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

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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

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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 eutanasiadas del linaje *Lewis*. 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; 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 commonly 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, some 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 different 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 establishing 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) mainly due to the presence of other cell types such as fibroblasts, which are morphologically identical and present surface markers similar to 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 new ideas, concepts, and technologies. Regenerative medicine, for example, has advanced as a result 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 very 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 2, 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*.

16 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 (2 females and 2 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 sufficient quantities in a given 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 ºC for 5 minutes in a centrifuge (Thermo Scientific™ Heraeus Biofuge Primo R 41272826). Cell cultures were carried out in 25 cm^2 culture flasks (Corning 3053) and kept at 37 ºC 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 ºC 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, DMi1) and culture medium changes were performed every two days.

Obtaining Primary Cultures

Aseptic dissection of the skin, muscle tissue, and periosteum of the femur and tibia bones of the hind limbs of euthanized rats was performed, following the Ridzuan protocol (44). The epiphyses of these bones were removed and the medullary lavage was performed at one end with a syringe loaded with culture medium. The collected lavage was mechanically disintegrated with the same syringes and washed twice with culture medium. The total number of cells were counted and seeded in culture flasks at a concentration of 5x105 cells for each flask. At the time of completing 95 % confluence, the primary culture (P0) was considered established.

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

Cells in PO were trypsinized, collected by centrifugation, counted and incubated with 10µl/1x106 cells of an antiCD45 PE (BD Pharmingen™ clone Ox-1, 554878) for 15 minutes at 4 °C to purify the CD45+ population (45), then were incubated for 15 minutes at 4 °C with 10µl 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) through which the CD45+ were retained and the CD45- were collected in the eluate, corroborating the purity of the separation by flow cytometry, the CD45- population were counted and subcultured to advance towards the first passage (P1). Starting at P1, each culture that reached cell confluence was subcultured until reaching P5.

Immunophenotypic Characterization of Crops

The immunophenotypic characteristics of the cultures from P0-P5 were determined by 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). The 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

Cell proliferation was evaluated by means of a colorimetric method that determines the number of viable cells in proliferation, using the CellTiter 96® commercial kit (Promega G3580) for which the manufacturer's instructions were followed, briefly, the cells were seeded in plates. Multi-wells of 96 wells (SPL life sciences) at a concentration of 3.2x103cell/well, after the evaluation time 12, 24, 48, 72 and 96 hours, the culture medium was removed from each well and a solution of the kit aqueous reagent in culture medium in a 1:5 ratio and left incubating at 37 °C for 4 hours, after which the optical density was read in a plate reader (Biotek ELx 800). To determine the number of cells for each absorbance value, optical density measurements were made at different concentrations of adhered cells and a cell growth equation was determined, through which the number of cells was found for each determined absorbance.

Morphological Evaluation of Crops

The morphological characteristics of the cultures were evaluated every two days by means of an inverted microscope (Leica, DMi1) through which the adherence to the surface of the culture flasks, the growth pattern and the cell confluence were evaluated. When they reached confluence, this 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 evaluated was determined by seeding cells in 6-well multi-well plates (Corning 3506) at a concentration of 2.5x105cell/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 quantified microscopically. Colonies with more than 30 cells were considered CFU-F. The following equation was used to calculate the efficiency in CFU-F:

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

Crop Multi-Differentiation Potential

Cell cultures were subjected to 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 ratio of 1.5x105 cells/well. 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 of dexamethasone (Sigma-Aldrich D4902), 50 μM of ascorbic acid (Sigma-Aldrich A4544) and 10 μM of β glycerol phosphate (Sigma-Aldrich G9422), cultures were maintained for 21 days with the differentiation induction medium, after which they were 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 visualized 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), 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. times with PBS (Sigma P3813), then they were fixed with a 4 % formaldehyde solution for

30 minutes, then the cells were washed and finally visualized in a light microscope and the 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 of 3-isobutyl-1-methylxanthine-IBMX (Sigma-Aldrich I5879), 5 μg/ml of insulin (Sigma-Aldrich I5500) and 200 μM of indomethacin (Sigma-Aldrich I7378. The stain used to identify this differentiation is Oil Red O (Sigma O1395) at 0.5 % added to the cultures induced, washed and fixed for 30 minutes, after which they were viewed under a light microscope and photographic images were taken.

