

Short Communication

Reduced Proliferative Potential with Conserved Stem/Stromal Phenotype of Human Umbilical Cord Mesenchymal Stem Cells in Placental Syndromes: A Prospective Cohort Study

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Abstract

Background: Pathophysiology of placental syndromes is still unclear, and umbilical cord-derived mesenchymal stem cells (UC-MSCs) might play a role in the development of these syndromes. In this prospective cohort study, we evaluated proliferative abilities of two types of UC-MSCs, Wharton’s Jelly MSCs (WJ-MSCs) and cord blood MSCs (CB-MSCs), in placental syndromes. **Methods:** A total of 16 cord blood and umbilical cord samples were seeded and cultured until MSC growth potential exhaustion. Cumulative population doublings were employed for studying growth potential, and flow cytometry immunophenotyping for verification of mesenchymal markers. **Results:** In our prospective cohort study, on one hand CB-MSCs from pathological pregnancies showed a significant reduction of growth potential, on the other hand WJ-MSCs showed a trend toward higher growth potential. This trend is consistent with the well-known faster-growing phenotype of WJ-MSCs under low oxygen atmosphere. Moreover, it’s well understood that chronic hypoxia is a main feature of both intrauterine growth restriction (IUGR) and preeclampsia, thus, our data perfectly match with the well-known clinical characteristics. **Conclusions:** Growth potential of CB-MSCs obtained from placental syndromes tended to be reduced compared to that of MSCs from healthy pregnancies. Our results need to be confirmed in larger *in vitro* studies, as a higher number of CB- and WJ-MSC would better clarify pathophysiology of placental syndromes.

Keywords: mesenchymal stem cells; Wharton’s Jelly; cord blood; placental syndromes; intrauterine growth restriction

1. Introduction

Inadequate placental development and subsequent inappropriate mother-fetus exchanges are responsible for preeclampsia and intrauterine growth restriction (IUGR) linked to elevated maternal and fetal morbidity and mortality, recently defined as “placental syndromes” [1]. The exact pathophysiology behind these syndromes is still unclear, however, an incomplete uterine spiral artery remodeling with the lack of uteroplacental vessel development is one of the driver events [1–4]. Physiologically, uteroplacental vessels have the function of showing down arterial pressure, lowering vessel resistance and increased the flow at the placental level [2]. Therefore, the absence of this system causes tissue damage, preeclampsia, IUGR, and umbilical cord flow changes detected by doppler ultrasound are typically found in placental syndromes [2–5].

Wharton’s Jelly mesenchymal stem cells (WJ-MSCs) are stem cells with high differentiative potential, which also play a role in placental syndrome development. WJ-MSCs might act as adventitia for umbilical vessels and might differentially express extracellular components such as growth factors during preeclampsia, oligohydramnios, IUGR, low birth weight, or the life-threatening condition known as “lean umbilical cord” with birth growth restriction and distress [6,7]. Mesenchymal stem cells with high proliferative and differentiation potentials can be isolated from umbilical cord blood (cord blood mesenchymal stem cells (CB-MSCs)). Collectively, WJ- and CB-MSCs are termed umbilical cord-derived mesenchymal stem cells (UC-MSCs), a class of extra-fetal stem cells present in the placenta, amniotic lining, and amniotic fluid [8–10]. UC-MSCs deeply interact with component of the maternal-fetal in-



Table 1. Group comparison of the 35 patients enrolled in the study.

	Pathological group (n = 10)	Physiological control group (n = 25)	p value
Maternal age (years)	32.78	32.77	>0.05
Mean gestational age (weeks)	37.86	38.96	>0.05
Mean birthweight (g)	2743.18	3058.95	>0.05
Delivery route (%)			
Cesarean section	92.3	82.6	>0.05
Vaginal route	7.7	17.4	>0.05

A *p* value < 0.05 was considered statistically significant.

terface, as WJ-MSCs cultured with trophoblast cells promote trophoblastic proliferation, migration, and invasion, and modification in the expression of human chorionic gonadotropin, placental growth factor, and endoglin [11–13]. Moreover, CB-MSC infusion in rats with preeclampsia improves weight at birth, improves uterine spiral arteries remodeling and renal function, and reduces tumor necrosis factor (TNF)- α while increases interleukin (IL)-10 levels [14].

