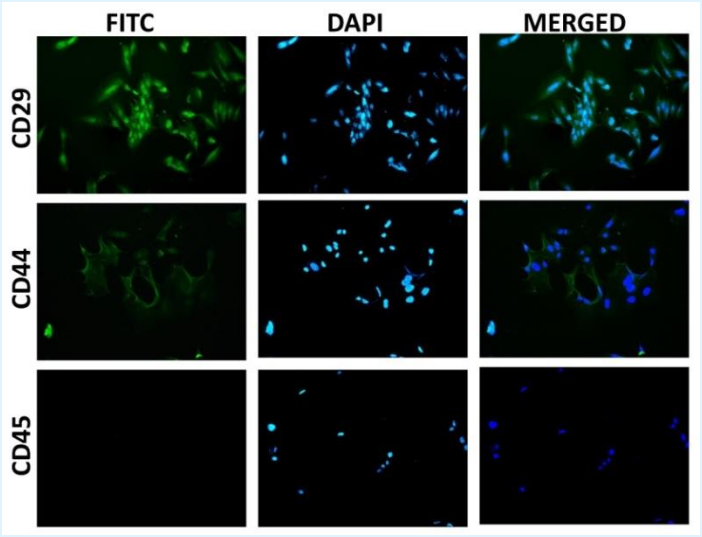

Figure 3: Immunofluorescence staining. The FITC filter shows that CD29 and CD44 are positive and CD45 is negative. Cell nuclei are shown by DAPI. Microscope objective 20x.

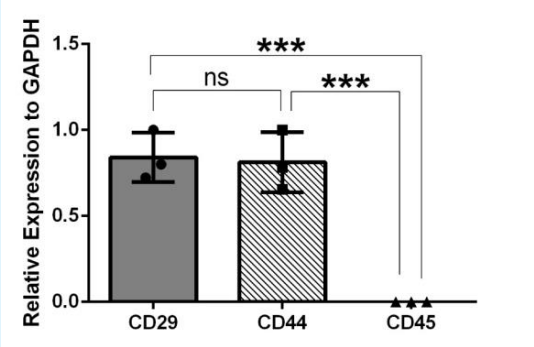

Figure 4: Quantitative Real Time PCR analysis. It was determined that relative expressions of CD29 and CD44 normalized to GAPDH were significantly higher than that of CD45, and that CD45 expression was not observed in rbDPSCs. As CD29 and CD44 expression values were very close to each other, no statistically significant difference was found; however, a statistically significant difference was found when the expressions of both CD29 and CD44 were compared with CD45 expression.

Colony Forming Units-Fibroblast (CFU-F) Assay

The clonogenic characteristics of the isolated cells in the culture medium were shown by making CFU-F. The colonies were counted after they were stained with crystal violet. The colony counts were 127.7+13.98 in 500 cells/well cultured and 211.3+10.40 colonies in 1000 cells/well cultured group (n=3, p=0.0086) (Figure 5).

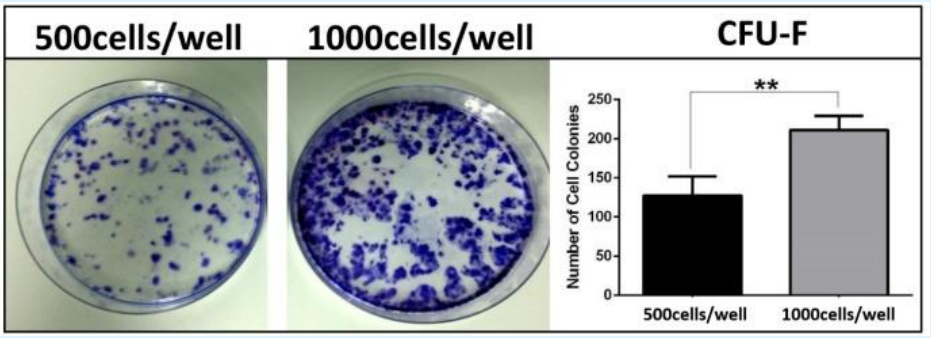

Figure 5: CFU-F Assay. Macroscopic view and CFU-F counts of colonies at the end of 14 days at 500 cells/well and 1000 cells/well cultured group.

Multiline age Differentiation of Rabbit DPSCs

It was determined that the cells reached 90-100% sub-confluency prior to induction, and 21 days of stimulation was optimal for adipogenic differentiation. At the beginning of the third week, lipid droplets in the cells proliferated and expanded, covering the entire cytoplasm. After 21 days, adipocyte-differentiating cells that were stained positively with AdipoRed and triglycerides were shown with fat droplets in the cells (Figure 6). AdipoRed fluorescence readout revealed that inducing cells differentiated significantly when compared to control group cells ($p<0.001$). After induction with osteogenic medium, proliferation increased significantly on days 5 and 6, and the cells began to form nodule aggregates. Ca^{2+} mineral deposits increased towards culture ends on days 13 and 14 in the cells. These Ca^{2+} mineral deposits in the cells were positively stained with Alizarin Red (Figure 7A-B). Osteogenic-differentiated cells showing alkaline phosphatase activity were observed in red-purple color in the plates (Figure 7C). Three weeks after chondrogenic induction, hypertrophic cells showing several

characteristic features of chondrogenic differentiation histologically were observed in pellets. It was also shown that the cells resembled cubic hyaline chondrocytes, and formed extracellular matrix. The forming glycosaminoglycan (GAG) and extracellular matrix stained positively with safranin-o (Figure 7D-F).