Statistical Analysis

All experiments were repeated at least three times. Data are presented as the mean ± SD. Statistically significant values were defined when $p < 0.05$. By means of the Shapiro-Wilk test, the normality of the data was verified, by means of ANOVA and Tukey's *post hoc* test, the differences between the expression of cell surface markers, cell proliferation, efficiency in the formation of colonies and the concentration of alkaline phosphatase. Student's T test was used to determine differences in the percentage of cells positive for the expression of typical stem cell markers. For all the analyses, the SPSS version 20.0 program (IBM, New York, USA) was used.

RESULTS

Primary crops

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

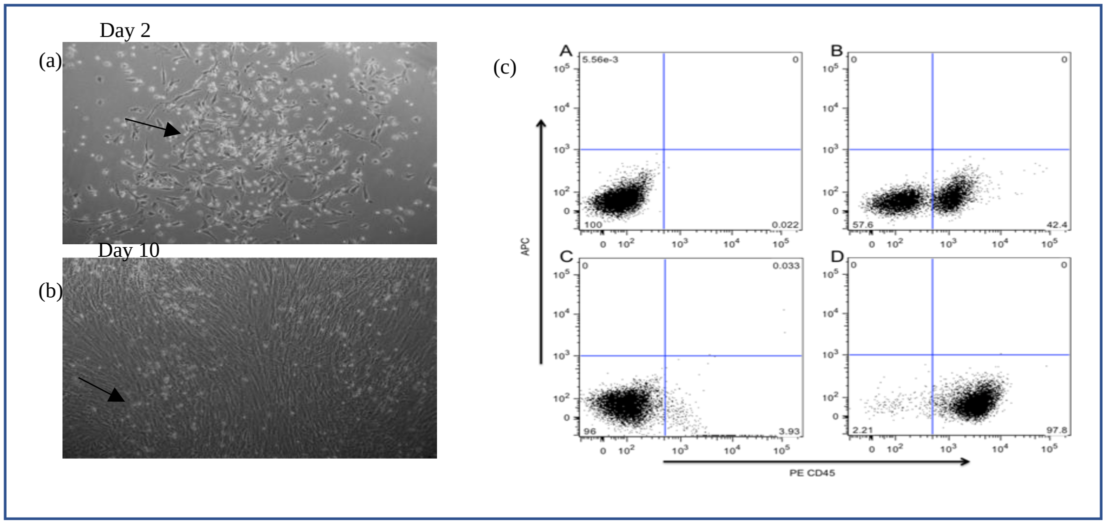

FIGURE 1

Characteristics of primary cultures derived from rat bone marrow. (a) Morphological characteristics of the primary cultures (10X), photomicrograph of the cells that grew adhered to the surface of the flask two days after starting the primary culture in which a population of small, round and refringent cells and another can be seen. population of stellate cells and fibroblastoid appearance. (b) Day 10 after starting the primary culture. In both (a) and (b) a growth pattern is observed in CFU-F (arrow). (c) Depletion of CD45+ cells, representative dotted image obtained by 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.

