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ORIGINAL ARTICLE

Characterization of human mesenchymal stem cell according to delivery route and its correlation with clinical-gynecological history

Caracterización de células troncales mesenquimales humanas según la vía de parto y su correlación con la historia clínica ginecológica

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Abstract

Objective: *To assess the pluripotency profile of human mesenchymal stem cells (hMSCs) as influenced by delivery mode and obstetric-gynecological background.* **Methods:** *Thirty-nine placentas were included. A fragment of the AM (5 cm) was utilized to obtain the AM-hMSCs. The cell cultures were monitored until 90% confluence, before carrying out expansion (passages). The cultures, cell morphology, adhesion, immunophenotyping characterization, and differentiation capacity were evaluated and compared with hMSC from bone marrow (BM-hMSC). T-student and Mann-Whitney U tests were carried out. Statistical significance was set at a $p < 0.05$.* **Results:** *No differences were observed in the proliferation and expansion of AM-hMSC obtained by vaginal or cesarean delivery route. Similar results were observed in the immunological characterization; however, CD105 was significantly lower compared with BM-hMSC. Nevertheless, cells from vaginal or cesarean delivery route showed great differentiation capacity to adipogenic, chondrogenic, and osteogenic lineages.* **Conclusions:** *The delivery route, clinical data, and obstetric and gynecologic histories are no limitations for using the AM to obtain hMSC and its possible application in Regenerative Medicine.*

Keywords: Delivery route. Obstetric history. Mesenchymal stem cells. Amniotic membrane. Immunophenotyping. Cell differentiation.

Resumen

Objetivo: *Caracterizar la pluripotencialidad de las CTMh de acuerdo con la vía de parto y los antecedentes obstétricos y ginecológicos.* **Métodos:** *Se incluyeron 39 placentas; las CTMh-MA fueron obtenidas de la MA. Los cultivos fueron monitoreados hasta el 90% de confluencia. La morfología, la adhesión, la caracterización inmunológica y la capacidad de diferenciación se evaluaron y compararon con CTMh de medula ósea (CTMh-MO). Se realizaron las pruebas t de Student y U de Mann-Whitney, y se consideró un valor $p < 0.05$.* **Resultados:** *No se observaron diferencias en la proliferación de las CTMh-MA obtenidas por vía vaginal o por cesárea. La caracterización inmunológica no mostró diferencias según la vía de parto; sin embargo, CD105 fue menor comparado con las CTMh-MO. No obstante, las células mostraron capacidad de diferenciarse al*

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linaje adipogénico, condrogénico y osteogénico sin importar su origen. **Conclusiones:** *La vía de resolución y los antecedentes obstétricos no son limitantes para el uso de CTMh de MA y su posible aplicación en medicina regenerativa.*

Palabras clave: *Canal de parto. Antecedentes obstétricos. Células troncales mesenquimales. Membrana amniótica. Inmunotipificación. Diferenciación celular.*

Introduction

Organ demand and regeneration strategies are currently a relevant topic, worldwide. Reports show many Mexicans on transplant waiting lists every year. Even though said transplantations are successfully carried out in Mexico, the shortage of organs remains constant[1].

Regenerative medicine (RM) and tissue engineering (TE) are a milestone in the replacement or regeneration of cells, organs, and tissues[2,3]. TE is mainly composed by three fundamental elements: autologous or allogenic cells with a multipotent or pluripotent regenerative potential, scaffolds as cellular support, and growth factors[4-8].

Human mesenchymal stem cells (hMSCs) are cells with the potential to differentiate into mesenchymal lineages (osteoblasts, chondroblasts, adipocytes), and under special culture conditions, they can trans-differentiate into endodermal (liver, lung, pancreas) and ectodermal (neuron-type) lineages[9-11]. The use of hMSCs and their application in organ restoration has attracted the attention of scientists and the clinical community[12-14]. In the past decade, the number of clinical trials related to hMSCs has seen a significant increase worldwide (1,138 records on clinicaltrial.gov). Several medical fields have shown promising results; for example, in cardiology, the administration of hMSCs has demonstrated improvements in dilated cardiomyopathy. Similar benefits have been observed in both ischemic and non-ischemic heart failure. Other medical specialties such as traumatology, pneumology, hepatology, nephrology, cartilage lesions, osteoarthritis, and immunomodulation of immune diseases (e.g., host versus graft reaction, rheumatoid arthritis) have also considered the use of hMSCs as a promising approach to restore tissue and organ function[14,15].

At present, hMSCs obtained from bone marrow (BM) are considered the gold standard. However, their retrieval through BM aspiration is an invasive procedure that must be performed in an operating room by an expert physician. In recent years, sources other than BM for obtaining hMSCs have been reported that include adipose tissue, muscle, dental pulp, amniotic membrane (AM), Wharton's jelly, and umbilical cord blood; even the number of cells from those sources has been described to be equal to or higher than the quantities directly retrieved from BM[16]. There are currently few standardized protocols for hMSCs collection. Moreover, the administration routes and the optimized number of cells are currently under evaluation in accordance with the pathology[17]. Furthermore, before its use as a clinically recommended procedure, pre-clinical and clinical studies need to be carried out.

