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Platelet-rich plasma as an Alternative Supplement for Enhancing CD Marker Expression in Umbilical Cord-Derived Mesenchymal Stem Cells

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Abstract

This study aimed to assess the effectiveness of platelet-rich plasma (PRP) and fetal bovine serum (FBS) as culture supplements for mesenchymal stem cells (MSCs) isolated from umbilical cord tissue. The study focused on the efficiency of xeno-free PRP in enhancing cell adhesion and proliferation and looked at cell surface markers for MSC characterization. Until the cells achieved 80% confluency, three repeats of FBS and PRP were added to the culture medium of mesenchymal stem cells derived from umbilical cord tissue in passage 10. Following harvesting, an assay kit for human mesenchymal stem cells was used to color the cells. Using flow cytometry, the surface markers CD73, CD90, CD105, and the negative marker CD44 were evaluated in the samples. Cells supplemented with PRP expressed more positive indicators and fewer negative markers. Whereas the results for CD90, CD105, and the negative marker were insignificant, the expression of CD73 increased significantly. PRP can enhance CD marker expression by acting as a substitute for FBS in the culture of xeno-free umbilical cord-derived MSCs.

Keywords: FBS; PRP; MSC; Stem Cells; Xeno-free.

Abbreviations: platelet-rich plasma (PRP), fetal bovine serum (FBS), mesenchymal stem cells (MSCs), Cluster of differentiation (CD)

INTRODUCTION

As of yet, stem cells serve no particular purpose in the human body. However, they can self-renew and specialize in several cell lineages. These cells play a crucial role as mediators in a newborn's growth and help them heal from illnesses or wounds. This is due to stem cells' capacity to proliferate and differentiate into a broad range of cell types and lineages required for continued development throughout the neonatal period. The potential is the ability of stem cells to differentiate into different kinds of cells. Based on their potential, stem cells can be divided into totipotent, pluripotent, heterogeneous, and multipotent groups. (Poliwoda et al., 2022) Adult tissue (fat, bone marrow, skeletal muscle, skin, and blood), fetal tissue (fetus, placenta, amniotic fluid, and umbilical cord), and differentiated somatic cells are the four primary sources of stem cells employed in research and therapy. (Zakrzewski et al., 2019)

Multipotent stem cells called mesenchymal stem cells (MSCs) are derived from various organs, including the umbilical cord. Adipocytes, osteoblasts, and chondroblasts are only a few mesodermal lineages into which mesenchymal stem cells can differentiate. Because of this, stem cells are now often used in regenerative medicine. MSC is a widely available substance because of its therapeutic properties. It has been documented to differentiate into various cell types, including stem cells from umbilical cord tissue. (Joo et al., 2020) Because of their unique qualities, mesenchymal stem cells can be employed in clinical settings. These traits include the ability to secrete trophic factors and proangiogenic stress and anti-inflammatory, immunomodulatory, and antioxidant effects. (Park & Jung, 2021)

Mesenchymal stem cells may be isolated and cultivated from umbilical cord tissue (UC-MSC), an excellent source of these stem cells. The non-invasive approach to obtaining these cells gives them an edge over other sources of mesenchymal stem cells. This prevents the risk of infection and is ethically more acceptable. These cells can be promising because of several properties: higher proliferation rates, cryogenic storage for long periods, hypoimmunogenic phenotypes, immune-suppressive effects, and being harvested from matrices rich in growth factors. These cells can be extracted from the umbilical cord tissue in various locations, including perivascular areas, veins, arteries, amniotic fluid, and sub-amniotic fluid.(Shang et al., 2021) Three approaches are available for this cell isolation technique: tissue explant, collagenase digestion,

and collagenase/trypsin digestion. (Hendijani, 2017; Nagamura-Inoue & He, 2014; Varaa et al., 2019)