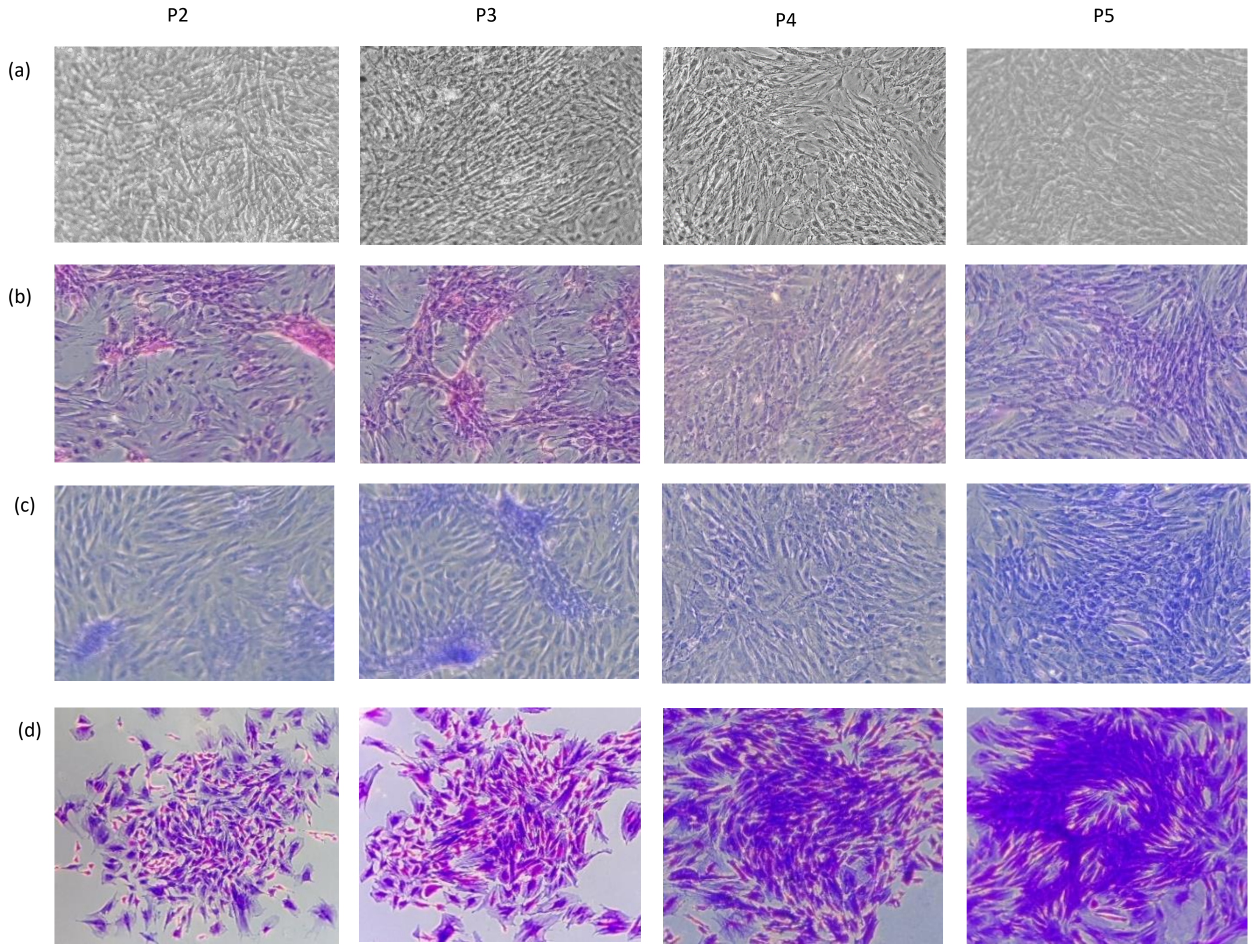

FIGURE 2
Characteristics of rat bone marrow stem cell cultures. (a) Microphotographs (10X) representative of the cultures in the different passages, two populations were identified, the first with large and rounded cells and the second with a predominance of spindle cells and fibroblastoids growing in 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, which had a value of 86.03 % ± 1.95. For CD45, the value was 42.46 % ± 3.75, this being a typical marker of the hematopoietic cell portion of the marrow, a positivity value was considered and through a separation column with magnetic beads, these cells were isolated from the 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, finding that the purity of the selection was approximately 97 % (Figure 1c). From the separation of the subpopulations with positivity for CD45, the CD45- population was sub-cultured to advance in 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 the surface of the culture flasks was identified, which, as the 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). Masson's Trichrome staining showed the presence of collagen deposits at the level of the intercellular spaces, characteristic of the formation of a cellular monolayer 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).