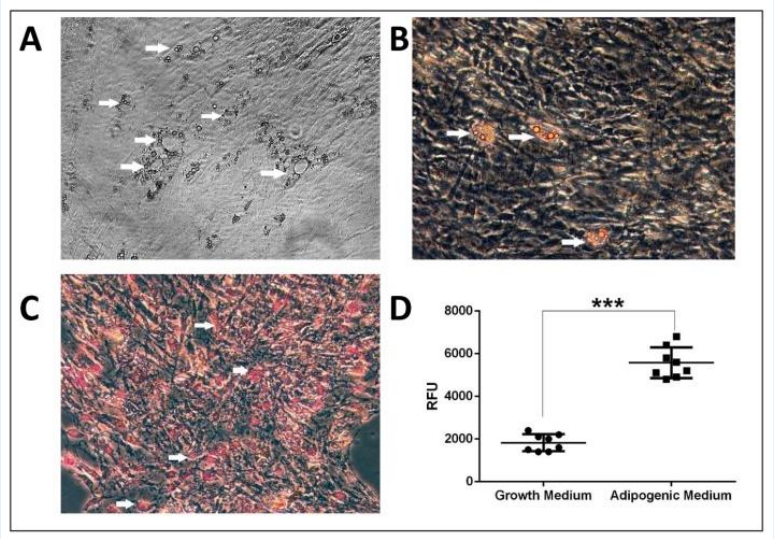

Figure 6: (A): Lipid droplets covering the entire cytoplasm at the end of day 21 are shown with white arrows, (B-C): triglycerides and fat droplets, which accumulate in the cells after AdipoRed staining, (D): AdipoRed fluorescence readings in growth medium and cells induced by adipogenic differentiation medium.

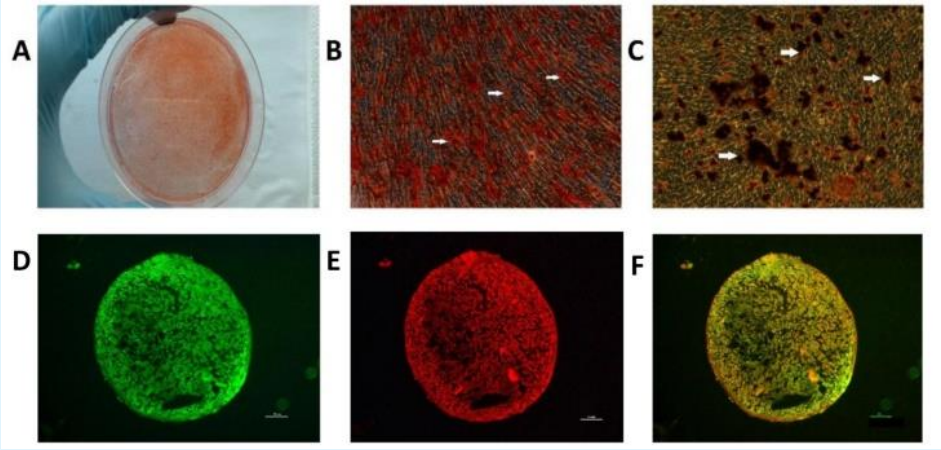

Figure 7: (A): Macroscopic appearance of osteogenic-differentiated cells and Ca2+ deposits as a result of Alizarin Red staining. (B): Microscopic image of Alizarin Red staining, 10X, (C): Alkaline phosphatase staining, 10x. (D): FITC, (E): Texas Red, (F): Merged images of chondrogenic differentiated pellet sections stained positive by safranin-O stain on fluorescence microscope. Fluorescent images were taken using Safranin-O's ability to emit radiation in both FITC and Texas Red fluorescent wave lengths. In non-chondrogenic differentiating cells, no emission was observed in filters of this wavelength. In the merged image, it is seen that the cells that differentiate into chondrocytes gain cubic morphology, and there are extra cellular matrix and fibers between the cells, as well as the perichondrium-like connective tissue structure surrounding the cross-sections. The magnification of the microscope is 20x.