The quality of the donated tissue must also be considered in relation to donor age, the timing of tissue collection, and tissue preservation, before collecting the hMSCs[18].

Controversial data regarding placenta-derived hMSCs and the association between the quality of the placenta and the age of the mother has been reported; placentas corresponding to mothers above 35 years of age are presumed to have poor vascular perfusion and delayed villous maturation[19].

In Mexico, a considerable number of pregnant women are overweight and obese. They gain even more weight per month during pregnancy, conditioning the presentation of hypertensive diseases associated with pregnancy. That pathophysiologic condition alters the blood flow and the exchange of gases (oxygen and carbon dioxide) and nutrients between the mother and fetus, not only affecting the product but also the epithelial and mesenchymal cells of the placental membranes[20-22].

The placenta tends to be thought of as biologic waste, with no ethical implications regarding its processing; it can be donated through signing a statement of informed consent. Different studies have considered the AM an alternative for retrieving hMSCs[23-25]. Nevertheless, at present, there are no studies associating the capacity of *in vitro* hMSCs proliferation with donor age and body mass index (BMI) or the delivery route of the pregnancy (vaginal delivery versus cesarean section). Therefore, the aim of the present work was to analyze the epidemiologic characteristics of the population seen at the Department of Obstetrics and Gynecology and the relation of those characteristics to the proliferation and

expansion, expression of multipotential markers, and the capacity differentiation of hMSCs obtained from placentas donated through signed statements of Informed Consent.

Methods

Patients

A cross-sectional study was conducted that included patients seen at the Department of Obstetrics and Gynecology of the Hospital General de México "Dr. Eduardo Liceaga." Inclusion criteria were primiparous or multiparous women between 20 and 36 years of age, at 38-40 weeks of gestation.

For the present study, some donors with comorbidities, such as arterial hypertension and diabetes mellitus (DM) were included, as well as donors with negative serology for human immunodeficiency virus, hepatitis B virus, and hepatitis C virus. Placentas with a gestation period of > 40 weeks or < 38 weeks, membrane rupture of 12 or more hours of progression, patients with fever (> 38°C) or with diagnosis of fetal suffering, and positive serology test were excluded.

BMI was calculated, complete blood count, blood chemistry (BC), and prothrombin time were determined at the central laboratory of the Hospital General de México "Dr. Eduardo Liceaga." The AM of the placental tissue was processed for hMSCs retrieval. All the patients signed statements of informed consent The present study was approved by the research and ethics committees of the Hospital General de México "Dr. Eduardo Liceaga" (CI/315/15) and the School of Medicine at the Universidad Nacional Autónoma de México (DI/115/2015) and was conducted in accordance with the principles described in the 1975 Declaration of Helsinki.

AM collection and processing

Regardless of the delivery route (vaginal or cesarean) of the placenta obtained, the placental tissue was placed in a metallic sterile bowl to identify the periumbilical zone of the fetal surface. A circular fragment of approximately 5 cm^2 was obtained. The AM was placed in sterile phosphate buffer solution (PBS), pH 7.2 (1X, Gibco, USA), and then transferred to PBS + antibiotic-antimycotic (1X, Gibco, USA). After 10 min, the AM was recovered and the processing was performed, following the protocol reported by León-Mancilla et al., 2021[17].

hMSCs retrieval

To obtain the hMSCs, the AM was digested with Trypsin-ethylenediaminetetraacetic acid (EDTA) (0.05%) (Gibco, USA) at 37°C for 1 h. Once the digestion time was over, the tissue was incubated with Collagenase Type II (0.01%) (Gibco, USA) for 2 h at 37°C. The samples were then centrifuged at 2,000 rpm/10 min and the cell pellet was resuspended in 5 mL of DMEM-HG (high in glucose) culture medium (Gibco, USA). The cell count was carried out using an automated system (Bio-Rad, TC20™). A density of 2.5×10^4 cells/cm^2 was adjusted and harvested in culture bottles with 5 mL of DMEM culture medium, supplemented with 10% fetal bovine serum (Biowest, France), HEPES (Sigma Aldrich, Germany), and antibiotic-antimycotic ×1 (Gibco, USA). The cells were maintained at 37°C in a 5% CO_2 atmosphere. After 24 h, the culture medium was changed, to remove the cells that did not adhere to the culture flask.

The cell cultures were monitored, until reaching confluence above 80% (approximately 15 days), to perform the first culture passage (P1). Briefly, the cells were incubated with trypsin-EDTA (0.05%) for 5 min at 37°C, the cell suspension was centrifuged at 2,000 rpm/10 min, and the cell pellet was resuspended in 5 mL of DMEM medium. The cell count was performed, and repeat harvesting was carried out with 2.5×10^4 cells/cm^2. This procedure was repeated until the cells no longer had the capacity to proliferate.