Immuno-Phenotyping

The positivity of the typical stem cell markers increased from P1, especially CD90 showed high positivity in all passages evaluated and CD45 was exceptionally low after selection of CD45- cells in the primary culture. The value of the CD90 marker was maintained within those recommended for the 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. Due to the above, in passages four and five it can be considered that due to the expression of the markers CD90, CD29, CD146 (Table 1) said populations meet the stem criteria. Regarding the markers CD71 and CD106 (Table 1), although they present positivity, said 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 the passages, a progressive increase in the number of cells was found from 12 h to 96 h, it was observed that as the passage increased, the number of cells increased at each evaluated time, P5 being better than the previous ones (Figure 3a). The determination of the number of cells in each passage and in each evaluated time allowed establishing the necessary time in each passage to obtain a given quantity of a population of stem cells that can be used in a tissue regeneration experiment, in passage five per for example, and after 96 hours of culture, 16,000 cells can be recovered for each well of a 96-well multi-well plate (Figure 3b).

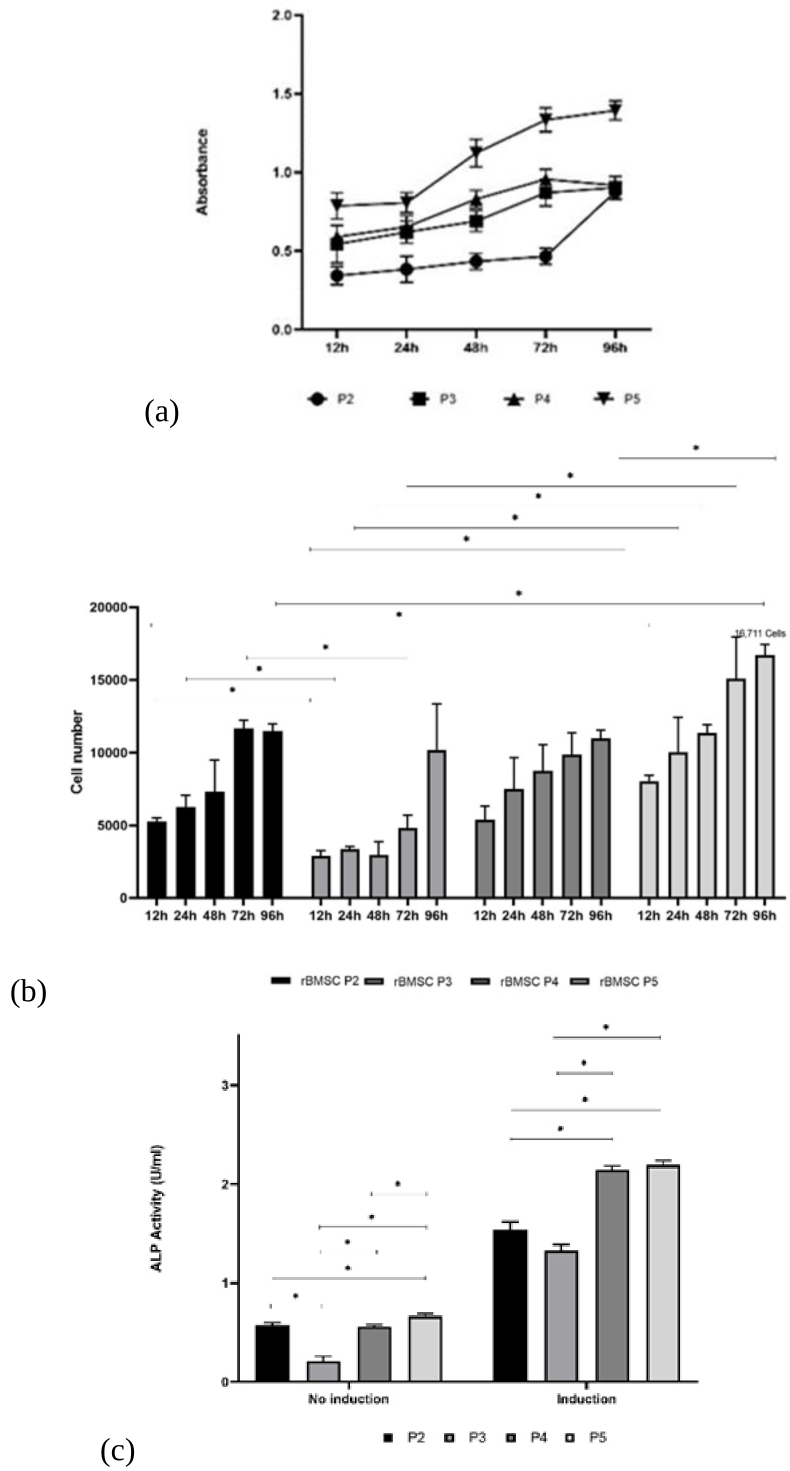

FIGURE 3

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 through the inverted microscope, the colonies were counted and their formation efficiency was determined, finding that in all the passages the culture presented a morphological pattern of cells that converge towards a center with a predominance of spindle cells and fibroblastoids with an efficiency of around 60 %, with P2 being the passage in which the highest value was found (Table 1).

