Rat Bone Marrow Stem Cell Culture for Use in Tissue Regeneration *

Cultivo de células troncales de médula ósea de ratas para uso en regeneración de tejidos

Cultura de células-tronco de medula óssea de rato para uso na regeneração de tecidos

Ángel Eduardo Pirela Labrador
Pontificia Universidad Javeriana, Colombia
apirela@javeriana.edu.co
ORCID: https://orcid.org/0000-0001-9534-994X

Luis Felipe Tangarife Tobón
Pontificia Universidad Javeriana, Colombia
ltangarife@javeriana.edu.co
ORCID: https://orcid.org/0000-0001-5802-0651

Nelly Stella Roa Molina
Pontificia Universidad Javeriana, Colombia
nelly.roa@javeriana.edu.co
ORCID: https://orcid.org/0000-0002-9884-9242

Camilo Durán Correa
Pontificia Universidad Javeriana, Colombia
Camilo.duran@javeriana.edu.co
ORCID: https://orcid.org/0000-0002-5355-3413

Lorenza María Jaramillo Gómez
Pontificia Universidad Javeriana, Colombia
lorenzaj@javeriana.edu.co
ORCID: https://orcid.org/0000-0002-0087-7879

DOI: https://doi.org/10.11144/Javeriana.uo41.rbms

Received: 05 April 2021
Accepted: 14 december 2021
Published: 02 november 2022

Abstract:

Background: Stem cells are considered a promising therapeutic agent in tissue regeneration. The application of stem cells in regenerative medicine procedures requires a previous and rigorous process of obtention, and the use of animal models is essential for the application. Purpose: To obtain stem cell populations from rat bone marrow, with preservation of stem characteristics in culture.Methods: This was an experimental study that used euthanized male and female rats of the Lewis lineage. Posterior leg bones were dissected, and primary cultures were obtained from their bone marrow and depleted of CD45+ populations. The CD45-free populations were sub-cultured until passage five and their morphological, immunophenotypic, proliferation, and differentiation capacity to three lineages was evaluated. Results: Morphological evaluation of the cultures showed a predominance of spindle-shaped and fibroblastic cells that grew adherent and in CFU-F. The immunophenotype was characterized by positive expression of CD90, CD29, and CD146. Cultures induced to osteogenic, chondrogenic, and adipogenic lineages showed a change in morphology and positivity to Alizarin Red, Alcian Blue, and Oil Red O staining, respectively, increased alkaline phosphatase activity corroborated osteogenic differentiation in subcultures induced to this lineage. Conclusion: Bone marrow stem cell populations were obtained from rats that retain the stem characteristics and therefore the possibility of being used in preclinical studies.

Keywords: biotechnology, bone marrow stem cells, cell culture, cell differentiation, Lewis rats, tissue engineering.

Resumen:

Antecedentes. Las células troncales se consideran un agente terapéutico prometedor en regeneración de tejidos. El uso de éstas requiere un proceso previo y riguroso de obtención y para su aplicación es esencial el uso de modelos animales. Objetivo: Obtener poblaciones de células troncales de médula ósea de ratas con la conservación en cultivo de las características de troncalidad. Métodos: Este fue un estudio experimental en el que se usaron ratas macho y hembras del linaje Lewis a las que se les realizó eutanasia. Se disecaron los huesos de las extremidades posteriores y, a partir de la médula ósea de estos, se obtuvieron los cultivos primarios a los cuales se les hizo la depleción de las poblaciones CD45+. Las poblaciones libres de CD45 se subcultivaron hasta

el pasaje cinco y se evaluaron sus características morfológicas, inmunofenotípicas, de proliferación y la capacidad de diferenciación a tres linajes. Resultados: La evaluación morfológica de los cultivos mostró un predominio de células ahusadas y fibroblastoides que crecieron adheridos y en UFC-F. El inmunofenotipo se caracterizó por la expresión positiva de CD90, CD29 y CD146. Los cultivos inducidos a los linajes osteogénico, condrogénico y adipogénico mostraron un cambio en la morfología y positividad a las tinciones de Rojo de Alizarina, Azul Alcian y Aceite Rojo O, respectivamente. El aumento en la actividad de fosfatasa alcalina corroboró la diferenciación osteogénica en los subcultivos inducidos a este linaje. Conclusión: Se obtuvieron poblaciones de células troncales de médula ósea de ratas que conservaban las características de troncalidad y por lo tanto la posibilidad de usarlas en estudios preclínicos de regeneración de tejidos.

Palabras clave: biotecnología, células troncales de médula ósea, cultivo celular, diferenciación celular, ingeniería de tejidos, odontología, ratas Lewis.

Resumo:

Antecedentes: As células-tronco são consideradas um promissor agente terapêutico na regeneração tecidual. A aplicação de células-tronco em procedimentos de medicina regenerativa requer um processo prévio e rigoroso de obtenção, sendo imprescindível o uso de modelos animais para a aplicação. Objetivo: Obter populações de células-tronco de medula óssea de rato, com preservação das características de tronco em cultura. Métodos: Trata-se de um estudo experimental que utilizou ratos machos e fêmeas eutanasiados da linhagem Lewis. Ossos da perna posterior foram dissecados e culturas primárias foram obtidas de sua medula óssea e depletadas de populações CD45+. As populações livres de CD45 foram subcultivadas até a passagem cinco e sua capacidade morfológica, imunofenotípica, proliferativa e de diferenciação para três linhagens foi avaliada. Resultados: A avaliação morfológica das culturas mostrou predominância de células fusiformes e fibroblásticas que cresceram aderentes e em UFC-F. O imunofenótipo foi caracterizado pela expressão positiva de CD90, CD29 e CD146. Culturas induzidas para linhagens osteogênicas, condrogênicas e adipogênicas mostraram uma mudança na morfologia e positividade para coloração Alizarin Red, Alcian Blue e Oil Red O, respectivamente, aumento da atividade da fosfatase alcalina corroborou diferenciação osteogênica em subculturas induzidas para esta linhagem. Conclusão: As populações de células-tronco da medula óssea foram obtidas de ratos que mantêm as características de tronco e, portanto, a possibilidade de serem utilizadas em estudos pré-clínicos.

Palavras-chave: biotecnologia, células-tronco da medula óssea, cultura de células, diferenciação celular, engenharia de tecidos, ratos Lewis.

INTRODUCTION

In regenerative medicine, stem cells are considered a very promising therapeutic agent; thus, they intensely studied (1). Translational research with stem cells seeks to find therapeutic alternatives that contribute to the solution of tissue and organ loss (2). In many cases, the study of these alternatives includes the use of animal models (3).

Stem cells exist in different pre- and postnatal tissues, both in humans and in animals. Unlike other cell types, such as somatic cells, stem cells possess a self-renewal capacity, non-differentiated status, and potential to differentiate into almost any type of cell (4,5). Additionally, great therapeutic potential in regenerative medicine has been attributed to stem cells due to their secretion of cytokines and growth factors (6). To guarantee the safe use of these cells in clinical practice, the scientific community is interested in deepening the knowledge of stem cell origin, location, isolation methods, *in vitro* and *in vivo* characterization, applications, and mechanisms of therapeutic action (7).

Stem cells have received different names. The most used term mesenchymal stem cells (MSCs) (8-10), which was first introduced in 1991 by Arnold Caplan (11). Currently, because its main functions relate to its immunomodulatory properties, scientific societies have suggested changing the meaning of MSC (12,13) to medicinal signaling cells (14) while retaining the acronym MSC through which they have been widely known.

MSCs were first described in bone marrow, which is why this tissue has been the main source for obtaining them and has been one of the most intensively studied (11,15,16). Currently, MSCs can be recovered and isolated from various organs and tissues (12,14), such as placenta (17), umbilical cord blood (18,19), umbilical cord (20), adipose tissue (21), skin (22), testicles (23), brain (24), and dental pulp of permanent

and deciduous teeth (25-27). Even though common characteristics between MSC populations have been reported from various sources, there is not yet an updated consensus of specific criteria to define them (28, 29). Stem cells derived from adult tissues have the ability of immunomodulating and establish cells without the risk of generating teratomas, in contrast to embryonic stem cells or induced pluripotent stem cells.

MSCs from bone marrow have been widely investigated and used in cell-based therapies (12, 30). The U. S. Food and Drug Administration has approved their use in autologous applications for the treatment of various diseases (31). For the application of MSCs in regenerative medicine, a rigorous process of obtaining, isolating, and characterizing stem cells seeks to guarantee their conservation (8), in addition to maintaining established MSC cultures from a primary culture to their successive passages. This is still a complex process (29) due to the presence of other cell types such as fibroblasts, which are morphologically identical and present surface markers like those of MSCs (32). The characterization of stem cells must be completed with the determination of their multi-differentiation potential (8,33,34) and, to guarantee their translation, studies seek to determine their efficacy and safety in different therapeutic applications (29).