Evaluation of hMSCs cultures through phase contrast microscopy

The hMSCs morphology was evaluated through phase contrast microscopy and scanning electron microscopy. For phase contrast microscopy viewing, the cells were observed directly on the culture bottles (T25), employing the Nikon Eclipse TE 2,000-S (Tokyo, Japan) inverted microscope. The images were obtained with a Nikon DMX 1,200 (Tokyo, Japan) camera and analyzed using the equipment software. In addition, adhesion capacity was evaluated through scanning microscopy.

Evaluation of hMSCs cultures through the collagen matrix (cell scaffold)

Approximately 2.5×10^4 cells/cm^2 were placed in a collagen scaffold obtained from Nukbone®. After 1 h

of incubation, the samples were placed in Zamboni (Newcomer supply, USA) fixative solution for 24 h. At the end of the fixation time, the samples were washed and fixed with 1% osmium tetroxide in 0.1M sodium cacodylate and covered with colloidal gold. The images were obtained using DSM-950 and EVO 10 (Zeiss, Germany) scanning electron microscope (SEM).

Immunophenotyping characterization

The AM-hMSCs membrane immune markers were analyzed by flow cytometry as was previously reported[17]. Briefly, AM-hMSCs from the third passage were selected and trypsinized. A total of 4×10^4 cells was incubated with the human MSCs analysis kit (BD Biosciences, Franklin Lakes, NJ, USA), following the supplier's recommendations. The kit contains antibodies anti-CD90, anti-CD73, anti-CD44, and anti-CD105, as multipotential markers. Whereas CD34, CD11b, CD45, CD19, and HLA-DR were used as negative immunophenotype markers (hematopoietic stem cells, HSC). The BM-hMSC immunophenotype characterization was used as a positive control. 20,000 events were analyzed by FACSCanto II flow cytometer and the data were processed using the FACSDiva software package (BD Biosciences, Franklin Lakes, NJ, USA). Three independent experiments were performed.

Differentiation of AM-hMSCs to specific lineages

Approximately, 4×10^4 cells were seeded in culture dishes (3 mm), after 21 days of culture the cells were incubated with the specific differentiation media,[24]. For chondrogenic differentiation was used chondrogenic medium (Cambrex Bio Science, NJ, USA) supplemented with transforming growth factor-β (TGF-β) (Cambrex Bio Science, NJ, USA).

The osteogenic medium was supplemented with ascorbic acid and β-glycerolphosphate and the adipogenic medium was supplemented with pre-mix ITS (Stem Cell Technologies Inc., Vancouver, BC, Canada). The specific differentiation was revealed using Alcian blue (Sigma-Aldrich, St. Louis, MO, USA) for chondrogenic lineage, von Kossa stain for the osteogenic phenotype, and red-O oil (Sigma-Aldrich, Germany) for the adipogenic differentiation. Images were obtained by Nikon Microphoto FDA, a Nikon DMX camera, and Nikon ACT software (Tokyo, Japan) Three independent experiments.

Statistical analysis

The quantitative variables were expressed as mean and standard deviation and the qualitative variables as median and percentage. The sample total was divided by age group and delivery route (vaginal versus cesarean). The comparisons were made using the t-test or the Mann-Whitney test for independent groups. The X^2 test was utilized to analyze the categorical variables. A 95% α significance level and statistical significance at a $p < 0.05$ were considered. All the statistical analyses were carried out using the IBM statistics package for Windows, version 21.0, Armonk, N.Y. IBM Corp.

Results

Demographic data of the placental tissue donors

Thirty-nine pregnant women were included in the study, 17 of whom were under 25 years of age (range from 18 to 25 years) and 22 of whom were above 25 years of age (range from 25 to 37 years). Under the selected age criterion, 11 placentas were recovered from vaginal deliveries and 28 from cesarean sections, with no differences between the age range and type of delivery route at which the placentas were obtained. The mean BMI of the two groups was in the obese range. The main occupation was housewife, with no differences in smoking or alcohol use, with respect to age (Table 1).

Regarding the biochemical data, there were only differences in uric acid between vaginal deliveries and cesarean sections, whereas the biochemical parameters, such as leukocytes, platelets, glucose, and others, were similar in the two groups (Table 2).

Obstetric and gynecologic data according to delivery route

In the obstetric and gynecologic history analysis, there were no differences between the criteria analyzed, with respect to vaginal delivery or cesarean section (Table 3).

Regarding the comorbidity analysis, no diagnosed clinical condition in the vaginal delivery donors was found, whereas 46.4% of the placenta donors that had a cesarean section presented with some type of comorbidity (Table 4).