Multi-Differentiation Potential

After the cultures reached 60 %, they were maintained for 21 days with the differentiation media and in each culture dish wells with culture medium without induction were maintained for the same 21 days to be used as controls for the differentiation tests. Regarding osteogenic differentiation, bone marrow stem cell cultures were positive for Alizarin Red staining, through which the formation of calcium nodules separated from each other was observed; said nodules were observed better defined and with a more intense coloration in passages four and five (Figure 4a). Alizarin red staining made it possible to visualize the morphological change of the cultures induced with osteogenic medium, through which the prevalence of spindle cells and fibroblastoids decreased and the appearance of polygonal cells on the cultures was observed. Additionally, osteogenic differentiation in cultures was corroborated by determining alkaline phosphatase activity, finding that it increased with induction time and that as passages progressed, alkaline phosphatase activity also increased (Figure 3c).

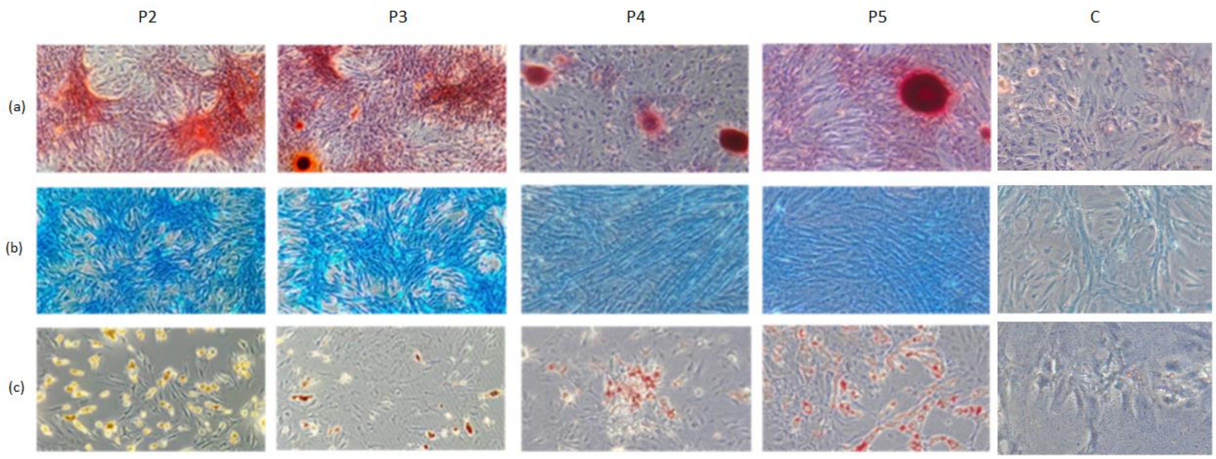

FIGURE 4
Multi-differentiation of rat bone marrow stem cells. (a) Cultures in passages P2-P5 and negative control (C) stained with Alizarin Red. (b) Cultures in passages P2-P5 and negative control (C) with Alcian Blue staining. (c) Cultures in passages P2-P5 and negative control (C) with Oil Red O staining.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

The induction with chondrogenic differentiation medium produced a change in the morphology of the cells that was identified as a change in the orientation of the growth of the cells, additionally, the positivity of the Alcian Blue staining that was greater to the extent that 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 that acquired an oval shape and in these the Oil Red O staining allowed the identification of lipid droplets., indicating positivity for this lineage (Figure 4c).