Because *in vitro* models do not fully mimic the complexity of an *in vivo* environment or predict the clinical efficacy of stem cell treatments, many studies use animal models (35). Animal models are necessary to assess innovative ideas, concepts, and technologies. Regenerative medicine, for example, has advanced because of experiments conducted with animal models (35). Rats are a model organism to study physiological functions *in vivo*; in both pathological and disease conditions the rat model is more comparable to humans than mice (36). Rats were the first species among mammals to be domesticated for scientific research with work dating back to before 1850 (37). The first homozygous inbred strain obtained from brother-sister mating was established in rats by King in 1909. Inbred work in mice began that same year (37).

Recent advances in genetic engineering have resulted in the development of various immunodeficient rat models to transplant and regenerate human tissues and cells (38,39). Due to their size and tissue density, these models caused the advancement of techniques such as multiphoton microscopy (36) and electrocardiography (40). Currently, there are models of immunocompromised rats transplanted with human immune cells that are known as humanized rats, which provide a means for robust longitudinal studies on the safety and efficacy of therapeutic agents aimed at the treatment of HIV and modeling of cardiovascular, neurocognitive, and pulmonary diseases (41).

Considering that rat is a species that allows applying preclinical models close to humans (42), it has been the selected model for our research group to develop *in vitro* and preclinical methods, which require identification, characterization, and proper manipulation of bone marrow-derived stem cells. Therefore, the research question that motivated this study was: What are the procedures to maintain rat's bone marrow cell cultures that maintain an immunophenotypic predominance of stemness, differentiation potential to different lineages, and allows obtaining it in high cell densities to be used in regeneration experiments? With the completion of this study, we developed laboratory methods to isolate, cultivate, expand, and functionally characterize bone marrow-derived stem cells, which contribute to develop dental tissue-regeneration protocols in the scientific community interested in the use of rats as an experimental animal model.

MATERIALS AND METHODS

This study followed the scientific, technical, and administrative standards for health research, "Biosafety of research (Title IV, Chapter III) and biomedical research with animals (Title V)" of resolution No. 008430 of 1993, of the Colombian Ministry of Health. Laboratory procedures were governed by the biosafety standards to manage biological specimens of the Dental Research Center of Pontificia Universidad Javeriana's Dental School from Bogotá, Colombia. This study was experimental and was approved by the Ethics and Research

Committee of the Dental School and financed by the Pontificia Universidad Javeriana's Office of the Vice-President for Research (projects 4027 and 4279).

Animals and Housing Conditions

The rats were housed under standardized conditions in ventilated cages (ONE CAGE 2000, Lab Products) under SPF conditions in the Pontificia Universidad Javeriana's Comparative Biology-Dentistry Unit, where a Lewis colony (LEW/SsNHsd) is housed, whose founding nucleus was obtained commercially from Envigo RMS, Inc. (Indianapolis, USA). All animals were housed in groups of two, in autoclavable plastic cages (425 mm x 266 mm x 155 mm), fed *ad libitum* with an autoclave diet (Labdiet # 1013) and free access to purified drinking water. The light/dark cycle is 12/12, the 12 hours of light are supplied with artificial light (approximately 40 lx in the box). The temperature was 22 ± 2 °C, the relative humidity 60 ± 10 %, the environment was kept free of noise and odors. The rats were free of viruses, bacteria, and parasites listed for SPF colonies according to FELASA (43), except for *Helicobacter spp*.

Sixteen rats, 8 males and 8 females, 4-6 weeks of age (120-160 g of weight) were part of the study. Euthanasia was applied to the animals by means of a mixture of CO_2 and air to obtain bone marrow as a source of stem cells. Cell groups of four individuals (two females and two males) were considered as a batch and the variables measured at each moment were established as a repetition until completing at least three repetitions per variable and for each batch. The characteristics of these populations were evaluated in each passage, which can provide useful information for different applications, as well as the time to obtain enough in each experiment.

Cell Culture Conditions

In all cases where the culture medium is mentioned, it refers to α-MEM (Gibco® 12000-014), supplemented with 2.2 g of sodium bicarbonate (Merck 106329), 10 % fetal bovine serum (Gibco ® 16000 -044), 1 % Glutamax (Gibco® 35050-061), and 1 % of a commercial solution of two antibiotics (penicillin 10,000 U/ ml and streptomycin 10,000 µg/ml) and an antifungal (amphotericin B 25 µg/ml) (Gibco® 15240-062). Serum -free medium refers to the same α-MEM culture medium, but without the supplementation with fetal calf serum. All cell centrifugation steps were performed at 300 g (2000 rpm) and 4 °C for 5 minutes in a centrifuge (Thermo Scientific™ Heraeus Biofuge Primo R 41272826). Cell cultures were conducted in 25 cm culture fl asks (Corning 3053) and kept at 37 °C and 5 % CO_2 in an incubator (NAPCO S400 CO_2)
until reaching 95 % confluence, at which time the cells were detached. The detachment of cells from the surface of the flask or trypsinization was performed with 0.5 M trypsin/EDTA (Merck 108418) for 3 minutes at 37 °C and 5 % CO_2. Cell counting was performed in a Neubauer chamber with 0.4 % Trypan Blue (Sigma T8154). Cultures were evaluated under an inverted light microscope (Leica, DMi 1) and culture medium changes were performed every two days.

Obtaining Primary Cultures

A technical expert performed an aseptic dissection of the skin, muscle tissue, and periosteum of the femur and tibia bones of the hind limbs of euthanized rat, following the Ridzuan protocol (44). The next steps included the removal of bone epiphyses and medullary irrigation at one end with a culture medium-loaded syringe. The collected lavage was mechanically disintegrated with the same syringes and washed twice with culture

medium. The entire cells were counted and seeded in culture flasks at a concentration of 5 x 105 cells for each flask. The primary culture (P0) was considered established when 95 % confluence was complete.

Depletion of CD45+ Populations and Sub-cultures up to P5

P0 cells were dissociated with trypsin, collected through centrifugation, counted, and incubated with 10 µl/1 x 106 cells of an antiCD45 PE (BD Pharmingen™ clone Ox-1, 554878) for 15 minutes at 4 °C to purify the CD45+ population (45). Then they were incubated for 15 minutes at 4 °C with 10 µl of a secondary antibody coupled to anti-PE magnetic beads (Miltenyi Biotech, 130048801) and this solution was passed through a magnetic separation column (Miltenyi Biotec, 120-000-472). This process allowed to retain CD45+ and collect CD45- in the eluate to corroborate the purity of the separation through flow cytometry. The CD45-population was counted and subcultured to advance towards the first passage (P1). Starting at P1, each culture that reached cell confluence was subcultured until reaching P5.

Crop Immunophenotypic Characterization

The immunophenotypic characteristics of the cultures from P0-P5 were determined through flow cytometry, using specific antibodies coupled with PE, APC and FITC fluorochromes and directed to the molecules CD45PE (554878, DB Biosciences), CD29Biot/Strep-APC (555004, DB Biosciences), CD90FITC (130-094-527, Miltenyi Biotec), CD71PE (554891, BD Biosciences and CD106PE (559229, BD Biosciences). Antibody titers in the labeled cultures were CD90: 1/100, CD29: 1/100. 1/200, CD45: 1/200, CD71: 1/100 and CD106: 1/200.

Cell Proliferation Assays

To evaluate cell proliferation, we used a colorimetric method that determines the number of viable cells in proliferation (CellTiter 96®, Promega G3580), following the manufacturer's instructions and then seeding the cells in plates (multi-wells of 96 wells—SPL Life Sciences—with a concentration of 3.2 x 103 cell/well). Evaluations took place at 12, 24, 48, 72 and 96 hours, in which the culture medium was removed from each well and a solution of the kit's aqueous reagent in culture medium at a 1:5 ratio was added and left incubating at 37 °C for 4 hours. After that time, the optical density was read in a plate reader (Biotek ELx 800). To determine the number of cells for each absorbance value, we measured the optical density at different concentrations of adhered cells to determine a cell growth equation, through which we found the number of cells for each absorbance.

Crop Morphological Assessment

We assessed the morphological characteristics of the cultures every two days by means of an inverted microscope (Leica®, DMi1) to determine adherence to the surface of culture flasks, growth pattern, and cell confluence. After the cultures reached confluence, characterization was completed by means of Masson's Trichrome and H&E stains.