Table 1. Demographic data according to age group

Delivery route and anthropometric data	Under 25 years of age, n = 17 (%)	Over 25 years of age, n = 22 (%)	p
Vaginal delivery, n (%)[ϐ]	4 (23.5)	7 (31.8)	0.568
Cesarian section, n %)[ϐ]	13 (76.5)	15 (68.2)	
BMI (kg/m^2)[†]	30.01 ± 5.0	32.7 ± 5.8	0.659
Normal[ϐ]	1 (5.9)	2 (9.2)	0.153
Overweight[ϐ]	8 (47.1)	4 (18.2)	
Obese[ϐ]	8 (47.1)	16 (72.7)	
Educational level			
Years (Q3 and Q1)	12 (6,16)	12 (6,16)	0.624
(Max and min)[¥]	(12,9)	(12,9)	
Occupation, n (%)[ϐ]			
Housewife	17 (100)	18 (81.8)	0.179
Businesswoman	-	2 (9.1)	
Student	-	2 (9.1)	
Drug addictions and smoking[ϐ]			
Alcohol use (no)	16 (94.1)	19 (84.6)	0.429
Smoking	15 (88.2)	17 (77.3)	0.376

[†]Significant t-test for independent samples.
[ϐ]Significant X^2 test for contingency tables.
[¥]Significant Mann-Whitney U test for independent samples. Significance was a $p < 0.05$ in all the statistical tests. BMI: body mass index.

Table 2. Biochemical values of the placenta donors, by delivery route (vaginal or cesarean)

Hematological and biochemical parameters	Vaginal delivery, n = 11 (%)	Cesarean section, n = 28 (%)	p
Leukocytes (10^3/μL)[†]	11.55 ± 2.32	11.47 ± 3.21	0.692
Neutrophils[†]	74.54 ± 16.84	73.83 ± 14.21	0.284
Hemoglobin (g/dL)[†]	11.65 ± 1.70	21.57 ± 55.18	0.237
[†]Platelets×10^3	258.3 ± 86.5	196.1 ± 62.8	0.181
Urea[†]	15.79 ± 2.66	17.2 ± 5.31	0.104
Creatinine[†]	0.64 ± 0.11	0.60 ± 0.14	0.626
Uric acid[†]	5.62 ± 0.59	5.35 ± 1.55	0.015
Glucose, n (%)[ϐ]			
< 80	6 (54.5)	15 (53.6)	0.713
80-90	2 (18.2)	8 (28.6)	
> 91	3 (27.3)	5 (17.9)	

[†]Significant t-test for independent samples. [ϐ]Significant X^2 test for contingency tables. Statistical significance, $p < 0.05$.

In vitro proliferation of the mesenchymal stem cells retrieved from the AM

The proliferation of the AM-hMSCs retrieved from the vaginal delivery and cesarean section placentas was evaluated through bright field microscopy. In general, all the cultures demonstrated similar behavior. Adaptation was apparent from 24 h and 85-90% confluence was reached at culture day 14 (Fig. 1).

The SEM results revealed hMSCs adhesion to a scaffold, as well as filopodium projection in the cells, confirming the adhesion capacity, and indirectly, the metabolic activity of the hMSCs (Fig. 2).

The cultures that did not progress to the subsequent passages were registered and categorized in groups by age and delivery route of the placentas obtained (Tables 5 and 6). The percentage of the P0-P3 cultures in the placentas obtained and organized by age group (under 25 years of age and over 25 years of age) was 88.2% and 72.7%, respectively, with no statistically significant difference between the two age groups (Table 5). Similarly, upon evaluating the culture passages, with respect to the delivery route of the placentas obtained, 81.8% of the cell cultures from vaginal deliveries and 78.6% from the cesarean section were between P0 and P3 (Table 6). Importantly, there were no statistically significant differences related to age group or delivery route of the placenta, with respect to the capacity of the cells to progress to the next passages.

AM-hMSCs from vaginal and cesarean through showed express similar levels of multipotential markers

After to observe that delivery route and the obstetric condition do not contribute on the cell proliferation of AM-hMSCs, we evaluated the expression of multipotential markers on cell obtained from vaginal and cesarean via. Interestingly, the multiple comparisons did not show differences in the expression of CD90+, CD73+, CD44+, and CD105+ in vaginal delivery versus cesarean (Fig. 3). However, hMSCs from BM display higher levels of endoglin (CD105+) compared with AM-hMSCs from both delivery routes. Moreover, the hematopoietic stem cell (HSC) markers showed an insignificant population in all the cases evaluated (Fig. 3).

