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LHPL as Substitute for FBS in Stem Cell Culture by FC

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Abstract

With the advent of regenerative medicine, new methods for treating many incurable diseases might be used to replace damaged, dead, or diseased cells using cell-based methodologies. Mesenchymal stem cells possess immunomodulatory, tissue-protective, and pro-angiogenic properties that make them highly promising for cell-based therapies. Umbilical cord tissue (UCT) is the most common source of mesenchymal stem cells (MSCs) used for explant culture because of its potential for ex-vivo proliferation. Fetal bovine serum (FBS) is the conventional and promising present medium used to culture the MSCs. It is derived from the blood of cow fetus on cardiac puncture without anaesthesia that causes a lot of pain and discomfort to the fetus. This makes its availability limited for research work. Also using a xenogeneic supplement like a foetal bovine serum (FBS) to grow MSCs outside the body increases the risk of zoonotic infection transmission and unfavourable immunological reactions. The self-life of MHC is also very limited. A lot of research is going on to find an alternate to FBS of the above reasons like, human serum, adult bovine serum, lyophilized human platelet lysate (LHPL and chemical formulations. Our research here experimented the potency of lyophilized human platelet lysate (LHPL) as an alternate for FBS in culturing MSCs by using flow cytometry and proved, LHPL is a non-xenogeneic growth supplement with a longer self-life that will sustain the development of MSCs effective and can be stored and reconstituted on demand.

Keywords: Foetal Bovine Serum, Immunomodulatory, Lyophilized Human Platelet Lysate, Mesenchymal Stem Cells, Zoonotic.

Introduction

In today's scientifically advanced world, human umbilical cord mesenchymal stem cells (MSC) are evolving as one of the highly promising cell-based therapies (1) because of their homing capacity, immunomodulatory ability (2), tissue-protective as well as anti-fibrotic and anti-apoptotic actions and so are presently being tested in the treatment of numerous clinical trials like diabetes, Parkinson's disease, Crohn's disease, osteoarthritis, liver failure, acute kidney injury and orthopaedic medical conditions (3). It is easily available as it is regarded as a biological waste (4). The quantity of MSC's required for cell-based treatment ranges from $0.5–2.5\times10^6$ cells/kg of body weight and the umbilical cord tissue (UCT) MSC's are better candidates to produce the necessary amount cells in ex-vivo proliferation. Alternative culture media supplements are under investigation now a days, because of the non-availability of FBS in required amount and its adverse effects (5). Iron-fortified calf serum is one such substitute (ICS). Larger volumes may be produced since the serum is taken from calves. Nevertheless, iron supplementation was necessary since calves are fed a diet deficient in iron. Despite the fact that ICS contains growth factors and cytokines that help the formation of MSC, batch-to-batch variation is observed. Because MSCs are derived from xenogeneic sources, there is some concern that the host may experience an immune reaction after MSC transplantation (6, 7). Other promising alternative growth supplements under test are the fresh platelet lysate and umbilical cord blood serum. Platelets from blood banks are regarded as medical waste after five days due to their aggregation and elevated risk of microbial contamination, but the process lyophilisation can

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eliminate the limitations of HPL and allowing for an extended shelf life (8). Furthermore, recent studies saying the LHPL can be reconstituted as needed for cell culture and so we planned to focus our research on LHPL as an alternative for FBS. The culture of MSC's can't be promoted in basal medial media without any supplement and was proved by Diez *et al.,* (9). The International Society for Cellular Therapy (ISCT) long set its standards for culture medial supplements and as of now FBS is the gold standard supplement for culturing MSC's with its own flaw and so ISCT called to develop a human based supplement in culturing MSC's. In this present study we analysed the potency of human LHPL as an alternate for FBS, examined and compared the parameters like cell proliferation and immuno phenotypic analysis of hUCT-MSCs, grown with FBS and LHPL. Parameters like Cell proliferation including, plastic surface adherence, cell yield and viability were analysed by using inverted microscope with trypan blue staining (10). The immuno phenotypic analysis and comparison between FBS and LHPL grown MHCs was done using flow cytometry. Our study focused and proved on the advantages of LHPL over FBS in cultivating and differentiating hUCT-MSCs in required amount.