Based on this evidence, we evaluated proliferative potentials of UC-MSCs from pregnant women with placental syndromes to better understand the pathophysiology behind these maternal-fetal disorders.

2. Materials and Methods

2.1 Patients

A total of 38 pregnant women were considered eligible for this prospective cohort study conducted at the University Hospital “San Giovanni di Dio e Ruggi d’Aragona”, Salerno, Italy, and at the Hospital “San Giuseppe Moscati”, Avellino, Italy, and 35 of them were enrolled (Table 1). Study protocol and written informed consent were reviewed and approved by local Institutional Review Board (Ethics Committee “Campania Sud”, Brusciano, Naples, Italy; prot./SCCE n. 24988). Subjects have given their written informed consent before sampling. 10 of them were diagnosed with placental syndromes: 6 with preeclampsia and 4 with IUGR. 28 patients had physiological pregnancies. Inclusion criteria for the pathologic group were an antepartum diagnosis of preeclampsia or IUGR.

2.2 WJ-MSCs Isolation

Human WJ-MSCs were isolated from fresh human umbilical cords placed in 0.9% NaCl physiological solution supplemented with 1 g of ampicillin (Sigma Aldrich, Milan, Italy) and 500 mg of sulbactam (Sigma Aldrich, Milan, Italy), stored at 4 °C, and processed within 4 h after collection. Briefly, samples were cut in small segments, washed in fresh medium to remove blood and debris, cut open lengthwise with sterile scissors, and arteries and veins were removed. Each piece was then transferred to a 175 cm² tissue culture flask (BD Falcon, Bedford, MA, USA) containing alpha-MEM medium (Corning Cellgro, Manassas, VA, USA) supplemented with 10% fe-

tal bovine serum (FBS, Corning Cellgro, Manassas, VA, USA), GlutaGro™ (Corning Cellgro, Manassas, VA, USA) and Penicillin-Streptomycin (Sigma Aldrich, Milan, Italy), and incubated at 37 °C in a humidified atmosphere containing 5% CO₂. Cell growth was daily monitored, culture medium was changed twice a week, and cells were detached using 0.05% trypsin (Sigma Aldrich, Milan, Italy) and 0.53 mM ethylenediaminetetraacetic acid (EDTA) (Corning Cellgro, Manassas, VA, USA) upon reaching 100% confluence. Subsequently, cells were re-seeded at a density of 4 × 10³ cells/cm² and cultured until reaching a plateau in the growth curve.

2.3 CB-MSCs Isolation

After vaginal or caesarean delivery, umbilical cord was clamped and disinfected; and 35 mL of blood was collected in sterile BD Vacutainer Z (no Additive) collection tubes (BD Diagnostic, Oxford, UK) containing 1 mL of citrate-phosphate dextrose as anticoagulant (MacoPharma, Tourcoing, France). Fresh whole blood was centrifuged for 15 minutes at 1900 rpm at 20 °C, and then the buffy coat interphase layer was collected, placed in a 15 mL falcon tube (BD Biosciences, Oxford, UK), and mixed with an equal volume of 0.9% NaCl physiological solution, and mononuclear cells were isolated by Ficoll-Paque density gradient centrifugation as per manufacturer’s instructions. Cells were then seeded at a density of 5 × 10⁵ cells/cm² in a 35 × 10 mm dish (BD Biosciences, Oxford, UK) in complete culture medium consisting of alpha-Minimum Essential Medium (MEM) supplemented with 20% FBS, glutamine (Sigma Aldrich, Milan, Italy), and Penicillin-Streptomycin Solution, and incubated at 37 °C in a humidified atmosphere containing 5% CO₂ with complete culture medium change twice a week. At 90% confluence, cells were detached using 0.05% trypsin and 0.53 mM EDTA, washed with phosphate buffer saline (PBS; Corning Cellgro, Manassas, VA, USA), re-seeded at a density of 4 × 10³ cells/cm², and cultured until reaching a plateau in the growth curve. Adherent cells that did not form colonies after 40 days were eliminated.