AM-hMSCs can differentiated to adipogenic, chondrogenic, and osteogenic lineages

After observed that the delivery route does not show differences in the proliferation, adhesion, expression of multipotent markers, we evaluated the differentiation capacity. The data showed that each differentiation

Table 3. Obstetric and gynecologic history, according to the delivery route

Obstetric and gynecologic parameters	Vaginal delivery, n = 11 (%)	Cesarean section, n = 28 (%)	p
Pregnancy history (# pregnancies)[8]			
One pregnancy	4 (36.4)	15 (53.6)	0.200
Two pregnancies	6 (54.5)	7 (25.0)	
Multiple pregnancies	1 (9.1)	6 (21.4)	
Weight before pregnancy[†]	66.64 ± 13.12	66.22 ± 13.45	0.874
Weight after pregnancy[†]	78.27 ± 16.98	76.64 ± 15.34	0.786
Capurro[8]			
Pre-term	-	2 (7.1)	0.417
Full term	11 (100)	24 (85.7)	
Post-term	-	2 (7.1)	
Sex of the product (female), n (%)	5 (45.5)	10 (35.7)	
Weight of the product (g)[†]	3281.8 ± 448.8	2950.7 ± 544.7	0.249
Fetal pathology n (%)	5 (12.8)	0	
Prenatal control (yes), n (%)[8]	7 (63.6)	23 (82.1)	0.217
Multivitamin use (yes), n (%)[8]	8 (72.7)	22 (78.6)	0.697

[†]Significant t-test for independent samples. [8]Significant X^2 test for contingency tables. Statistical significance, $p < 0.05$.

Table 4. Comorbidities in the group of placenta donors, by delivery route

Condition	Vaginal birth, n = 11 (%)	Cesarean section, n = 28 (%)	p[8]
Absent	11 (100)	15 (53.6)	0.662
Gestational diabetes		2 (7.1)	
Cytomegalovirus		1 (3.6)	
Asthma		2 (7.1)	
Deep vein thrombosis		1 (3.6)	
Gestational thrombocytopenia		1 (3.6)	
Diabetes mellitus		1 (3.6)	
Intellectual disability		1 (3.6)	
Primary hypothyroidism		1 (3.6)	
Severe pre-eclampsia		1 (3.6)	
Left renal agenesia		1 (3.6)	

[8]Significant X^2 test for contingency tables. Statistical significance is a $p < 0.05$.

Table 5. Comparison of culture passages with donor age

Passage, n (%)[8]	Under 25 years of age, n = 17 (%)	Above 25 years of age, n = 22 (%)	p
P0	6 (35.3)	3 (13.6)	0.592
P1	5 (29.4)	6 (27.3)	
P2	2 (11.8)	3 (13.6)	
P3	2 (11.8)	4 (18.2)	
P4	-	2 (9.1)	
P5 or more	2 (11.8)	4 (18.2)	
P0-3	15 (88.2)	16 (72.7)	0.234
p > 3	2 (11.8)	6 (27.3)	

[8]Significant X^2 test for contingency tables. Statistical significance is a $p < 0.05$.

media allowed the correct differentiation to the three compromised phenotypes. In other words, AM-hMSCs obtained from the placenta of vaginal or cesarean delivery have the same capacity to differentiate at adipogenic, chondrogenic, and osteogenic lineages (Fig. 4).

Discussion

The amnion, or human AM, is the most internal of the fetal membranes, and so is in direct contact with the amniotic fluid and the fetus[23]. The AM is known to have an epithelial layer, a basal membrane, and a mesenchymal layer. Mesenchymal cells are multipotent and pluripotent cells that can differentiate into the three germ cell layers[26]. The hMSCs retrieval from the AM is advantageous, compared with hMSCs retrieval from BM, given that the placenta is obtained non-invasively and requires only the informed consent of the donor. In previous studies, our working group described the differentiation capacity and the expression of surface markers, as well as the genes (*oct-4, nanog, sox-2*) expressed by the hMSCs obtained from the AM but did not report the proliferation and expansion of the cells obtained or their relation to obstetric and gynecologic data[17].

Due to social, economic, educational, and medical factors, there has been an increase in recent years in