Materials and Method

Materials

Umbilical cord samples, Platelet lysate, Alpha-MEM, FBS, Inverted microscope, CD90, CD105, CD13, and HLA DR Antibodies, BD FACS Canto II Flow Cytometer, FlowJo Software (Tree Star), Image J software.

Huct Collection and Explant Culture

The total experimental design was duly approved by the Institutional Human Ethical Committee, Committee for the Purpose of Control and Supervision of Experiments on Human, Sri Lakshmi Narayana Institute of Medical Sciences, with the approval number IEC/C-P/18/2021. Umbilical cord samples (n=3) from caesarean deliveries were collected from Sri Lakshmi Narayana Institute of Medical Sciences in compliance with Helsinki Declaration (11). Umbilical cord samples were collected on caesarean deliveries to reduce the potential risk of contamination with vaginal delivery after getting consent from mothers. Within 4 hours post collection, the materials were brought to the laboratory at 4ºC in tubes containing 1X PBS with Gentamycin, (Gibco, USA). In the laboratory the samples were cut into 5 cm^2 segments after being rinsed with PBS and cultured in Minimum Essential Medium Eagle with Alpha Modification (alpha-MEM) with antibiotics. The samples were divided into two groups; one supplemented with 20% FBS and the other with 10% LHPL in primary cell culture - passage 0 (P0).

Collection of Lyophilized Platelet Lysate

The expired blood platelets from blood bank were used as the basis for this investigation. Blood platelets from types AB and O were pooled, processed in a pathogen free environment and the lysate was collected in the expected concentration (12). Following that the platelet lysate was lyophilized, gamma sterilized, and stored at 4 ºC until use (13).

Parameter Analysis - Proliferation Study

The MSC characterization of the LHPL-supplemented cultures complied with the standards established by ISCT, including Proliferation and Immuno phenotypic analysis were selected as parameters for this study. Rate of proliferation and the characteristic of cells after proliferation are important indicators of a proper culture media supplement as we need 3-8×10^6 cells/kg body weight (14), to be transplanted during cell therapy. So by analysing and comparing the cultured hUCT-MSCs supplemented with LHPL and FBS, we can conclude the ability of LHPL in substituting FBS in tissue culture. Each sample was cultured in two different medium compositions; one supplemented with 20% FBS and the other with 10% LHPL in P0. The tissue culture flasks were incubated in a humidified environment with 5% CO_2 and incubated at 37 °C for seven (15) days without being disturbed. Following the first week-long incubation period, the medium was replaced for the first time, and then every 3-4 days thereafter. At passages 1, 2, and 3, the basal medium of each batch was supplemented with 10% FBS and 5% LHPL - "Figure 1". After two weeks, the hUCT explants were removed, and the adhering cells were allowed to proliferate until day 21, at which point the cells were separated using 0.05% trypsin-EDTA. The trypan blue staining of subculture cells was used to quantify the cells.