2.4 Flow Cytometry

Flow Cytometry immunophenotype of WJ- and CB-MSCs was performed on cells at first culture passage.

Briefly, samples were incubated with the following mouse anti-human antibodies: cluster of differentiation (CD)90-fluorescein (FITC), CD45-Phycoerythrin (PE)-Cyanine7 (PC7), CD105-PE, CD56-PE-Cyanine5 (PC5), CD14-PC7, CD45-PC7, human leukocyte antigen (HLA)-DR-FITC, CD34-PE (Beckman Coulter, BC, Fullerton, CA, USA), and CD73-APC (Miltenyi Biotec, Gladbach, Germany), as described elsewhere [15]. Sample acquisition was carried out using a BD FACSVerser flow cytometer (BD Biosciences) equipped with blue (488 nm) and red lasers (628 nm) and BD FACSuite software (BD Biosciences, Oxford, UK). PMT voltages setting, and compensation were performed using single-color controls for each fluorochrome and an unstained sample as negative control. All samples were run with the same PMT voltages, and a minimum of 10,000 events were recorded.

2.5 Cumulative Population Doublings

Population doubling was calculated for WJ-MSCs and CB-MSCs using the following equation: population doubling (PD) = $\log_{10}(N)/\log_{10}(2)$; where N is the number of cells harvested at the end of the culture or the number of seeded cells. To calculate expansion potential, cumulative population doublings (CPD) were calculated by recording cell counts and cellular dilution factors at each passage (P). Cell counting was performed using Trypan Blue to discriminate dead cells.

2.6 Statistical Analysis

Data were analyzed using SPSS software (v.2.0; SPSS Inc., Chicago, IL, USA) and Prism (v.9.3.1; GraphPad Software, San Diego, CA, USA).

A power calculation was performed to determine an appropriate sample size for this noninferiority study. We set the mean population doubling numbers at 10. The standard deviation was set as 1. This power calculation indicated that 32 patients in each group would be necessary to detect a 15% difference in with a power of 80% at a 0.5% level of significance.

For flow cytometry data, results are presented as percentage of positive cells, and expression of each marker on single cells is also reported as histograms and using unstained samples as negative controls. For characterization of mesenchymal cells, percentage of positive cells. Shapiro-Wilk's test for parametrical distribution and *t*-test were employed for statistical comparisons between groups. A $p < 0.05$ was considered statistically significant.

3. Results

3.1 Umbilical Cord Samples as a Source of MSCs

From the 35 patients enrolled in this prospective cohort study, a total of 28 umbilical cord samples and 31 cord blood samples were obtained. Of the 28 umbilical cord blood samples, 11 resulted in WJ-MSC growth: (i) 10 from physiological pregnancies; (ii) 1 from patholog-

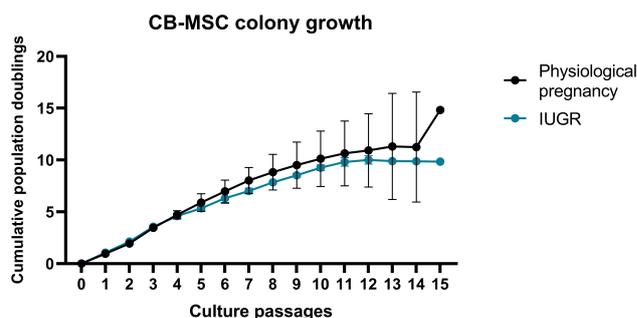


Fig. 1. Cord blood derived mesenchymal stem cell (CB-MSC) growth curves. Each CB-MSC growth curve from every healthy pregnancy is reported as shades of blue curves, while each CB-MSC growth curve for each pathological pregnancy is shown as shades of red curves. IUGR, intrauterine growth restriction.