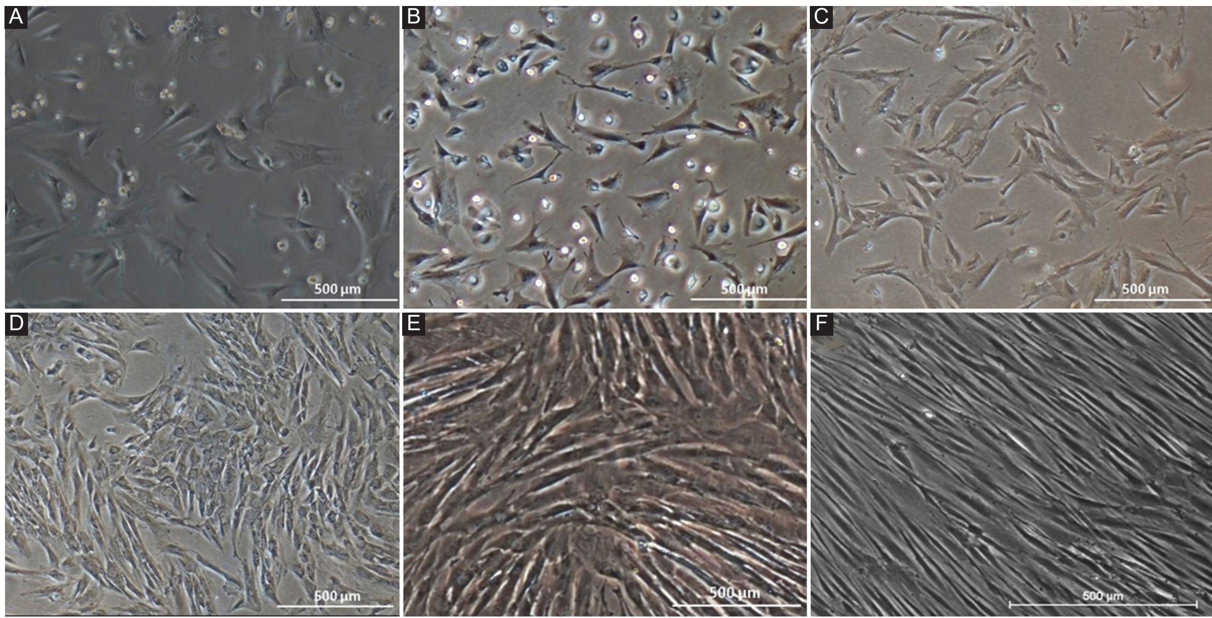

Figure 1. *Growth progression of mesenchymal stem cells from the placenta. Representative images of cell proliferation evaluated at,* **(A)** *24 h,* **(B)** *3 d,* **(C)** *7 d,* **(D)** *10 d, and* **(E)** *14 d from the cells retrieved from placentas at vaginal delivery, showing an increase in confluence. Similar pattern is observed in hMSCs from bone marrow at* **(F)** *day 14. hMSCs: human mesenchymal stem cells.*

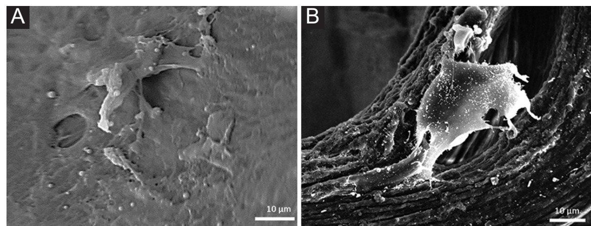

Figure 2. *Electron microscopy evaluation of hMSCs on a collagen structure of bovine origin. The adhesion capacity of the cells retrieved from placentas at cesarean section* **(A)** *and at vaginal delivery* **(B)** *is observed. Cytoplasmic projections and mesenchymal cell morphology type are also observed. hMSCs: human mesenchymal stem cells.*

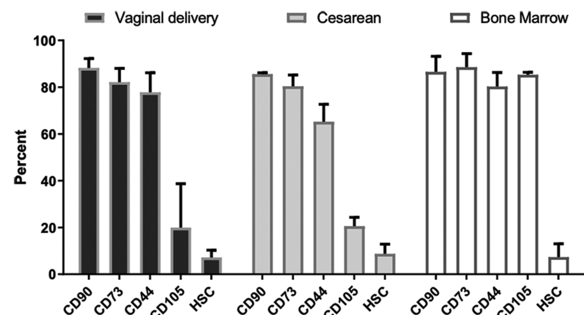

Figure 3. *Expression of pluripotential markers in AM-hMSCs according with the delivery route. Cells obtained from AM obtained from vaginal and cesarean route. The immunophenotyping was performed using CD90, CD73, CD44 and CD105. Hematopoietic stem cells (HSC), markers: CD34, CD11b, CD45, CD19, and HLA-DR were used as negative immunophenotype markers. Bone Marrow cells were used for multiple comparison of each marker. Data were expressed as the mean ± standard deviation. $p < 0.001$ for multiple comparison was consider. AM-hMSCs: amniotic membrane-human mesenchymal stem cells.*

the number of older pregnant patients[20]. In 2010, through multivariate analysis, the age of older placenta donors (above 35 years of age) was shown to be related to a significant decrease in the quantity of total protein, bFGF, HGF, KGF, NGF, and TGF-β1, compared with younger donors[27-29]. The same behavior was observed with respect to gestational stage, finding a decrease in the abovementioned factors, mainly from 280 gestational days to 294 gestational days in white women[27]. Contrastingly, in our study population, there were no differences in the women divided into groups of below 25 years of age and above 25 years of age, with respect to the demographic data or the proliferation and expansion capacity of the hMSCs in culture. We also found no statistically significant differences regarding weight between the two age groups (data not shown). In both the vaginal delivery and cesarean section groups, the BMI of the women revealed some degree of obesity. Those data are correlated with the predominant condition in the Mexican population[30,31]. In addition, the risk for presenting with maternal obesity and susceptibility to its associated diseases has been reported to increase during pregnancy[31].