Discussion

A number of studies have been reported in the literature in which mesenchymal stem cells from dental pulp are isolated [15-17]. In 2000, the concept of "Dental Pulp Stem Cells" was added to the literature by Gronthos, et al. [4] with pulpal stem cells obtained from human 3rd molar teeth. It has become very popular as a promising, potential cell in terms of its rich biologic features, which have been studied on many occasions as of that date [4]. The greatest advantages of these cells are that they can be reproduced without any surgical procedure and without any ethical problems. In addition to these advantages, the interest in dental pulp tissue is now increasing due to the rich stem cell population and differentiation to many cell types [11]. Studies have shown that Dental Pulp Stem Cells are predominantly isolated from human dental pulp [18,19]. Because rabbits are widely used in preclinical models to evaluate regenerative medicine applications, studies are needed to evaluate the potential of the stem cells obtained from this species as a live translational model. Studies on mesenchymal stem cells obtained from rabbit dental pulp tissue have been limited in number, and are generally limited to bone marrow tissue [20-22]. For this reason, we evaluated the immunophenotypic characteristics and *in vitro* osteogenic, adipogenic and chondrogenic differentiation capabilities of rabbit DPSCs. In our study, rabbit pulp tissue was treated with collagenase and dispose enzymes, and the isolation was successfully performed. Two commonly used methods for isolating Dental Pulp Stem Cells (enzymatic and explanation techniques) have been reported in the literature as advantages and disadvantages relative to each other [18,23]. Similar to the results of Noce, et al. [23] the cells were individually lowered to allow the flask to attach faster, and the flow of the culture process was accelerated to allow flow cytometric analysis in early passages. The separation of the cells in explant culture is relatively difficult and requires longer culture processes. El-Backly, et al. [24] reported in 2008 that they easily isolated Dental Pulp Stem Cells from rabbit teeth as isolated from other species. These cells are presented with typical fibroblast-like morphology and high proliferation and clonogenic abilities. The immunophenotypic characterization has been reported to be positive only in CD271 [24]. In the light of these data, it is seen that the rabbit DPSCs we isolated in our study are in accordance with the literature. However, according to our own findings, it was determined that the expression of immune phenotypically specific markers and the colony-forming abilities of rabbit DPSCs in the literature are compatible with the results we found in our study; and that CD29 and CD44 expressions are higher. Given the characteristics of CFU-F formation in rabbit DPSCs, El-Backly, et al. [24] reported that 47-70 colonies per 1000 cells were formed. In our study, we found 211+10 colonies per 1000 cells. This means that we achieved a highly efficient population of clonogenic properties. Furthermore, when compared with the human bone marrow and dental pulp MSCs given in the literature, it was seen in our study that rabbit DPSCs are a much more active cell group than the clonogenic side [4,25]. However, this may vary according to the age of the organism and the state of the cells and the media used [26]. Minimal criteria designed to identify multipotent mesenchymal stem cells have been reported by the international community of cellular therapies. MSC markers specifically known for the identification of immunophenotypic characteristics have been described [27]. MSCs should be negative for CD34 and CD45, while surface markers such as CD44, CD73, CD90, CD105, and CD29 are expected to be positive. Because CD45, CD34 are basically hematopoietic stem cell markers [27]. However, these markers may vary even between species or even between different populations of the same cells. Some differences in human and rabbit cell identification have been identified [3,20]. CD90, known to be positive in human MSCs, is not expressed in rabbit cells [20]. This result was reported by Lapi, et al. [22] and Bakhtina, et al. [20]; and in this regard, CD90 was not used as a marker in our study. In our study, CD29 and CD44 markers were positive, and CD45 was negative. In this respect, three different assays have been demonstrated for characterization not only by flow cytometry but also by immunofluorescence staining and qRT-PCR, in which CD29 and CD44 are strongly expressed in rabbit DPSCs and are negative for CD45. We found that the results we have in our study are higher for CD29 and CD44 and lower for CD45 than the percentages of expression in the studies presented in the literature for rabbit DPSCs [20,21]. The most specific feature of MSC is the differentiation into adipocytes, osteoblasts and chondrocytes [3]. In our experiments, rabbit DPSCs were shown to easily differentiate in these three lines. It has been reported in literature that Dental Pulp Stem Cells are a better source of mineralized tissue regeneration than mesenchymal stem cells derived from bone marrow [28]. In this respect, the osteogenic differentiation of Dental Pulp Stem Cells is better than that of other stem cell types. In addition, other studies have emphasized that Dental Pulp Stem Cells are capable of expressing chondrogenic markers [29]. DPSCs with neuroectoderm origins besides mesoderm origin have the ability of differentiation into adipocyte, osteoblast, chondrocyte, muscle cells,

hepatocytes and neurons [11,30]. As a result, rabbit DPSCs have been successfully isolated from rabbits, and have been proven to be MSCs of the obtained cells. It has been determined that there are high potency cells according to other stem cell sources. Rabbit is a model organism frequently used in *in vivo* studies. Hence, rabbit Dental Pulp Stem Cells can be used in both in *in vitro* and *in vivo* clinical, surgical and tissue engineering studies. DPSCs are a new research topic in the literature in terms of biological behavior and their potential for differentiation. DPSCs have gained considerable importance in stem cell research nowadays with their ease of access, their immature nature and their wide repertoire of differentiation. In the clinical sense, these are special cells that can be used in the fields of dentistry, regenerative medicine, tissue engineering in the next few years. The biggest disadvantage for these cells now is that they cannot be fully characterized. Future studies are needed to isolate and characterize these cells in a specific, pure population, and to bring their niches to light.

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Potential Conflict of Interest

The authors have no conflicting financial interest.

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