DISCUSSION

In the present study, an efficient methodology for the isolation, expansion, and characterization of stem cells derived from the bone marrow of Lewis rats was validated, by means of which populations are obtained that maintain the characteristics of stemness in culture and, therefore, the possibility of being used in preclinical studies. Obtaining populations of stem cells that preserve the stem immunophenotype, proliferation and differentiation potentials during their expansion, are of great interest in regenerative medicine, these characteristics allow their use in studies that seek to replicate the cellular environment and its processes in vivo. in a safe and reliable way (13,51), both in 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 some of the 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). and although there are some limitations in humans due to its low availability (54) and the invasiveness of the process of obtaining it (55), in animal models these limitations can be reduced since the marrow tissue can be acquired from experimental colonies, being the implementation of cell cultures from animal tissues is the basis for the establishment of reliable and predictable clinical protocols (44).

Based on the fact that 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 their isolation and expansion may be the basis For dental tissue regeneration projects such as those being carried out in our group (57), the cytokines and extracellular particles found in their conditioned media are also very useful for these cultures (58), which is why, in regenerative medicine their paracrine activity has been found to be an alternative approach for their use (59).

The bone marrow, which is especially useful, since the prototype mesenchymal stem cell is found in it, has been recognized for more than three decades and has therefore 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). Because rats are a model of choice in studies of physiology, behavior, and complex human diseases (63), several research groups have selected them; In this study, functional populations of stem cells derived from bone marrow were obtained, which were homogenized with the depletion of CD45+ cell subpopulations.

CD45 or leukocyte common antigen is expressed exclusively in the nucleated cells of the hematopoietic fraction of the marrow and is key in the activation of immune system cells (64), its expression, although at a low level, is not desirable, therefore in our populations were depleted in the first passage, achieving that the subcultures were negative in the expression of this marker and presenting an expression profile very similar to 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 and although this depletion method had only been reported for blood cells (45,68) in the present study it shows its effectiveness. in the separation of subpopulations of the hematopoietic fraction that may interfere with the characterization of stem cells.

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). Highly positive for CD29, which acts 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. Also, highly positive for CD146, whose expression in MSCs of multiple organs has been associated with the potential for differentiation into three lineages (71). As in other studies, the findings of this one show that both the cell surface protein CD71, related to intracellular iron transport, 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 the start of 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 and in the initial subcultures a subpopulation of polygonal cells was also identified as some that have been reported (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 of the cells were elongated, fibroblastoid in appearance, aligned and grouped, each cell group of these, with a different direction, findings also reported in other studies with this type. of cells (72,73). H&E staining showed that in cultures most of the cells are mononucleated and that these nuclei are large, elongated and centrally located, as described in some other studies (65,72), while with trichrome staining of Masson, type I collagen fibers were clearly visualized in the extracellular spaces, fibers that may be associated with the formation of an extracellular matrix and this matrix with the ability of these populations to form cell sheets, which are a very useful tool in tissue engineering experiments (74).

The subculturing showed that the 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 the increase in alkaline phosphatase activity

Studies with stem cells have shown that although they are derived from the same tissue, they have enormous variability from donor to donor (75). In this study, in order to reduce this variability, Lewis rats belonging to an inbred lineage were used. syngeneic, the members of the colony are the result of mating in the first degree of consanguinity. Additionally, the marrows used as stem cell source tissue were grouped into batches to include the same number of males and females in each batch, in accordance with what was indicated by the National Institute of Health of the United States, regarding the need to include both to males and females in all clinical and preclinical research (76,77). During many years of research, females were not included in the studies considering that hormonal variations due to the estrous cycle could affect the results (77,78), however some studies have shown that there are no differences in the sex of the rats involved. in neuroscience research (76) or that females show greater variability between individuals in immunological traits while males in morphological traits (77), being a subject that is still not completely clear and in order to achieve the greatest homogeneity in the populations characterized in this study were used together with tissues from males and females.

Carrying out this study allowed the validation of a methodology for culturing populations of rat bone marrow stem cells, at the right densities to be used in cell-based therapies or in in vitro cell models, in which the collection is expected. of a biological asset such as stem cells with defined characteristics, which, being heterogeneous, contain highly proliferative cells, with the potential for differentiation into multiple lineages and which can be collected in sufficient numbers for the development of tissue engineering protocols; additionally, this study contributes 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.

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