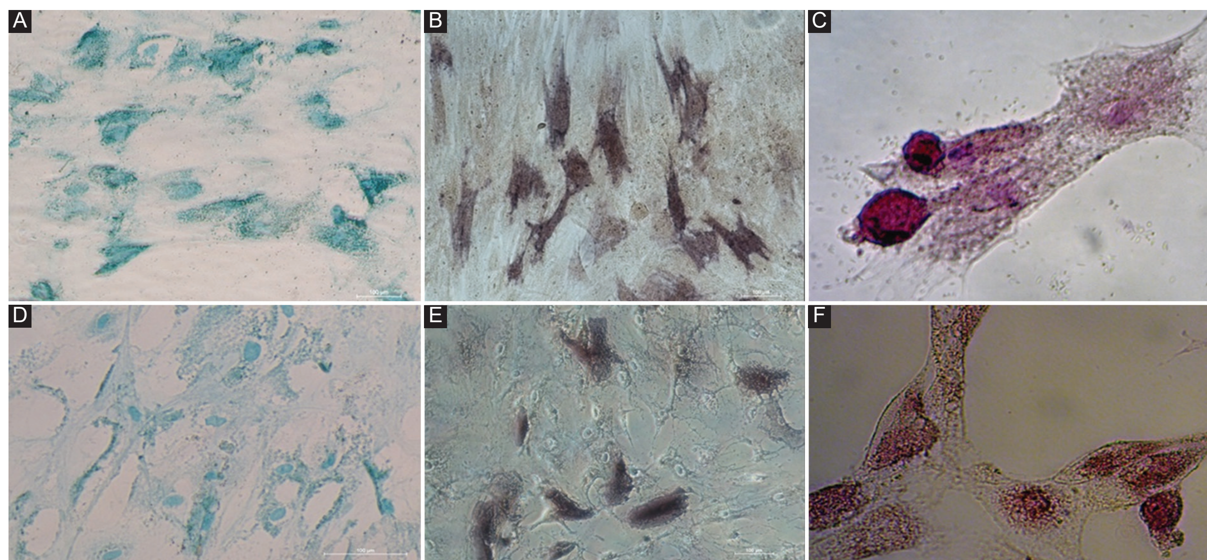

Figure 4. *Differentiation capacity of AM-hMSCs to chondrogenic, osteogenic and adipogenic in accordance with delivery via. Cells were stained using* **A** and **D:** *alcian blue,* **B** and **E:** *Von Kossa and* **C** and **F:** *oil red, to confirm chondrogenic, osteogenic, and adipogenic lineages, respectively.* **A:** *AM-hMSCs from placenta obtained of vaginal delivery incubated with chondrogenic,* **B:** *osteogenic and* **C:** *adipogenic differentiation media.* **D:** *AM-hMSCs from placenta obtained of cesarean delivery incubated with chondrogenic,* **E:** *osteogenic, and* **F:** *adipogenic differentiation media. AM-hMSCs: amniotic membrane-human mesenchymal stem cells.*

Table 6. Comparison of culture passages with the type of amniotic membrane mesenchymal stem cell retrieval

Passage, n (%)[§]	Vaginal delivery, n = 11 (%)	Cesarean section, n = 28 (%)	p
P0	5 (45.5)	4 (14.3)	0.287
P1	3 (27.3)	8 (28.6)	
P2	1 (9.1)	4 (14.3)	
P3	-	6 (21.4)	
P4	1 (9.1)	1 (3.6)	
P5 or more	1 (9.1)	5 (17.8)	
P0-3	9 (81.8)	22 (78.6)	0.821
p > 3	2 (18.2)	6 (21.4)	

[§]Significant X^2 test for contingency tables. Statistical significance is a $p < 0.05$.

A proteomic study on AM-hMSCs from donors that had a cesarean section revealed that obesity (BMI: 42.7 ± 7.7) produced the dysregulation of 62 proteins. Those proteins participate in the regulation pathways of oxidative stress and the regulation of the cytoskeleton and metabolic routes. However, the implications in the newborn or the mother are still being studied[31]. Despite the fact that our study groups of vaginal delivery and cesarean section did not have a BMI above 40, the possible alterations in AM-hMSCs proteins or genes are important to consider. We observed no apparent changes in proliferation or expansion of the cell cultures in either group. From the reported evidence and our results, we consider that to preserve good conditions in the hMSCs retrieved from the placenta, they should be collected from donors under 30 years of age, with a BMI no higher than 30 years. Similarly, in our study, we found that the obstetric and gynecologic variables analyzed had no effect on the proliferation or clonal expansion of the AM-hMSCs. Even though donors with comorbidities were included in the cesarean section group, and are intrinsic in that group, no direct role in the proliferation conditions was found in the AM-hMSCs cultures. Nevertheless, future studies with a larger population size need to be conducted, to determine possible changes at the cellular and genetic levels.