Serum or medium supplementation is crucial for cell survival and encourages the growth of cells in vitro. Usually, because fetal bovine serum (FBS) is the gold standard, it is utilized. When used clinically, rejection is dangerous because this serum is obtained from animals and contains xeno-proteins, including N-glycolylneuraminic acid (Neu 5GC), which may elicit an immune response in patients. (M. Hesler et al., 2019; Liu et al., 2023; Suryani et al., 2013; Tonarova et al., 2021) The development of alternative xeno-free medium supplementation has attracted the attention of researchers worldwide. (Abdel Moniem et al., 2019; Michelle Hesler et al., 2019) Platelet-rich plasma (PRP) is a medium supplementation different from FBS. Platelet-rich plasma is xeno-free with components from humans and does not include foreign species such as animals. PRP as a medium supplementation in umbilical cord MSC cultures is more effective because it increases cell proliferation and makes cell attachment to the bottom of the culture dish faster than conventional medium supplementation such as FBS. (Lang et al., 2018)

Cluster of differentiation (CD) is an antigen that can be used to identify cell surface molecules that provide targets for cell immunophenotyping. In a physiological sense, CD antigens can function as ligands or receptors that are crucial for cells. Cell adhesion, cell activation, and cell inhibition are among the roles played by CD antigens. (Ghaneialvar et al., 2018) Mesenchymal stem cells are defined by the criteria set forth by the International Society for Cellular Therapy (ISCT): they express CD105, CD73, and CD90 positively, and CD34, CD45, CD14, and CD79 negatively. Positive signals for CD34, CD45, CD14, and CD79 are expressed by hematopoietic cells. (Kaveh Baghaei1, 2017) The ISCT has identified three criteria for mesenchymal stem cells. The first need is that the cells in standard culture conditions be able to cling to plastic surfaces (plastic-adherent). The second need is the ability of the cells to develop into osteocytes, adipocytes, and chondrocytes under typical in-vitro differentiation circumstances. Third, cells must be able to express antigens on the surface of stem cells; hematopoietic cell markers such as CD45, CD34, and CD14, or CD11b, CD79a, or CD19, must be expressed by fewer than 2% of cells, whereas more than 95% of MSCs must express CD29, CD105, CD73, and CD90. (Renesme et al., 2022)

A liquid that concurrently passes via a laser beam can have several physical properties of cells or individual particles measured and analyzed using flow cytometry. This device creates dispersed fluorescent light signals and can be read by detectors like photodiodes or photomultiplier tubes utilizing a laser as the light source. These light impulses will be transformed into electrical signals, which a computer will then analyze and write into data files in a standard manner. Analyzing and purifying cell populations according to their light-scattering or fluorescent properties is possible. Moreover, immunology, molecular biology, bacteriology, virology, cancer biology, and infectious disease monitoring are all beneficial to flow cytometry. (McKinnon, 2018) Consequently, the expression of surface markers (CD73, CD90, and CD105) in mesenchymal stem cells derived from umbilical cords grown with fetal bovine serum and platelet-rich plasma medium supplements interests researchers.

MATERIALS AND METHODS

The ingredients used in this research include Gibco Phosphate Buffer Saline (PBS), Gibco Dulbecco's Modified Eagle Medium (DMEM), Gibco Fetal Bovine Serum (FBS), Platelet-Rich Plasma (PRP) with blood type AB, Gibco glutamax , 70% alcohol, Gibco trypan blue, Gibco penicillin-streptomycin, and Gibco amphotericin B, Gibco tryple select, heparin sodium, Gibco cryopreservation medium (synth-a-freeze), distilled water, isopropyl alcohol, FITC Mouse Anti-Human CD90, PE Mouse Anti-Human CD44, PerCP-Cy™5.5 Mouse Anti-Human CD105, APC Mouse Anti-Human CD73, hMSC Positive Isotype Control Cocktail, PE hMSC Negative Isotype Control Cocktail, hMSC Positive Cocktail, PE hMSC Negative Cocktail.

The design employed in this study was an in vitro experiment with three repeats for each treatment. This work employed human umbilical cord mesenchymal stem cells (UC-MSCs) acquired from Stem Cell and Tissue Engineering (SCTE). In addition to control cocktails including mouse anti-human CD90, PE, and hMSC positive and negative isotypes, there are also mouse anti-human CD90, mouse anti-human CD44, PerCP-Cy™5.5 mice anti-human CD105, mouse anti-human CD73 APC, and mouse anti-human CD90 FITC.