Determination of Fibroblastoid Colony-Forming Units (CFU-F)

Growth in colony-forming units for each passage measured was determined by seeding cells in 6-well multi-well plates (Corning 3506) at a concentration of 2.5 x 105 cell/well. After 21 days of culture, the cells were fixed with 10 % buffered formalin (Merck, HT50112) and stained with Gram's violet (Merck, 94448) for 30 min. The growth potential in CFU-F was verified macroscopically and the number of cells in each colony was counted microscopically. We considered CFU-F colonies with more than 30 cells. The following equation was used to calculate the efficiency in CFU-F,

$$\text{UFC-F Formation Efficiency} = \frac{\text{Number of colonies counted}}{\frac{\text{Number of cells seeded}}{\text{cm}^2 \text{ of cultivated area}}} \text{ X } 100$$

Crop Multi-Differentiation Potential

Cell cultures underwent differentiation into three lineages: osteogenic, chondrogenic, and adipogenic. Cells in each passage were seeded in triplicate in 12-well multi-well plates (Corning 3512) at a 1.5 x 105 cells/well ratio. Once they reached 60 % confluence, the culture medium was changed to induction medium. differentiation prepared by supplementing the culture medium with the reagents for induction of osteogenic (46,47), chondrogenic (25,48,49) and adipogenic (25,50) differentiation.

The osteogenic differentiation medium was prepared by supplementing the culture medium with 0.1 μM dexamethasone (Sigma-Aldrich D4902), 50 μM ascorbic acid (Sigma-Aldrich A4544) and 10 μM β glycerol phosphate (Sigma-Aldrich G9422). Cultures were maintained for 21 days with the differentiation induction medium and then washed twice with PBS (Sigma P3813), fixed with paraformaldehyde solution (Sigma Aldrich ,158127) at 4 % for 15 min, and stained with a 2 % Alizarin Red (Sigma A5533) solution and with pH 4.2 for 30 min. Finally, the cells were observed in the light microscope and the images were captured with a camera (Canon EOS REBEL T51 18-55 IS STM). To quantify osteogenic differentiation, alkaline phosphatase activity was determined using a commercial colorimetric kit (Sigma Aldrich, APO100) by following the manufacturer's instructions.

For the induction of chondrogenic differentiation, the culture medium was supplemented with 10 ng/ml human transforming growth factor β3 (TGF-β3) (Sigma-Aldrich E. coli SRP3171), 100 nM dexamethasone (Sigma-Aldrich D4902), 200 μM ascorbic acid (Sigma-Aldrich A4544), 40 μg/ml L-proline (Sigma-Aldrich 81709), 1 mM pyruvate (Sigma-Aldrich P5280), 1 mg /ml bovine serum albumin (BSA) (Sigma-Aldrich 05470), and 50 mg/ml ITS+3 Liquid Media Supplement (100x) (Sigma-Aldrich I2771). Cultures were induced with this medium for 21 days and washed twice with PBS (Sigma P3813). Then, they were fixed with a 4 % formaldehyde solution for 30 minutes, and cells were washed and visualized in a light microscope. Images were captured with a photographic camera.

Cultures with adipogenic induction were maintained for 21 days with culture medium supplemented with 1 μM dexamethasone (Sigma-Aldrich D4902), 500 μM 3-isobutyl-1-methylxanthine-IBMX (Sigma-Aldrich I5879), 5 μg/ml insulin (Sigma-Aldrich I5500), and 200 μM indomethacin (Sigma-Aldrich I7378. The dye used to identify differentiation was 0.5% Oil Red O (Sigma O1395) that was added to the cultures induced, washed, and fixed for 30 minutes. Afterwards, images were obtained with a microscope and photographic camera.

Statistical Analysis

We conducted all experiments at least three times. Descriptive data consisted of mean ± SD ($p < 0.05$). The Shapiro-Wilk test verified data normality, and ANOVA and Tukey's *post hoc* tests determined differences between the expression of cell surface markers, cell proliferation, efficiency in the formation of colonies, and concentration of alkaline phosphatase. Student's t test established differences in the percentage of positive cells for expression of typical stem cell markers. Data analysis was performed with SPSS software, 20.0 version 20.0 (IBM, New York, USA).

RESULTS

Primary Crops

Morphology

Inspections with light microscopy allowed visualizing the surface characteristics of the culture flasks of two different cell populations. Observations of primary cultures after 48 h starting to show a population of small, round, and refractive cells that grew isolated and aggregated on a population of star-shaped fibroblastoid cells beginning to show a growth pattern in CFU-F (Figure 1a). Cellular confluence was reached 8-10 days after the beginning of the culture and showed a predominance of spindle-shaped cells with a fibroblastoid appearance (Figure 1b).

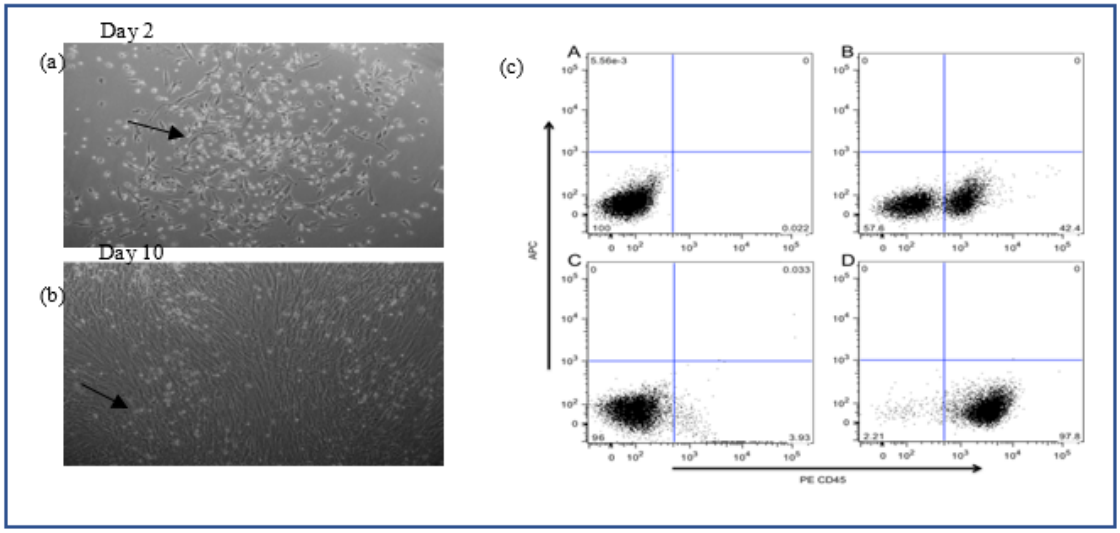

FIGURE 1

Characteristics of primary cultures derived from rat bone marrow. (a) Morphological characteristics of primary cultures (10X), microphotograph of cells that grew adhered to the surface of the flask 2 days after starting the primary culture in which a population of small, round, and refringent cells. Another population of star-shaped fibroblastoid cells was observed. (b) 10 days after starting the primary culture; in both images, (a) and (b), a growth pattern is identified in CFU-F (arrow). (c) Depletion of CD45+ cells, representative dotted image obtained through flow cytometry from primary rat bone marrow cultures. CD45+ cells were separated by immuno-magnetism. A. Cells not marked as control; B. Total population before separation of CD45+ cells; C. CD45- cells after the separation column by immuno-magnetism; D. CD45+ cells after the immunomagnetic separation column.

Source: the authors.