Even though the clinical indication for a cesarean section must be justified, the practice has become common worldwide in recent years, especially in low-income countries[32]. However, the fact that cesarean section has certain contraindications for both the mother and the product is important to consider[33,34]. Cultural implications and the promotion of reduced pain are the main reasons for choosing cesarean section versus vaginal delivery[35]. On the other hand, present health conditions worldwide have revealed an increase in the transmission of COVID-19 and other diseases through the vaginal delivery route[36].

At present, there are no studies on cellular and/or molecular changes with respect to AM-HSCs retrieved from placentas obtained at vaginal deliveries compared with those obtained at cesarean sections. The study most related to the type of delivery route and stem cell gene expression was conducted on cells retrieved from Wharton's jelly[37]. Those authors reported that there was higher expression of the *POU5F1* gene, (a gene associated with pluripotency that encodes for the OCT3/4 protein) in placenta donors that had vaginal deliveries (logRQ ± SE = 1.55 ± 0.31), compared with those that underwent cesarean section (logRQ ± SE = 0.66 ± 0.15) (p = 0.008). In addition, the working group reported that, with the progressive number of vaginal deliveries, there was reduced *POU5F1* expression[37]. Our working group is currently conducting studies at the molecular level for determining whether there are differences in the genes associated with pluripotency (*oct-4, nanog*, and *sox-2*) in AM-hMSCs, according to their collection method. However, in the present study, no alterations in the proliferative capacity, expression of pluripotential markers, and cell differentiation of the AM-hMSCs retrieved from placentas obtained at vaginal deliveries or cesarean sections were found.

The cell cultures revealed randomly distributed fibroblastoid morphology, but an organized arrangement like that observed in "marine currents" was apparent on day 14. The SEM analysis showed the adhesion capacity of the collagen matrix through the formation of lamellipodia or cytoplasmic extensions, which reflects their adaptation to the surrounding environment[38]. Our data are correlated with those already reported for hMSCs, regardless of origin[38,39].

Upon analyzing age, type of delivery route the hMSCs were obtained from, and proliferation and expansion capacity (passages), no differences with respect to the number of passages were found. Nevertheless, there was a higher percentage trend for cultures in the P0-P3 passages, whereas a lower percentage of cultures advanced beyond P3, which was observed in relation to age and type of delivery route. Pluripotent gene expression has been reported to be regulated differentially, according to culture passage, finding negative regulation in *nanog* gene expression in P4 and P5 culture passages[17]. In this context, we observed that cells from P3 express excellent levels of CD90, CD73, and CD44, which have been considered as multipotential markers; the expression was equal independently of the source that the cells were obtained. However, lower expression of CD105 was noticed in comparison with the gold standard source of hMSCs (BM). The difference in the expression of CD105 in AM-hMSCs versus BM-hMSCs, correlated with previous reports. Mark et al., observed and important reduction of CD105 expression in hMSCs obtained from BM, this phenotype was evident after that hMSCs were incubated in serum-free culturing condition, but those cells maintain multilineage potential to adipogenic, chondrogenic, and osteogenic lineages[40]. Endoglin plays an important role in angiogenesis and its expression in hMSCs has correlated with the regenerative potential in a murine model of myocardial infraction[41]. However, during the differentiation analysis we did not observe a correlation between CD105 expression osteogenic, adipogenic, and chondrogenic potential.

Our work is the first clinical study to present a relation between age, type of delivery route (vaginal delivery versus cesarean section), obstetric and gynecologic history, comorbidities, and AM-hMSCs proliferation, expansion, pluripotential markers, and differentiation capacity.

Conclusion

Our results showed no differences in AM-hMSCs in patients younger or older than 25 years of age, or in the vaginal or cesarean section delivery routes. The population seen at the Department of Obstetrics and Gynecology of the Hospital General de México is very heterogeneous with respect to age, BMI, and clinical history. We found no differences in AM-HSC, signifying that there are no limitations for using placental tissue from donors seen at the Department of Obstetrics and Gynecology, to obtain hMSCs and for their possible application in RM.

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Conflicts of interest

The authors declare no conflicts of interest.

Ethical considerations

Protection of humans and animals. The authors declare that the procedures followed complied with the ethical standards of the responsible human

experimentation committee and adhered to the World Medical Association and the Declaration of Helsinki. The procedures were approved by the institutional Ethics Committee.

Confidentiality, informed consent, and ethical approval. The authors have followed their institution's confidentiality protocols, obtained informed consent from patients, and received approval from the Ethics Committee. The SAGER guidelines were followed according to the nature of the study.

Declaration on the use of artificial intelligence. The authors declare that no generative artificial intelligence was used in the writing of this manuscript.

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