MSCs culture with FBS and PRP supplementation

The complete medium contains 10% Gibco Fetal Bovine Serum (FBS), 1% glutamate, 1% Gibco penicillin-streptomycin, and 1% amphotericin B in addition to Gibco Dulbecco's Modified Eagle Medium (DMEM) basal medium. Additionally, it contains a combination of 10% Platelet-Rich Plasma (PRP) containing blood type AB, 1% glutamate, 1% heparin, 1% amphotericin B, and 1% penicillin-streptomycin. Every two to three days, the medium is changed until the cell confluence reaches 80–90%. In 12 well plates, there are 100,000 seeded cells. The cells are placed in an incubator with 5% CO2 at 37°C.

Flow Cytometry Analysis

Cells from the healthy plate can be harvested directly, while cells in the scaffold must be given Gibco triple select for 5 minutes in the incubator. Then, they can be harvested and centrifuged at 1200 rpm for 10 minutes.

Re-suspend 100 µl of pellets at a 5x10⁶ cell/ml concentration in Gibco PBS. Using FITC Mouse Anti-Human CD90, PE Mouse Anti-Human CD44, PerCP-Cy™5.5 Mouse Anti-Human CD105, APC Mouse Anti-Human CD73, and hMSC Positive Isotype Control Cocktail, PE hMSC Negative Isotype Control Cocktail, hMSC Positive Cocktail, and PE hMSC Negative Cocktail. The cell solution can be divided or transferred into three 1.5 ml tubes (unstained, isotype, stain antibody human mesenchymal stem cell analysis kit).

Statistical analyses

Statistical analysis will be conducted to test the research hypotheses. Data will be presented as tables, images, and graphs. Statistical processing will be performed using GraphPad Prism 10. Normality tests will be conducted to determine whether the data are normally distributed. If the normality assumption is met (data are normally distributed and show homogeneous variance), parametric tests such as the t-test will be applied. If the parametric test assumptions are not satisfied, a non-parametric alternative, the Mann-Whitney test, will be employed.

RESULTS AND DISCUSSION

UC-MSC culture with FBS and PRP medium supplementation

Cells seeding in 12-well plates are cultured using complete FBS and PRP medium until they reach 80% confluence. Observation of the morphological development of UC-MSC culture on day 2 showed the results of cell changes from round to spindle-shaped fibroblasts. It appeared to have attached to the base of the culture dish (plastic adherent) according to the ISCT criteria (Figure 1). Observations on days 4 to 8 showed that more and more cells were attached with a slimmer fibroblastic image, had a visible nucleus, tapering cytoplasm along the axis of the cell nucleus (spindle shape), and processes similar to collagen bundles. Observations over time showed that the provision of FBS and PRP medium supplementation made cells denser, forming more robust aggregates. So that cells will appear to reach a confluence level of 80%.

In addition to observing the morphological development of UC-MSC, there are differences between UC-MSC cultured using FBS and PRP medium supplementation. The fibroblastic, nuclear, cytoplasmic, and process images appear more transparent and denser in UC-MSC cultures using PRP medium supplementation. Thus, the confluence level of UC-MSC cultured using PRP medium supplementation was higher than that of FBS.

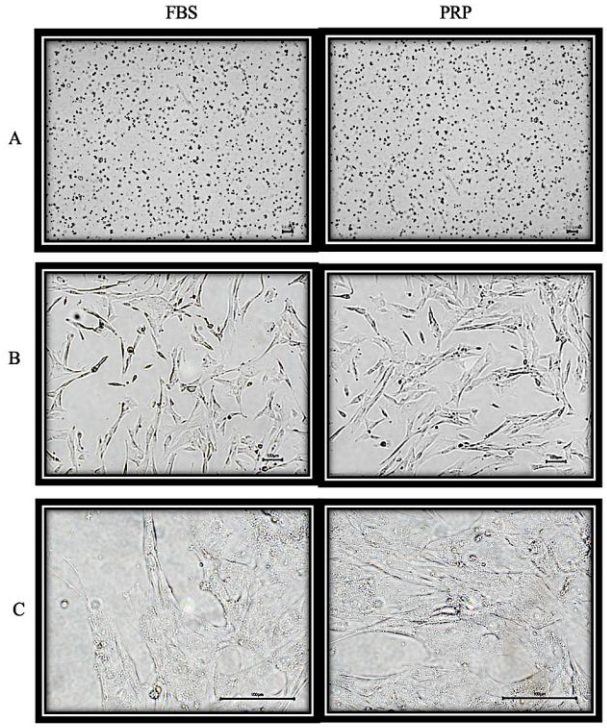

Figure 1. Microscopic photo results after cell implantation in the two treatment groups. Figure A. Macroscopic photo with 40x magnification. B. Macroscopic photo with 100x magnification. C. Macroscopic photo with 400x magnification.