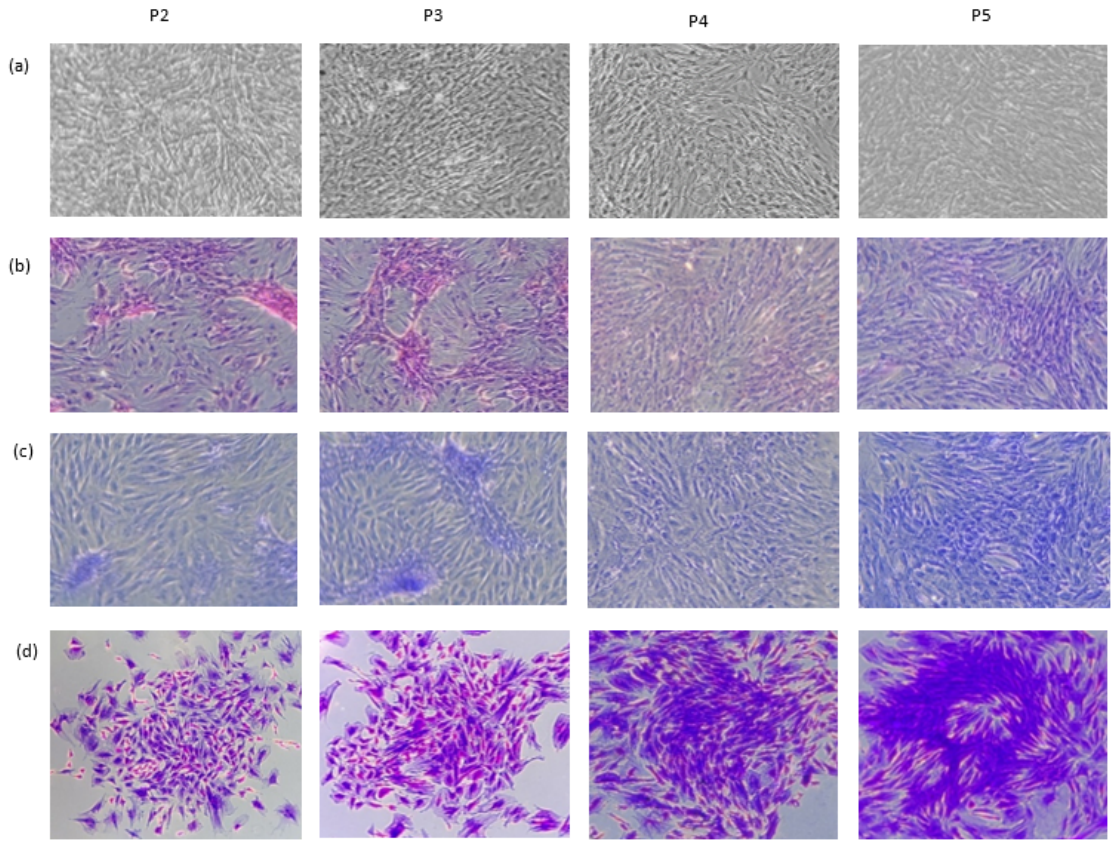

FIGURE 2
Characteristics of rat bone marrow stem cell cultures. (a) Microphotographs (10X) representative of the cultures in the different passages. Two populations were identified: the first with large and rounded cells and the second with a predominance of spindle cells and fibroblastoids growing in UFC-F. (b) Photomicrographs (10X) of H&E staining. (c) Photomicrographs (10X) of Masson's Trichrome staining. (d) Photomicrographs (10X) of Violet staining showing growth in CFU-F.
Source: the authors.

Immunophenotyping and Separation of CD45+ Populations

In primary cultures, low positivity values were found in the expression of typical stem cell markers, except for CD90 that was 86.03 % ± 1.95. For CD45, positivity was 42.46 % ± 3.75. Since this is a typical marker of the hematopoietic cell portion of the marrow, it was considered positive. Through a separation column with magnetic beads, these cells were isolated from those collected at the moment of the cell confluence of the primary culture, managing to lower its value to 3.63 % ± 0.34. The removal of CD45+ cells from the populations obtained in the primary culture was confirmed by flow cytometry, showing that the purity of the selection was approximately 97 % (Figure 1c). After the separation of subpopulations with positivity for CD45, the CD45- population was subcultured to advance the expansion of the rCTMO cultures.

Expansion of Bone-Marrow Stem-Cell Cultures from OrCTMO Rats

Crop Morphology

During the development of the subcultures, a population adhered to culture flask surfaces was identified, which, as culture time increased, also increased the prevalence of the population of spindle cells and fibroblastoids in relation to the population of small round cells (Figure 2a). Cultures were also evaluated with H&E staining, whereby these cells were found to have large, centrally located, elongated basophilic nuclei surrounded by acidophilic cytoplasm (Figure 2b). Staining with Masson's trichrome showed the presence of collagen deposits at the intercellular space level that is characteristic of cellular monolayer formation in which the cells are in direct contact with each other (Figure 2c). As the subculture increased, a more purified population was also observed in terms of small cells that maintained a pattern in UFC-F (Figure 2d).

Immunophenotyping

The positivity of typical stem cell markers increased from P1. Particularly, CD90 showed high positivity in all passages and CD45 was exceptionally low after the selection of CD45- cells in the primary culture. The levels of the CD90 marker remained within those recommended for cultures to be considered as heterogeneous populations of stem cells derived from rat bone marrow. CD29, especially from P2, maintained high values and CD146 from P3 presented medium positivity values from P3. Because of the above findings, in passages four and five it can be considered that, due to the expression of CD90, CD29, CD146 markers (Table 1) those populations meet the stem criteria. Regarding CD71 and CD106 markers (Table 1), although they presented positivity, such positivity is considered low.

TABLE 1

Expression of stem cell markers. Positive for CD90, CD29 and CD146 is found. There was no expression for CD71 and CD106. The depletion of the CD45+ populations in the primary culture made it possible to reduce this marker in the passages evaluated. The growth efficiency in CFU-F was also determined in the evaluated passages.

	CD90	CD29	CD146	CD71	CD106	CD45	UFC-F
P1	95,46 ± 0,25	37,50 ± 2, 15	16,16 ± 0,41	9,27 ± 0.63	1,21 ± 0,14	0,39 ± 0,02	ND
P2	93,83 ± 1,04	54, 73 ± 19,54	14,06 ± 0,15	2,08 ± 0,66	0,82 ± 0.35	0,72 ± 0,21	73,16±1,28
P3	97,83 ± 0,26	56,80 ± 0.45	32,23 ± 0,37	3,73 ± 0,41	2,07 ± 0,08	0,75 ± 0,10	61,34±0,32
P4	96,50 ± 0,55	58,10 ± 0,26	27,96 ± 1,76	15,09 ± 0.2	12,36 ± 1,18	2,30 ± 0,08	44,61±7,46
P5	94, 83 ± 0,86	59,26 ± 0,32	40,16 ± 0,35	3,17 ± 0.20	1,83 ± 0,5	3,09 ± 0,20	58,78±1,39

Source: the authors.

Cell Proliferation

In all passages, a progressive increase in the number of cells was evident between 12 h and 96 h. As the passage increased, the number of cells increased at each evaluated time, being P5 better than the previous ones (Figure 3a). The determination of the number of cells in each passage and in each time allowed establishing the necessary time in each passage to obtain a given quantity of a population of stem cells that could be used in a tissue regeneration experiment. For instance, in P5 after 96 hours of culture 16,000 cells per well could be recovered from 96-well multi-well plates (Figure 3b).

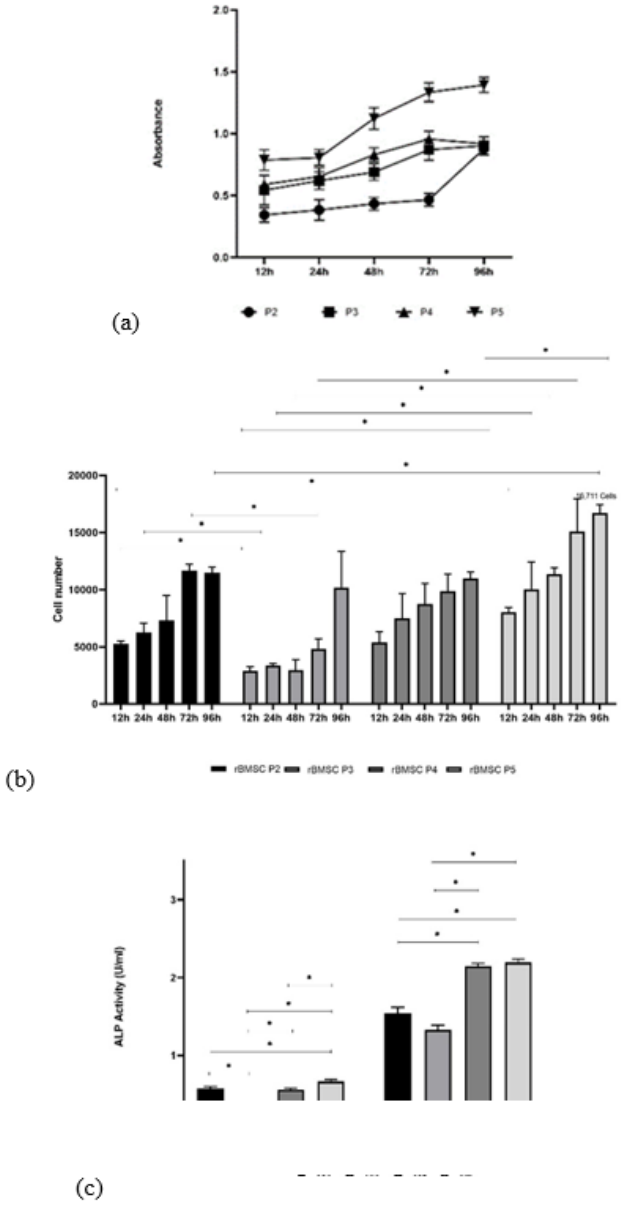

FIGURE 3

Proliferation kinetics and alkaline phosphatase activity of rat bone marrow stem cells. (a) Cell proliferation in passages 2-5; each point represents the average of the absorbance obtained at 12, 24, 48, 72 and 96 h. (b) Bars represent the average number of cells obtained at each of the times for passages 2-5. Bars represent the standard deviation of the mean and * is the significance when the p value < 0.05. (c) Alkaline phosphatase activity was evaluated in passages 3-5 with osteogenic induction and their respective controls for 21 days. Bars represent the standard deviation of the mean and * is the significance when the p value < 0.05.