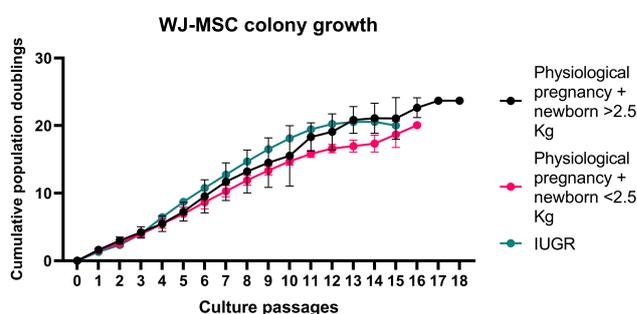


Fig. 2. Wharton's Jelly mesenchymal stem cell (WJ-MSC) growth curves. Each WJ-MSC growth curve from every healthy pregnancy is reported as shades of blue curves, while each CB-MSC growth curve for each pathological pregnancy is shown as shades of red curves. IUGR, intrauterine growth restriction.

ical pregnancy, specifically, from IUGR. Of the 31 collected cord blood samples, 5 resulted in CB-MSC growth: (i) 3 from physiological pregnancies; (ii) 2 from pathological pregnancy, specifically, 1 from a preeclampsia and 1 from IUGR. The overall growth percentage of CB- and WJ-MSCs was 16.1% and 39.2%, respectively. Percentage of cord blood and umbilical cord samples that failed in cellular growth for any reason was compared to that of samples that resulted in MSC colonies by Chi square test. Umbilical cord samples tended to have increased ability to result in MSC colonies compared to cord blood.

3.2 CB-MSCs from Pathological Placenta Show Slower Cellular Growth, while WJ-MSCs from Pathological Placenta Show Faster Cellular Growth

Next, cellular growth rates of MSCs obtained from cord blood or umbilical cord samples were analyzed. Of the 5 cord blood samples that originated o MSC colonies, CB-MSCs from pathological placenta showed a slower growth rate compared to those obtained from physiological pregnancies (Fig. 1 and Table 2). Moreover, CB-MSCs from pathological samples, ultimately resulted in cell death.

Table 2. CB- and WJ-MSC growth curve comparison.

Physiological vs. Pathological	CB-MSCs (n = 5, 3 physiological vs. 2 pathological)	WJ-MSCs (n = 11, 10 physiological vs. 1 pathological)
Mean ± SD	0.54 ± 0.48	1.09 ± 1.10
SD mean error	0.137	0.275
95% CI	0.2407 to 0.8449	0.5101 to 1.6826
T	3.954	3.986
p value	0.002	0.001

A *p* value < 0.05 was considered statistically significant. CB-MSCs, Cord Blood MSCs; WJ-MSCs, Wharton's Jelly MSCs; SD, standard deviation; CI, confidence interval; T, Student *t*-test.

Conversely, WJ-MSCs from the pathological group showed faster growth rate compared to that observed in the physiological group (Fig. 2 and Table 2). To confirm our result, we divided physiological pregnancies into two groups based on the newborn's weight (<2.5 kg - namely physiological small for gestational age (SGA) infants or >2.5 kg): WJ-MSCs from the physiological group with SGA newborns have a lower growth rate compared to that observed in the physiological group with newborns weighted >2.5 kg (Fig. 2).

3.3 Immunophenotype of Cord Blood and Umbilical Cord Derived MSCs

Finally, immunophenotype of CB- and WJ-MSCs was evaluated by flow cytometry (Fig. 3). Of the 5 cord blood samples that originated MSC colonies, flow cytometry immunophenotype was performed on all three CB-MSCs from healthy pregnancies, while no pathological samples were analyzed due to premature death of cultured cells. Of the 11 UC-MSCs, flow cytometry analysis was not performed on one sample of the control group and one of the pathological groups, because of earlier cell death that did not allow further immunophenotype analysis. Both CB- and WJ-MSCs showed a mesenchymal phenotype with positivity for CD90, CD73, and CD105, and negativity for CD45, HLA-DR, CD34, CD14, and CD56, and no differences were described between the two sources of stem cells and between healthy and pathological pregnancies.