Surface marker expression of UC-MSC cultured with FBS and PRP medium supplementation

The scatter plot results on PRP samples show granularity/complexity and cell size similar to the gold standard, namely FBS. However, the average cell count value in FBS samples is lower than in PRP samples. The FBS sample obtained results (10,767.8 ± 4,229), and the PRP sample (14,474.4 ± 8,469). This shows that UC-MSC culture with PRP supplementation has higher cell counts than FBS.

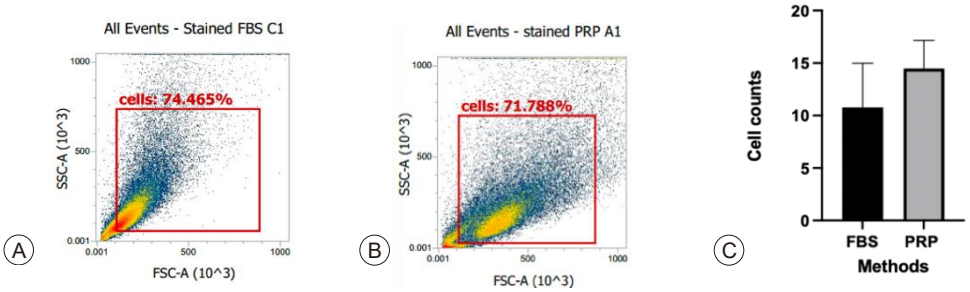

Figure 2. Histogram of Cell Count analysis results. A. Scatter plots of SSC-A/FSC-A UC-MSC with FBS serum. B. Scatter plots of SSC-A/FSC-A UC-MSC with PRP serum. C. Statistical analysis graph.

The flow cytometry analysis graph results showed positive expression of CD73 in both FBS and PRP samples with a gating percentage of ≥95% (Figure 3). The FBS sample obtained results (96.18 ± 1.175) and the PRP sample (99.03 ± 0.299). This shows that PRP expresses CD73 more highly, Contributing to UC's migration, differentiation, and proliferation-MSC. A significant difference was found in the T-test in CD73 ($p = 0.0302$).

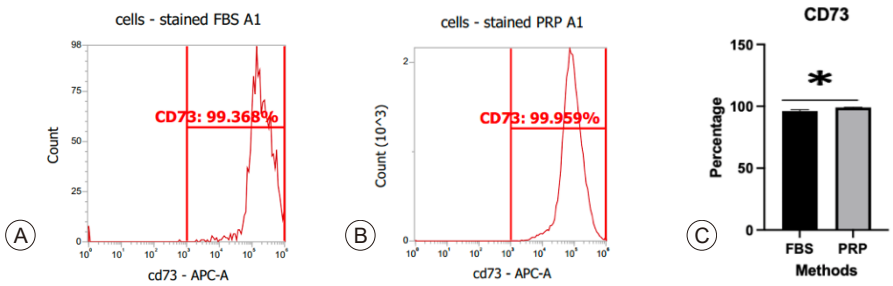

Figure 3. Histogram of CD73 analysis results. A. Flow cytometry analysis graph of UC-MSC with FBS serum. B. Flow cytometry analysis graph of UC-MSC with PRP serum. C. Statistical analysis graph.

The flow cytometry analysis graph results showed positive expression of CD90 in both FBS and PRP samples with a gating percentage of ≥95% (Figure 4). The FBS sample obtained results (98.33 ± 0.737) and the PRP sample (99.62 ± 0.097). This shows that PRP is higher in expressing CD90, which functions as UC-MSC adhesion. However, in the Mann-Whitney test, there was no significant difference in CD90 ($p = 0.1376$).