Source: the authors.

Colony Forming Units Assay (CFU-F)

After 14 days of culture, the colonies were stained and observed with an inverted microscope. Colonies were counted and CFU efficiency was determined. In all passages cultures presented a morphological cell

pattern that converged towards a center with a predominance of spindle cells and fibroblastoids. Efficiency was around 60 % with P2 being the passage with highest count (Table 1).

Multi-Differentiation Potential

After cultures reached 60 %, they were maintained for 21 days with the differentiation media. In each culture dish, wells with culture medium without induction were maintained also for 21 days to be used as controls for differentiation tests. Regarding osteogenic differentiation, bone marrow stem cell cultures were positive through Alizarin red staining. The formation of calcium nodules separated from each other was evident. Said nodules were better defined, with a more intense coloration in P4 and P5 (Figure 4a). Morphological changes of the cultures induced with osteogenic medium were visible through Alizarin red staining. The amount of spindle cells and fibroblastoids decreased and polygonal cells in the cultures became apparent. Additionally, osteogenic differentiation in cultures was corroborated by alkaline phosphatase activity. It increased with induction time and as passages progressed, as well as alkaline phosphatase activity (Figure 3c).

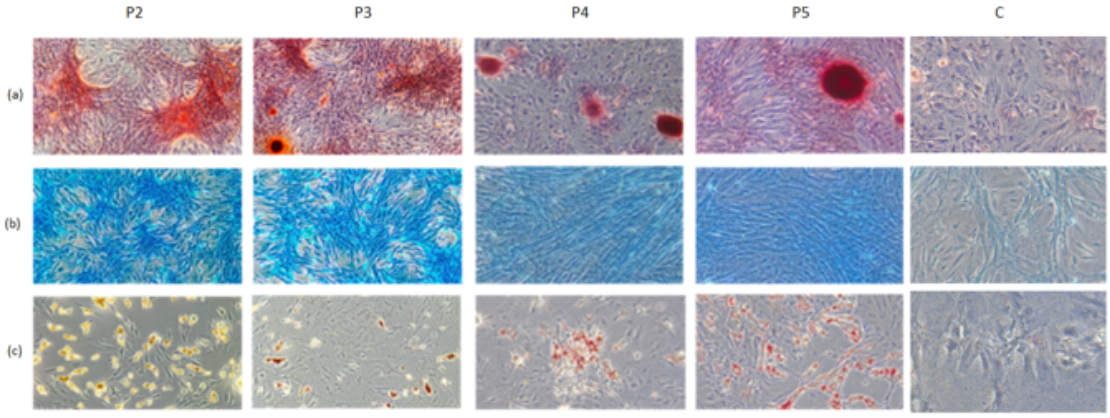

FIGURE 4
Multi-differentiation of rat bone marrow stem cells. (a) Cultures in passages P2-P5 and negative control (C) stained with Alizarin Red. (b) Cultures in passages P2-P5 and negative control (C) with Alcian Blue staining. (c) Cultures in passages P2-P5 and negative control (C) with Oil Red O staining.
Source: the authors.

The induction with chondrogenic differentiation medium produced a change in the morphology of the cells as a change in the orientation of the growth of the cells. Moreover, the positivity of the Alcian Blue staining was higher as the evaluation time advanced (Figure 4b). On the other hand, the cultures with adipogenic induction also showed a change in morphology; the initial tapered and fibroblastoid morphology had a change in some cells now showing an oval shape. Also, the Oil Red O staining allowed identifying lipid droplets, which indicated positivity for this lineage (Figure 4c).

DISCUSSION

In this study, an efficient methodology for isolating, expanding, and characterizing stem cells derived from bone marrow of Lewis rats was validated, through which populations maintaining the characteristics of stemness in culture were obtained. Therefore, such cultures could be used in preclinical studies. Obtaining populations of stem cells that preserve stem immunophenotype, proliferation, and differentiation potentials during their expansion are of great interest in regenerative medicine. These characteristics allow using them in

studies that seek to replicate the cell environment and its processes in vivo, in a safe and reliable way (13,51), in both cell and cell-free therapies (52).

Considering the limited regeneration potential of dental and periodontal tissues, in addition to the high incidence of pathologies that lead to their loss, translational research using stem cells and animal models seeks to find therapeutic alternatives that can be applied to humans (2) and that resolve limitations of current treatments. The characteristics displayed by bone marrow-derived stem cells, such as differentiation potential, self-renewal capacity, and immune regulation, have positioned them as a key element in gene therapy, tissue engineering, and replacement therapy applications (53). Although there are limitations in humans due to its low availability (54) and invasiveness of the process to obtaining it (55), in animal models these limitations can be reduced since the marrow tissue can be acquired from experimental colonies. Thus, the implementation of cell cultures from animal tissues becomes the basis for the establishment of reliable and predictable clinical protocols (44).

Because knowledge of the self-maintenance and differentiation mechanisms of stem cells helps to understand a variety of processes, from embryogenesis and oncogenic transformation (56), the development of methods for isolation and expansion may be the basis for dental tissue regeneration projects. They include those being conducted in our group (57). The cytokines and extracellular particles found in their conditioned media are also particularly useful for these cultures (58), which is why, in regenerative medicine, their paracrine activity has become an alternative approach for their use (59).

Bone marrow, which is especially useful because it is found in the prototype mesenchymal stem cell, has been recognized for more than three decades and has been the most studied source of stem cells (11,16,60,61). This tissue has been studied especially in mice (62) and not so much in rats (44). Rats are a model of choice in studies of physiology, behavior, and complex human diseases (63); therefore, research groups use them. In this study we obtained functional populations of stem cells derived from bone marrow, which were homogenized with the depletion of CD45+ cell subpopulations.

CD45 or leukocyte common antigen is expressed exclusively in the nucleated cells of the marrow hematopoietic fraction and is key in the activation of immune system cells (64), its expression, although at a low level, is not desirable. Therefore, we depleted our populations in the first passage, resulting in negative subcultures in the expression of this marker with an expression profile like that described in other studies (25,29,65-67). The separation method of these subpopulations did not affect the stem characteristics of the cultures since the cells were minimally manipulated. Although this depletion method had only been reported for blood cells (45,68) it shows effectiveness in the present study in the separation of subpopulations of the hematopoietic fraction that may interfere with stem cell characterization.

The immunophenotype found in the subpopulations after CD45+ depletion corresponds to that reported in other studies in which they are highly positive for CD90 (25,66), an important regulator of mesenchymal differentiation processes (69) with a proven critical role in deciding the fate of MSCs (70). It was highly positive for CD29, acting as a receptor for the union of the cell with the extracellular matrix and with other cells, whose positivity in stem cells has been related to greater cell survival when these are applied in regenerative therapies. It was also highly positive for CD146, whose expression in MSCs of multiple organs has been associated with the potential for differentiation into three lineages (71). Like in other studies, our findings show that both the cell surface protein CD71, related to intracellular iron transportation, and the cell surface protein CD106, which participates in cell immunomodulation, have extremely low positivity values (44,72).

Cellular confluence was reached approximately two weeks after starting the cultures, as has been reported in other studies (72,73). The greater the confluence, the greater the proportion of cells adhered to the surface of the culture flask with tapered and fibroblastoid morphology. This cell proliferation also showed the formation of fibroblastoid colony units, as has been reported (25,44), especially in the primary cultures. In initial subcultures, we also identified a subpopulation of polygonal cells as some have been reported

somewhere else (72), which shows that these cultures correspond to heterogeneous populations that can be purified as the expansion progresses, either due to the basic characteristic of stem cells adhering to plastic or due to the selectivity of the culture medium used.

In the P2-P5 subcultures, the cell morphology was more homogeneous, most cells were elongated, fibroblastoid in appearance, aligned, and grouped. Each cell group of the latter showed a different direction, findings that are also reported in similar studies (72,73). H&E staining showed that most cells in cultures are mononucleated with large, elongated, and centrally located nuclei, as described in other studies (65,72). On the other hand, with Masson trichrome staining, type I collagen fibers were clearly visible in the extracellular spaces. Those fibers may be associated with the formation of an extracellular matrix, which has the ability of forming cell layers, which are a useful tool in tissue engineering experiments (74).