4. Discussion

In this prospective cohort study, differences in growth potential between cord blood and umbilical cord derived-MSCs from healthy and placental syndromes affected pregnancies, such as preeclampsia and IUGR, were evaluated. Reported rates of CB-MSCs isolation range from 1% to 25%; therefore, our CB-MSCs isolation rate of 16.1% is consistent with that described in literature [14–16]. Conversely, our rate of WJ-MSC isolation of 39.2% was significantly lower than that reported in literature, which was described to be close to 100% [17]. This difference could be related to umbilical cord conditions and placental integrity that are higher for caesarean deliveries [18], while they could decrease in samples obtained from vaginal de-

liveries, as described in our study. Indeed, preeclampsia and IUGR are not an absolute indication for caesarean section (C-section) delivery that is chosen based on obstetrics situation and disease severity [5]. Therefore, sample collection only from C-section would have resulted in a bias, excluding mild cases from our study.

Growth potential of CB-MSCs obtained from placental syndromes were reduced compared to that of MSCs from healthy pregnancies. However, no differences in immunophenotyping were described, as previously reported [19]. Previous studies have documented a reduced proliferative capacity of placental MSCs obtained from pregnancies complicated by IUGR and preeclampsia [20,21]. This reduced potential might be related to impairment in number and proliferation capacity of endothelial cell progenitors, hematopoietic stem cells, and endothelial colony forming cells in umbilical cord from pregnancies complicated by placental syndromes [17,22,23]. Indeed, CB-MSCs infusion improves clinical outcomes of preeclampsia in rat models by reducing proteinuria, blood pressure, proinflammatory cytokine levels (e.g., TNF- α and IL-1) and by increasing anti-inflammatory IL-10 levels [14].

On the other hand, WJ-MSCs enhance trophoblasts *in vitro* migration and invasion capacities [19]. Therefore, highest proliferation capacity of WJ-MSCs is consistent with literature, stating that WJ-MSC has a faster growth potential under low oxygen tension [24–27]. These behaviors of both CB- and WJ-MSCs might be implicated in the development of placental syndromes, as documented in our study, showing decreased growth potential of CB-MSCs obtained from pregnancies complicated by IUGR and preeclampsia. Indeed, chronic hypoxia could be the missing factor of why preeclampsia and IUGR set on and/or develop, negatively impairing the known favorable effects of UC-MSCs on placentation [28–30]. This finding could be supported by the lower growth rate observed in those physiological pregnancies who delivered SGA newborns. Indeed, we hypothesize that, as SGA newborns are not affected with chronic hypoxia as IUGR newborns, their WJ-MSCs growth is not influenced by low tension O₂, resulting in a lower growth [27–29].

Limitations of our study are: (i) the small number of samples; (ii) lack of more in-depth analysis, such apoptosis rate by flow cytometry or differentiation potential to other