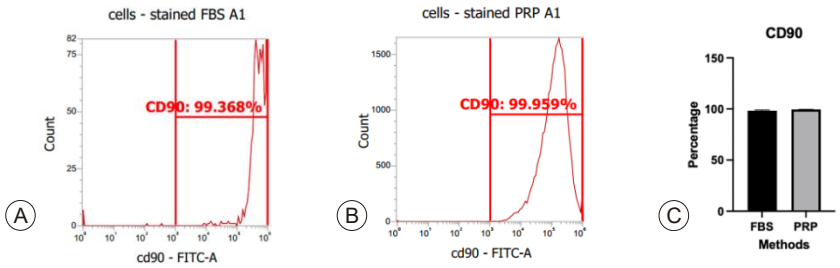

Figure 4. Histogram of CD90 analysis results. A. Flow cytometry analysis graph of UC-MSC with FBS serum. B. Flow cytometry analysis graph of UC-MSC with PRP serum. C. Statistical analysis graph.

The flow cytometry analysis graph results showed positive expression of CD105 in both FBS and PRP samples with a gating percentage of ≥95% (Figure 5). The FBS sample obtained results (99.45 ± 0.158) and the PRP sample (99.25 ± 0.255). This shows that FBS is higher in expressing CD105, which functions as proliferation, differentiation, and migration of UC-MSC. However, in the Mann-Whitney statistical test, there was no significant difference in CD105 ($p = 0.6698$).

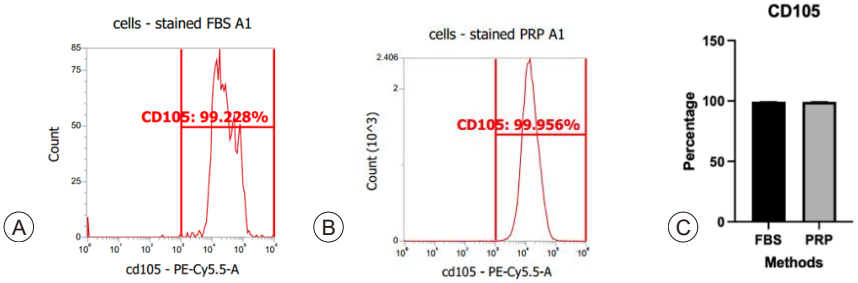

Figure 5. Histogram of CD105 analysis results. A. Flow cytometry analysis graph of UC-MSC with FBS serum. B. Flow cytometry analysis graph of UC-MSC with PRP serum. C. Statistical analysis graph.

The flow cytometry analysis graph showed negative expression (CD45, CD34, CD14, and CD19) in both FBS and PRP samples with a gating percentage of ≤2%. The FBS sample obtained results (0.07 ± 0.026) and the PRP sample (M = 0.009 ± 0.002). This shows that FBS is higher in expressing negative markers (CD45, CD34,

CD14, and CD19), which are hematopoietic cell surface markers. However, in the Mann-Whitney statistical test, negative markers did not differ significantly ($p = 0.1240$).

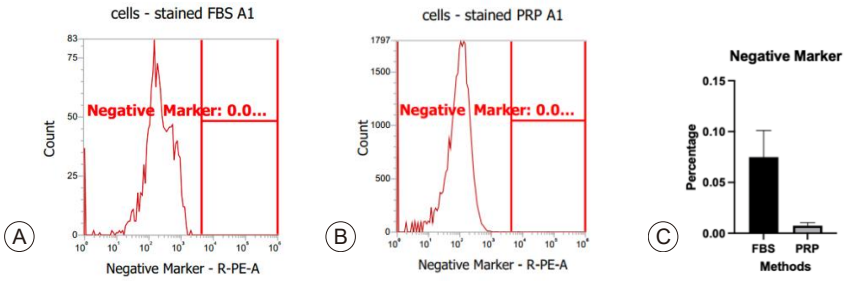

Figure 6. Histogram of *negative marker* (CD45, CD34, CD14, and CD19) analysis results. Figure A. Flow cytometry analysis graph of UC-MSC with FBS serum. Figure B. Flow cytometry analysis graph of UC-MSC with PRP serum. Figure C. Statistical analysis graph.

The scatter plot results on PRP samples show granularity/complexity and cell size similar to the gold standard, namely FBS. However, the average cell count value in FBS samples is lower than in PRP samples. The FBS sample obtained results (10,767.8 ± 4,229), and the PRP sample (14,474.4 ± 8,469). This shows that UC-MSC culture with PRP supplementation has higher cell counts than FBS.