The subcultures showed that populations of this study conserved the capacity for multi-differentiation. The induced cultures experienced a change in morphology accompanied by the formation of a mineralized extracellular matrix when they were induced to the osteogenic lineage. The presence of intracytoplasmic lipid vacuoles indicated differentiation to pre-adipocytes and in the chondrogenic lineage showed the formation of a cartilage matrix. Osteogenic differentiation was also confirmed by an increase in alkaline phosphatase activity.

Studies with stem cells have shown that, despite originating from the same tissue, they have enormous variability from donor to donor (75). In this study, we used Lewis rats belonging to an inbred lineage to reduce syngeneic variability; the members of the colony are the result of mating in the first degree of consanguinity. In addition, the marrows used as stem cell source tissue were grouped into batches to include the same number of males and females in each batch, following indications of the U.S. National Institutes of Health regarding the need to include both males and females in all clinical and preclinical research (76,77). For years of research, females were not part of studies for considering that hormonal variations due to the estrous cycle could affect findings (77,78). However, studies have shown that there are no sex-related differences in neuroscience research (76) or evidence that female rats show greater inter-individual variability than males in immunological traits (77). This issue is still not completely clear so, to achieve the greatest homogeneity in the populations characterized in this study, we used tissues from both males and females.

Conducting this study allowed us to validate a methodology to culture populations of stem cells from rat bone marrow at the right densities to use in cell-based therapies or in vitro cell models, in which the collection is expected. A biological asset such as stem cells with defined characteristics that are heterogeneous, contain highly proliferative cells with the potential for differentiation into multiple lineages, and are collectable in sufficient amounts to develop tissue engineering protocols. In addition, this study provides key elements that justify studies with populations of stem cells derived from human tissues.

CONCLUSIONS

We developed a simple and efficient method to isolate and expand bone marrow-derived stem cells from rats and dental pulp, lacking the expression of hematopoietic markers, and capable of differentiating *in vitro* up to passage five to osteogenic, chondrogenic, and adipogenic lineages. Additionally, this method allows obtaining MSCs at high densities for use in tissue bioengineering experiments.

References

1. Mizukami A, Swiech K. Mesenchymal Stromal cells: from discovery to manufacturing and commercialization. Stem Cells Int. 2018 Apr 11; 2018: 4083921. https://dx.doi.org/110.1155/2018/4083921

2. Tatullo M, Codispoti B, Paduano F, Nuzzolese M, Makeeva I. Strategic Tools in Regenerative and Translational Dentistry. Int J Mol Sci. 2019 Apr 16; 20(8): 1879. https://dx.doi.org/10.3390/ijms20081879

3. McGovern JA, Griffin M, Hutmacher DW. Animal models for bone tissue engineering and modelling disease. Dis Model Mech. 2018 Apr 23; 11(4): dmm033084. https://dx.doi.org/10.1242/dmm.033084

4. Kumar R, Sharma A, Pattnaik AK, Varadwaj PK. Stem cells: An overview with respect to cardiovascular and renal disease. J Nat Sci Biol Med. 2010 Jul; 1(1): 43-52. https://dx.doi.org/10.4103/0976-9668.71674

5. Goradel NH, Hour FG, Negahdari B, Malekshahi ZV, Hashemzehi M, Masoudifar A, Mirzaei H. Stem Cell Therapy: A New Therapeutic Option for Cardiovascular Diseases. J Cell Biochem. 2018 Jan; 119(1): 95-104. https://dx.doi.org/10.1002/jcb.26169

6. Faça VM. Human mesenchymal stromal cell proteomics: contribution for identification of new markers and targets for medicine intervention. Expert Rev Proteomics. 2012 Apr; 9(2): 217-230. https://dx.doi.org/10.1586/epr.12.9

7. Yelick PC, Sharpe PT. Tooth Bioengineering and Regenerative Dentistry. J Dent Res. 2019 Oct; 98(11): 1173-1182. https://dx.doi.org/10.1177/0022034519861903

8. De Luca M, Aiuti A, Cossu G, Parmar M, Pellegrini G, Robey PG. Advances in stem cell research and therapeutic development. Nat Cell Biol. 2019 Jul; 21(7): 801-811. https://dx.doi.org/10.1038/s41556-019-0344-z

9. Liu JQ, Li QW, Tan Z. New Insights on Properties and Spatial Distributions of Skeletal Stem Cells. Stem Cells Int. 2019 Jun 3; 2019: 9026729. https://dx.doi.org/10.1155/2019/9026729

10. Bianco P, Robey PG, Saggio I, Riminucci M. "Mesenchymal" stem cells in human bone marrow (skeletal stem cells): a critical discussion of their nature, identity, and significance in incurable skeletal disease. Hum Gene Ther. 2010 Sep; 21(9): 1057-1066. https://dx.doi.org/10.1089/hum.2010.136

11. Caplan AI. Mesenchymal stem cells. J Orthop Res. 1991 Sep; 9(5): 641-650. https://dx.doi.org/10.1002/jor.1100090504

12. Samsonraj RM, Raghunath M, Nurcombe V, Hui JH, van Wijnen AJ, Cool SM. Concise Review: Multifaceted Characterization of Human Mesenchymal Stem Cells for Use in Regenerative Medicine. Stem Cells Transl Med. 2017 Dec; 6(12): 2173-2185. https://dx.doi.org/10.1002/sctm.17-0129

13. Sipp D, Robey PG, Turner L. Clear up this stem-cell mess. Nature. 2018 Sep; 561(7724): 455-457. https://dx.doi.org/10.1038/d41586-018-06756-9

14. Caplan AI. Mesenchymal Stem Cells: Time to Change the Name! Stem Cells Transl Med. 2017 Jun; 6(6): 1445-1451. https://dx.doi.org/10.1002/sctm.17-0051

15. Friedenstein AJ, Petrakova KV, Kurolesova AI, Frolova GP. Heterotopic of bone marrow. Analysis of precursor cells for osteogenic and hematopoietic tissues. Transplantation. 1968 Mar; 6(2): 230-247

16. Friedenstein AJ, Chailakhyan RK, Latsinik NV, Panasyuk AF, Keiliss-Borok IV. Stromal cells responsible for transferring the microenvironment of the hemopoietic tissues. Cloning in vitro and retransplantation in vivo. Transplantation. 1974 Apr; 17(4): 331-340. https://dx.doi.org/10.1097/00007890-197404000-00001

17. Pelekanos RA, Sardesai VS, Futrega K, Lott WB, Kuhn M, Doran MR. Isolation and Expansion of Mesenchymal Stem/Stromal Cells Derived from Human Placenta Tissue. J Vis Exp. 2016 Jun 6; (112): 54204. https://dx.doi.org/10.3791/54204

18. Amati E, Sella S, Perbellini O, Alghisi A, Bernardi M, Chieregato K, Lievore C, Peserico D, Rigno M, Zilio A, Ruggeri M, Rodeghiero F, Astori G. Generation of mesenchymal stromal cells from cord blood: evaluation of in vitro quality parameters prior to clinical use. Stem Cell Res Ther. 2017 Jan 24; 8(1): 14. https://dx.doi.org/10.1186/s13287-016-0465-2

19. Bieback K, Netsch P. Isolation, Culture, and Characterization of Human Umbilical Cord Blood-Derived Mesenchymal Stromal Cells. Methods Mol Biol. 2016; 1416: 245-258. https://dx.doi.org/10.1007/978-1-4939-3584-0_14

20. Nagamura-Inoue T, He H. Umbilical cord-derived mesenchymal stem cells: Their advantages and potential clinical utility. World J Stem Cells. 2014 Apr 26; 6(2): 195-202. https://dx.doi.org/10.4252/wjsc.v6.i2.195

21. Li CY, Wu XY, Tong JB, Yang XX, Zhao JL, Zheng QF, Zhao GB, Ma ZJ. Comparative analysis of human mesenchymal stem cells from bone marrow and adipose tissue under xeno-free conditions for cell therapy. Stem Cell Res Ther. 2015 Apr 13; 6(1): 55. https://dx.doi.org/10.1186/s13287-015-0066-5

22. Orciani M, Di Primio R. Skin-derived mesenchymal stem cells: isolation, culture, and characterization. Methods Mol Biol. 2013; 989: 275-283. https://dx.doi.org/10.1007/978-1-62703-330-5_21

23. Choi WY, Jeon HG, Chung Y, Lim JJ, Shin DH, Kim JM, Ki BS, Song SH, Choi SJ, Park KH, Shim SH, Moon J, Jung SJ, Kang HM, Park S, Chung HM, Ko JJ, Cha KY, Yoon TK, Kim H, Lee DR. Isolation and characterization of novel, highly proliferative human CD34/CD73-double-positive testis-derived stem cells for cell therapy. Stem Cells Dev. 2013 Aug 1; 22(15): 2158-2173. https://dx.doi.org/10.1089/scd.2012.0385