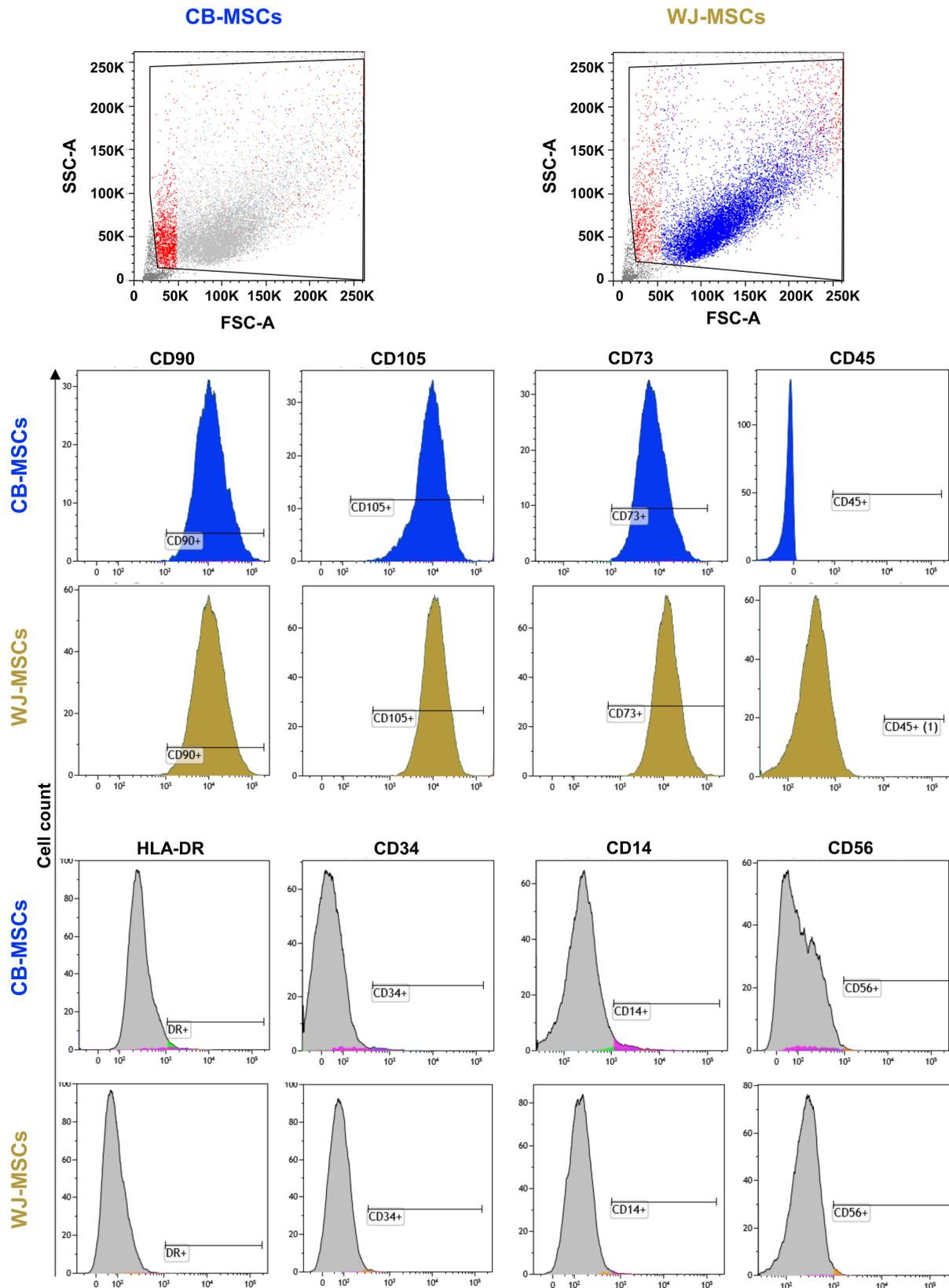


Fig. 3. Cord blood derived (CB-MSCs) and Wharton's Jelly-derived mesenchymal stem cell (WJ-MSCs) immunophenotype. Flow cytometry analysis was performed on CB- and WJ-MSCs, and cells were positive for mesenchymal markers, such as CD90, CD73, and CD105, and negative for lineage-specific markers, such as HLA-DR, CD45, CD34, CD14, and CD56. SSC-A, side scatter area; FSC-A, forward side scatter-area; CD, cluster of differentiation.

lineage of obtained MSCs; and (iii) confirmation of reduced proliferation capacity of CB-MSCs by comparison of early and late passages.

5. Conclusions

Our results open new scenarios on possible roles of UC-MSCs in placental development also suggesting these stem cells sources as potential therapeutic strategy for IUGR and preeclampsia; however, further studies are required to confirm our hypothesis.

Availability of Data and Materials

The data that support the findings of this study are available on request from the corresponding author.

Author Contributions

Conceptualization: LM, MAC and BS; methodology: LM, SGC, PM, FP, MRC, CS, and VG; clinical patients' sample and data collection: MAC, CF, MP, and MG; formal analysis: CF, PM, and VG; writing—original draft preparation: LM, MAC, and VG; writing—review and editing: CS and BS. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

Ethics Approval and Consent to Participate

This research was conducted ethically in accordance with the World Medical Association Declaration of Helsinki. Study protocol were reviewed and approved by local Institutional Review Board (Ethics Committee “Campania Sud”, Bruscianno, Naples, Italy; prot./SCCE n. 24988). Informed consent was obtained from all subjects involved in the study. Written informed consent has been obtained from the patients to publish this paper.

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Conflict of Interest

The authors declare no conflict of interest. Maria Antonietta Castaldi is serving as one of the Guest editors of this journal. We declare that Maria Antonietta Castaldi had no involvement in the peer review of this article and has no access to information regarding its peer review. Full responsibility for the editorial process for this article was delegated to Michael H. Dahan.

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