Cells were cultured with two different medium supplements, namely PRP and FBS. After seeding, a microscopic image of cells appeared round and had not yet been attached to the culture dish. This is consistent with studies (Suelzu et al., 2020), which states that cells not yet attached to the culture dish are round. The morphological development of the UC-MSC culture on the 2nd day showed the results of changes from round-shaped cells to spindle-like fibroblasts. It was seen attached to the base of the culture dish (plastic adherent) according to the ISCT criteria.(Guan et al., 2019; Kusnanto et al., 2023; Miryam Mebarki et al., 2021; Shang et al., 2021) Andrzejeskwa et al. (2019) also claimed in their study that huge, flat, spindle-shaped proliferating cells with a distinct cytoskeleton structure comprising many granules resembled fibroblasts. (Andrzejewska et al., 2019) Next, tiny, spherical cells with a strong potential for self-renewal are observed. Fibroblasts have a nucleus that looks real, cytoplasm tapering along the axis of the cell nucleus (spindle shape), and processes similar to collagen bundles.(Dick et al., 2019; Guan et al., 2019; Plikus et al., 2021) Observations from day 3 to day 8 demonstrate this, showing that more and more cells are attached with a slimmer fibroblastic image.

There are differences in development between UC-MSC cultured using FBS and PRP medium supplementation. (Pratama et al., 2020; Sukmawati et al., 2022) A clearer fibroblastic image, a more visible nucleus with a more pointed cytoplasm along the nuclear axis (spindle shape), and processes similar to collagen bundles are more clearly visible in UC-MSC cultures using PRP medium supplementation. So, the confluence level of UC-MSC cultured using PRP medium supplementation looks higher than that of FBS. Compared to FBS, the concentration of PRP in UC-MSC culture demonstrated the greatest efficacy in encouraging higher rates of cell attachment and proliferation. (Kandoi et al., 2018; Pratama et al., 2020; Sukmawati et al., 2022; Thaweesapphithak et al., 2019) PRP can protect against more extended chromosome instability than FBS. Platelet-rich plasma can also help the attachment of UC-MSC to the culture dish and help UC-MSC in the directional movement (cell migration) of a single cell or group of cells to chemical signals. The proliferation rate of UC-MSC can be assessed as higher in PRP from donors of a younger age. (Kandoi et al., 2018; Sheriff et al., 2018; Sukmawati et al., 2022; Trivanovic et al., 2023)

In contrast to FBS, platelet-rich plasma contains growth factors critical for wound healing. Growth factors found in platelet-rich plasma include essential fibroblast growth factor (bFGF), which stimulates angiogenesis and cell differentiation; platelet-derived growth factor (PDGF) and transforming growth factor-β1 (TGF-β1) are two factors that facilitate cell division. The growth factor for vascular endothelial cells (VEGF) encourages neovascularization and cell survival. Insulin-like growth factor-1 (IGF-1) stimulates cell migration and multiplication and is related to the hormone epidermal growth factor (EGF), which promotes cell division and expansion (proliferation). (Taniguchi et al., 2019; Trivanovic et al., 2023). Adding PRP to UC-MSC culture can also considerably enhance the quantity of chondroblasts generated. This is how PRP affects UC-MSC development into chondroblasts. This demonstrates that PRP is particularly successful at enhancing UC-MSC proliferation compared to FBS. (Wang et al., 2019; Zha et al., 2021)

PRP is derived from human plasma containing certain growth factors and blood with a high platelet concentration. PRP's growth factors, released during degranulation, promote the healing of different tissues and hasten the regeneration of new cells when given to wounds. (Khalisha et al., 2018) According to reports, PRP can promote the healing of musculoskeletal tissue,

including bones, muscles, tendons, and cartilage. (Boivin et al., 2023; Wang et al., 2019) PRP can influence cell proliferation and the migration and differentiation of several cell types, including chondrogenic stem cells and subchondral progenitor cells. Since PRP can influence cell proliferation, migration, and differentiation, it is commonly used in medical applications and cell cultures instead of FBS supplements. (Boivin et al., 2023; Nguyen & Pham, 2018; Rattanasuwan et al., 2018; Strauss et al., 2020)