24. Appaix F, Nissou MF, van der Sanden B, Dreyfus M, Berger F, Issartel JP, Wion D. Brain mesenchymal stem cells: The other stem cells of the brain? World J Stem Cells. 2014 Apr 26; 6(2): 134-143. https://dx.doi.org/10.4252/wjsc.v6.i2.134

25. Alge DL, Zhou D, Adams LL, Wyss BK, Shadday MD, Woods EJ, Gabriel Chu TM, Goebel WS. Donor-matched comparison of dental pulp stem cells and bone marrow-derived mesenchymal stem cells in a rat model. J Tissue Eng Regen Med. 2010 Jan; 4(1): 73-81. https://dx.doi.org/10.1002/term.220

26. Yamada Y, Nakamura S, Ito K, Sugito T, Yoshimi R, Nagasaka T, Ueda M. A feasibility of useful cell-based therapy by bone regeneration with deciduous tooth stem cells, dental pulp stem cells, or bone-marrow-derived mesenchymal stem cells for clinical study using tissue engineering technology. Tissue Eng Part A. 2010 Jun; 16(6): 1891-900. https://dx.doi.org/10.1089/ten.TEA.2009.0732

27. Kunimatsu R, Nakajima K, Awada T, Tsuka Y, Abe T, Ando K, Hiraki T, Kimura A, Tanimoto K. Comparative characterization of stem cells from human exfoliated deciduous teeth, dental pulp, and bone marrow-derived mesenchymal stem cells. Biochem Biophys Res Commun. 2018 Jun 18; 501(1): 193-198. https://dx.doi.org/10.1016/j.bbrc.2018.04.213

28. Kozlowska U, Krawczenko A, Futoma K, Jurek T, Rorat M, Patrzalek D, Klimczak A. Similarities and differences between mesenchymal stem/progenitor cells derived from various human tissues. World J Stem Cells. 2019 Jun 26; 11(6): 347-374. https://dx.doi.org/10.4252/wjsc.v11.i6.347

29. Guadix JA, Zugaza JL, Gálvez-Martín P. Characteristics, applications and prospects of mesenchymal stem cells in cell therapy. Med Clin (Barc). 2017 May 10; 148(9): 408-414. English, Spanish. https://dx.doi.org/10.1016/j.medcli.2016.11.033

30. Alhadlaq A, Mao JJ. Mesenchymal stem cells: isolation and therapeutics. Stem Cells Dev. 2004 Aug;13(4):436-48. https://dx.doi.org/10.1089/scd.2004.13.436

31. Chahla J, Mannava S, Cinque ME, Geeslin AG, Codina D, LaPrade RF. Bone Marrow Aspirate Concentrate Harvesting and Processing Technique. Arthrosc Tech. 2017 Apr 10; 6(2): e441-e445. https://dx.doi.org/10.1016/j.eats.2016.10.024

32. Soundararajan M, Kannan S. Fibroblasts and mesenchymal stem cells: Two sides of the same coin? J Cell Physiol. 2018 Dec; 233(12): 9099-9109. https://dx.doi.org/10.1002/jcp.26860

33. Dominici M, Le Blanc K, Mueller I, Slaper-Cortenbach I, Marini F, Krause D, Deans R, Keating A, Prockop Dj, Horwitz E. Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. Cytotherapy. 2006; 8(4): 315-7. https://dx.doi.org/10.1080/14653240600855905

34. Bianco P, Cao X, Frenette PS, Mao JJ, Robey PG, Simmons PJ, Wang CY. The meaning, the sense and the significance: translating the science of mesenchymal stem cells into medicine. Nat Med. 2013 Jan; 19(1): 35-42. https://dx.doi.org/10.1038/nm.3028

35. Nakashima M, Iohara K, Bottino MC, Fouad AF, Nör JE, Huang GT. Animal Models for Stem Cell-Based Pulp Regeneration: Foundation for Human Clinical Applications. Tissue Eng Part B Rev. 2019 Apr; 25(2): 100-113. . https://dx.doi.org/10.1089/ten.TEB.2018.0194

36. Sandoval RM, Molitoris BA, Palygin O. Fluorescent Imaging and Microscopy for Dynamic Processes in Rats. Methods Mol Biol. 2019; 2018: 151-175. https://dx.doi.org/10.1007/978-1-4939-9581-3_7

37. Jacob HJ. The rat: a model used in biomedical research. Methods Mol Biol. 2010; 597:1-11. https://dx.doi.org/10.1007/978-1-60327-389-3_1

38. Beldick SR, Hong J, Altamentova S, Khazaei M, Hundal A, Zavvarian MM, Rumajogee P, Chio J, Fehlings MG. Severe-combined immunodeficient rats can be used to generate a model of perinatal hypoxic-ischemic brain injury to facilitate studies of engrafted human neural stem cells. PLoS One. 2018 Nov 28; 13(11): e0208105. https://dx.doi.org/10.1371/journal.pone.0208105

39. He D, Zhang J, Wu W, Yi N, He W, Lu P, Li B, Yang N, Wang D, Xue Z, Zhang P, Fan G, Zhu X. A novel immunodeficient rat model supports human lung cancer xenografts. FASEB J. 2019 Jan; 33(1): 140-150. https://dx.doi.org/10.1096/fj.201800102RR

40. Konopelski P, Ufnal M. Electrocardiography in rats: a comparison to human. Physiol Res. 2016 Nov 23; 65(5): 717-725. https://dx.doi.org/10.33549/physiolres.933270

41. Agarwal S, Harter ZJ, Krishnamachary B, Chen L, Nguyen T, Voelkel NF, Dhillon NK. Sugen-morphine model of pulmonary arterial hypertension. Pulm Circ. 2020 Feb 4; 10(1): 2045894019898376. https://dx.doi.org/10.1177/2045894019898376

42. Liao J, Cui C. Generation and Characterization of Rat iPSCs. Methods Mol Biol. 2016; 1357: https://dx.doi.org/133-48. 10.1007/7651_2015_200

43. Mähler Convenor M, Berard M, Feinstein R, Gallagher A, Illgen-Wilcke B, Pritchett-Corning K, Raspa M. FELASA recommendations for the health monitoring of mouse, rat, hamster, guinea pig and rabbit colonies in breeding and experimental units. Lab Anim. 2014 Jul; 48(3): 178-192. https://dx.doi.org/10.1177/0023677213516312

44. Ridzuan N, Al Abbar A, Yip WK, Maqbool M, Ramasamy R. Caracterización y expresión del marcador de senescencia en pasajes prolongados de células madre mesenquimales derivadas de médula ósea de rata. Células Madre Int. 2016; 2016: 8487264. https://dx.doi.org/10.1155/2016/8487264

45. Ahmed N, Vogel B, Rohde E, Strunk D, Grifka J, Schulz MB, Grässel S. CD45-positive cells of haematopoietic origin enhance chondrogenic marker gene expression in rat marrow stromal cells. Int J Mol Med. 2006 Aug; 18(2): 233-240.

46. Davies OG, Cooper PR, Shelton RM, Smith AJ, Scheven BA. A comparison of the in vitro mineralisation and dentinogenic potential of mesenchymal stem cells derived from adipose tissue, bone marrow and dental pulp. J Bone Miner Metab. 2015 Jul; 33(4): 371-82. https://dx.doi.org/10.1007/s00774-014-0601-y

47. Wen Y, Yang H, Liu Y, Liu Q, Wang A, Ding Y, Jin Z. Evaluation of BMMSCs-EPCs sheets for repairing alveolar bone defects in ovariectomized rats. Sci Rep. 2017 Nov 29; 7(1): 16568. https://dx.doi.org/10.1038/s41598-017-16404-3

48. Sprio AE, Di Scipio F, Raimondo S, Salamone P, Pagliari F, Pagliari S, Folino A, Forte G, Geuna S, Di Nardo P, Berta GN. Self-renewal and multipotency coexist in a long-term cultured adult rat dental pulp stem cell line: an exception to the rule? Stem Cells Dev. 2012 Dec 10; 21(18): 3278-88. https://dx.doi.org/10.1089/scd.2012.0141

49. Kaibuchi N, Iwata T, Yamato M, Okano T, Ando T. Terapia de lámina de células estromales mesenquimales multipotentes para la osteonecrosis de la mandíbula relacionada con bisfosfonatos en un modelo de rata. Acta Biomater. 2016 15 de sep; 42: 400-410. https://dx.doi.org/10.1016/j.actbio.2016.06.022

50. Komada Y, Yamane T, Kadota D, Isono K, Takakura N, Hayashi S, Yamazaki H. Origins and properties of dental, thymic, and bone marrow mesenchymal cells and their stem cells. PLoS One. 2012; 7(11): e46436. https://dx.doi.org/10.1371/journal.pone.0046436