After running flow cytometry, it can be seen that UC-MSC has a cluster of differentiation that meets the ISCT criteria, namely positive CD73, CD90, CD105, and negative expressing CD34, CD45, CD14, and CD79. Approximately 95% of the hematopoietic markers CD105, CD73, and CD90 were expressed by a range of MSC populations; however, CD45, CD34, CD14, and CD19 were not expressed. According to the flow cytometry graph data, surface markers such as CD73, CD90, and CD105—positive indicators of UC-MSC—have an expression level above 95%. Conversely, HLA-DR (positive <2%), a marker of hematopoietic cells, and CD34, CD45, CD14, and CD79a, negative markers of UC-MSC, are found below 2%.(Khalisha et al., 2018; Widowati et al., 2019)

FBS samples obtained results (96.18 ± 1.175) and PRP samples (99.03 ± 0.299). This shows that PRP expresses CD73 more highly, which functions in the proliferation, differentiation, and migration of UC-MSC.(Kimura et al., 2021; Li et al., 2021; Kezhe Tan et al., 2019) These findings are inconsistent with earlier studies demonstrating that FBS expresses CD73 at a greater level than PRP. (Khalisha et al., 2018) However, it is in line with research that PRP expresses CD73 more than FBS. (Martinez et al., 2019) Most people agree that CD73 is a traditional surface marker that characterizes mesenchymal stem cells as multipotent. It was discovered, nevertheless, that the expression of the CD73 protein varied throughout sources. Bone marrow contains 19.2%, subcutaneous adipose 51.8%, and umbilical cord 84.2%. (Kimura et al., 2021; K. Tan et al., 2019)

FBS samples obtained results (98.33 ± 0.737) and PRP samples (99.62 ± 0.097). This shows that PRP is higher in expressing CD90, which functions as a UC-MSC adhesion. (Guan et al., 2019) PRP has a higher value in expressing CD90 than FBS. CD90 variable in PRP was higher than FBS, and the CD90 surface marker functioned as cell adhesion. (Rashid et al., 2023; Rosadi et al., 2024)

FBS samples obtained results (99.45 ± 0.158) and PRP samples (99.25 ± 0.255). Research indicates that UC-MSCs grown in FBS express CD105 more than those grown in PRP. This is significant since CD105 is a well-known marker linked to MSCs and essential to their operation. (M. Mebarki et al., 2021; Pham et al., 2019) The functions of CD105 include migration, differentiation, and proliferation of cells. (M. Mebarki et al., 2021; Wang et al., 2020) Increased MSC proliferation rates are correlated with higher levels of CD105, which is necessary for growing cell populations for therapeutic uses. (Qu et al., 2020), The homodimeric transmembrane protein CD105, encoded by endoglin, is produced by most organs and is a component of the growth factor beta receptor transformation complex (TGFBR). One prerequisite for identifying MSCs is the expression of CD105. Regarding these biomarkers' dependability, there are, nevertheless, conflicting results. (Pham et al., 2019; Rashid et al., 2023; Wu et al., 2023)

CONCLUSIONS

Mesenchymal stem cells (MSCs) from the umbilical cord were used in the study, and PRP and FBS were used as culture supplements to evaluate the expression of surface markers in PCs. The CD73 surface marker was expressed by MSCs grown with FBS (96.18±1.175), CD90 (98.33±0.737), and CD105 (99.45±0.158), while those cultured with PRP exhibited higher expression levels of CD73 (99.03±0.299), CD90 (99.62±0.097) and CD105 (99.25±0.255). A significant difference in CD73 expression ($p=0.0225$) was observed between the two groups using the t-test, with no significant differences in CD90, CD105, or negative markers (CD45, CD34, CD14, CD19) based on the Mann-Whitney test. These results suggest that PRP could be a viable alternative to serum supplementation in MSC culture, particularly for enhancing CD73 expression while maintaining stable expression of other key markers.

Author's Contributions: PAP: Performed the experiment; Analyzed and interpreted the data; Wrote the paper WLS: Performed the experiment; Analyzed and interpreted the data; Wrote the paper; Contributed reagents, materials, analysis tools or data, reviewed the manuscript, HJ: Performed the experiment; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data, reviewed the manuscript; SWA: Performed the experiment; Analyzed and interpreted the data; reviewed the manuscript.

Competing Interests: The authors declare that there are no competing interests.

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