51. Prowse AB, Chong F, Gray PP, Munro TP. Stem cell integrins: implications for ex-vivo culture and cellular therapies. Stem Cell Res. 2011 Jan; 6(1): 1-12. https://dx.doi.org/10.1016/j.scr.2010.09.005

52. Hu L, Liu Y, Wang S. Stem cell-based tooth and periodontal regeneration. Oral Dis. 2018 Jul; 24(5): 696-705. https://dx.doi.org/10.1111/odi.12703

53. Wang J, Liu S, Li J, Zhao S, Yi Z. Roles for miRNAs in osteogenic differentiation of bone marrow mesenchymal stem cells. Stem Cell Res Ther. 2019 Jun 28; 10(1): 197. https://dx.doi.org/10.1186/s13287-019-1309-7

54. Chahla J, Dean CS, Moatshe G, Pascual-Garrido C, Serra Cruz R, LaPrade RF. Concentrated Bone Marrow Aspirate for the Treatment of Chondral Injuries and Osteoarthritis of the Knee: A Systematic Review of Outcomes. Orthop J Sports Med. 2016 Jan 13; 4(1): 2325967115625481. https://dx.doi.org/10.1177/2325967115625481

55. Mazini L, Rochette L, Amine M, Malka G. Regenerative Capacity of Adipose Derived Stem Cells (ADSCs), Comparison with Mesenchymal Stem Cells (MSCs). Int J Mol Sci. 2019 May 22; 20(10): 2523. https://dx.doi.org/10.3390/ijms20102523

56. Lagarkova MA. Such Various Stem Cells. Biochemistry (Mosc). 2019 Mar; 84(3): 187-189. https://dx.doi.org/10.1134/S0006297919030015

57. Jaramillo L, Briceño I, Durán C. Odontogenic cell culture in PEGDA hydrogel scaffolds for use in tooth regeneration protocols. Acta Odontol Latinoam. 2012; 25(3): 243-254

58. Chen W, Sun Y, Gu X, Cai J, Liu X, Zhang X, Chen J, Hao Y, Chen S. Conditioned medium of human bone marrow-derived stem cells promotes tendon-bone healing of the rotator cuff in a rat model. Biomaterials. 2021 Apr; 271: 120714. https://dx.doi.org/10.1016/j.biomaterials.2021.120714

59. Rodríguez-Saenz Á, Martínez-Carreño M, Munévar JC. The therapeutic potential of stem cells secretome. Rev CES Odont. 2018; 31(2): 10.

60. Pittenger MF, Mackay AM, Beck SC, Jaiswal RK, Douglas R, Mosca JD, Moorman MA, Simonetti DW, Craig S, Marshak DR. Multilineage potential of adult human mesenchymal stem cells. Science. 1999 Apr 2; 284(5411): 143-147. https://dx.doi.org/10.1126/science.284.5411.143

61. Yianni V, Sharpe PT. Perivascular-Derived Mesenchymal Stem Cells. J Dent Res. 2019 Sep; 98(10): 1066-1072. https://dx.doi.org/10.1177/0022034519862258

62. Mashimo T, Sato Y, Akita D, Toriumi T, Namaki S, Matsuzaki Y, Yonehara Y, Honda M. Bone marrow-derived mesenchymal stem cells enhance bone marrow regeneration in dental extraction sockets. J Oral Sci. 2019; 61(2): 284-293. https://dx.doi.org/10.2334/josnusd.18-0143

63. Smith JR, Bolton ER, Dwinell MR. The Rat: A Model Used in Biomedical Research. Methods Mol Biol. 2019; 2018: https://dx.doi.org/1-41.d10.1007/978-1-4939-9581-3_1

64. Chamberlain G, Fox J, Ashton B, Middleton J. Concise review: mesenchymal stem cells: their phenotype, differentiation capacity, immunological features, and potential for homing. Stem Cells. 2007 Nov; 25(11): 2739-2749. https://dx.doi.org/10.1634/stemcells.2007-0197

65. Yusop N, Battersby P, Alraies A, Sloan AJ, Moseley R, Waddington RJ. Isolation and Characterisation of Mesenchymal Stem Cells from Rat Bone Marrow and the Endosteal Niche: A Comparative Study. Stem Cells Int. 2018 Mar 22; 2018: 6869128. https://dx.doi.org/10.1155/2018/6869128

66. Harrington J, Sloan AJ, Waddington RJ. Quantification of clonal heterogeneity of mesenchymal progenitor cells in dental pulp and bone marrow. Connect Tissue Res. 2014; 55 Suppl 1: 62-67. https://dx.doi.org/10.3109/03008207.2014.923859

67. Yu J, Wang Y, Deng Z, Tang L, Li Y, Shi J, Jin Y. Odontogenic capability: bone marrow stromal stem cells versus dental pulp stem cells. Biol Cell. 2007 Aug; 99(8): 465-474. https://dx.doi.org/10.1042/BC20070013

68. Nicodemou A, Danisovic L. Mesenchymal stromal/stem cell separation methods: concise review. Cell Tissue Bank. 2017 Dec; 18(4): 443-460. https://dx.doi.org/10.1007/s10561-017-9658-x

69. Moraes DA, Sibov TT, Pavon LF, Alvim PQ, Bonadio RS, Da Silva JR, Pic-Taylor A, Toledo OA, Marti LC, Azevedo RB, Oliveira DM. A reduction in CD90 (THY-1) expression results in increased differentiation of mesenchymal stromal cells. Stem Cell Res Ther. 2016 Jul 28; 7(1): 97. https://dx.doi.org/10.1186/s13287-016-0359-3

70. Saalbach A, Anderegg U. Thy-1: more than a marker for mesenchymal stromal cells. FASEB J. 2019 Jun; 33(6): 6689-6696. https://dx.doi.org/10.1096/fj.201802224R

71. Crisan M, Yap S, Casteilla L, Chen CW, Corselli M, Park TS, Andriolo G, Sun B, Zheng B, Zhang L, Norotte C, Teng PN, Traas J, Schugar R, Deasy BM, Badylak S, Buhring HJ, Giacobino JP, Lazzari L, Huard J, Péault B.

A perivascular origin for mesenchymal stem cells in multiple human organs. Cell Stem Cell. 2008 Sep 11; 3(3): 301-313. https://dx.doi.org/10.1016/j.stem.2008.07.003

72. He Q, Ye Z, Zhou Y, Tan WS. Comparative study of mesenchymal stem cells from rat bone marrow and adipose tissue. Turk J Biol. 2018 Dec 10; 42: 477-489. https://dx.doi.org/10.3906/biy-1802-52
73. Li C, Wei G, Gu Q, Wen G, Qi B, Xu L, Tao S. Donor Age and Cell Passage Affect Osteogenic Ability of Rat Bone Marrow Mesenchymal Stem Cells. Cell Biochem Biophys. 2015 Jun; 72(2): 543-9. https://dx.doi.org/10.1007/s12013-014-0500-9
74. Jiang Z, Xi Y, Lai K, Wang Y, Wang H, Yang G. Laminin-521 Promotes Rat Bone Marrow Mesenchymal Stem Cell Sheet Formation on Light-Induced Cell Sheet Technology. Biomed Res Int. 2017; 2017: 9474573. https://dx.doi.org/10.1155/2017/9474573
75. McLeod CM, Mauck RL. On the origin and impact of mesenchymal stem cell heterogeneity: new insights and emerging tools for single cell analysis. Eur Cell Mater. 2017 Oct 27; 34: 217-231. https://dx.doi.org/10.22203/eCM.v034a14
76. Becker JB, Prendergast BJ, Liang JW. Female rats are not more variable than male rats: a meta-analysis of neuroscience studies. Biol Sex Differ. 2016 Jul 26; 7: 34. https://dx.doi.org/10.1186/s13293-016-0087-5
77. Zajitschek SR, Zajitschek F, Bonduriansky R, Brooks RC, Cornwell W, Falster DS, Lagisz M, Mason J, Senior AM, Noble DW, Nakagawa S. Sexual dimorphism in trait variability and its eco-evolutionary and statistical implications. Elife. 2020 Nov 17; 9: e63170. https://dx.doi.org/10.7554/eLife.63170
78. Beery AK. Inclusion of females does not increase variability in rodent research studies. Curr Opin Behav Sci. 2018 Oct; 23: 143-149. https://dx.doi.org/10.1016/j.cobeha.2018.06.016

Notes

* Original research.

How to cite this article: Pirela Labrador AE, Tangarife Tobón LF, Roa Molina NS, Durán Correa C, Jaramillo Gómez LM. Rat Bone Marrow Stem Cell Culture for Use in Tissue Regeneration. Univ Odontol. 2022; 41. https://doi.org/10.11144/Javeriana.uo41.rbms