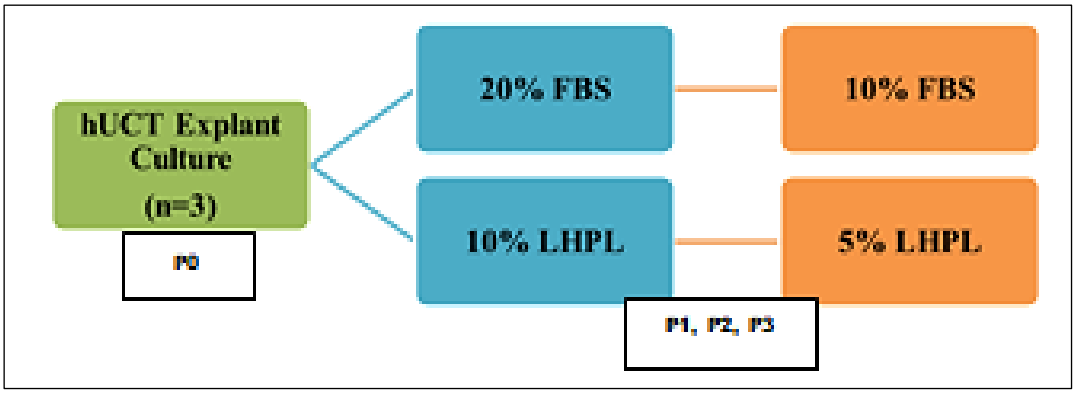

Figure 1: Showing the Overall Representation of hUCT MSC Explant Culture using FBS and LHPL

Plastic surface adherence - The explant cultures obtained from hUCT supplemented with FBS and LHPL were morphologically analysed for plastic surface adherence by using an inverted microscope.The cell yield and cell viability- was analysed by staining the subculture cells by trypan blue and counting the live and dead cells in a Neubauer chamber (16) using an inverted microscope.

Parameter Analysis - Immuno Phenotypic Analysis - Flow Cytometry

This parameter analyses the expression of hematopoietic markers over the surface of cultured hUCT-MSCs supplemented with FBS and LHPL. According to International Society for Cell and Gene Therapy (ISCT), expression of certain cell marker (positive for CD90, CD105, CD13, and negative for HLA DR) (17), are one of the standard product specifications for MSCs responsible for immune modulation. They modulate the immune response of the recipient and so involve in the management of graft rejection. So as to analyse and compare the immuno modulatory effect of the cells cultured, we have planned to analyse 4 markers in FBS and LHPL supplemented hUCT-MSCs. CD90 is a potential marker for cancer cells as it regulates cell death. CD105 regulates the integrity of vascular endothelium and so involve in wound healing and tissue regeneration. CD13 regulates the formation and activities of immune cells like phagocytosis and secretion of cytokines. All the above hematopoietic markers (18) represent the hUCT-MSCs blood forming ability. HLA DR (negative marker) is an antigen presenter that elicits the immune response.Analysis of the cultured hUCT-MSCs surface markers was done using flow cytometry. Post-harvest, a single-cell suspension was taken, followed by hUCT-MSCs incubation in dark for 15 minutes with fluorescent conjugated antibodies. In this analysis, antibodies against CD90-FITC (BD Biosciences), CD13-FITC (Bio-legend), HLA DR, and CD105-FITC (R&D Systems) were utilized. After being washed in 1XPBS and centrifuged for five minutes at 1500 rpm, the cells were resuspended in 1XPBS in preparation for flow cytometry analysis. The samples were then examined in BD FACSCanto II Flow cytometer (19) and the data obtained was analysed using FlowJo Software (Tree Star).

Results

The ISCT (20) has established minimal requirements for MSCs to proliferate and have fibroblast like morphology, including their ability to adhere to plastic surfaces and to express specific surface markers. All the above criterions were easily met by LHPL grown MSCs.

Parameter Analysis - Proliferation Study

The explant cultures obtained from human umbilical cord tissue @ P2 from FBS and LHPL were morphologically identified through microscopic magnification. When maintained for long-term culture, the hUCT-MSC primary cultures with 20% FBS and 10% PL supplementation were noted for fibroblast-like morphology. Plastic surface adherence- is one of the properties of MSC that enable the MSC to adhere over the surface of plastic to form a homogenous layer of culture. This property was observed with FBS and LHPL supplemented MSC. Under 4X magnification, plastic adherence of MSC to form a homogenous layer of culture was seen. The “Figure 2” displays the explant cultures taken from human umbilical cord tissue at P2

using FBS and LHPL at 4X magnification. On analysis the fibroblast like morphology and plastic surface adherence property of hUCT-MSCs grown in FBS and LHPL were looking similar with more cells in LHPL supplemented culture media.

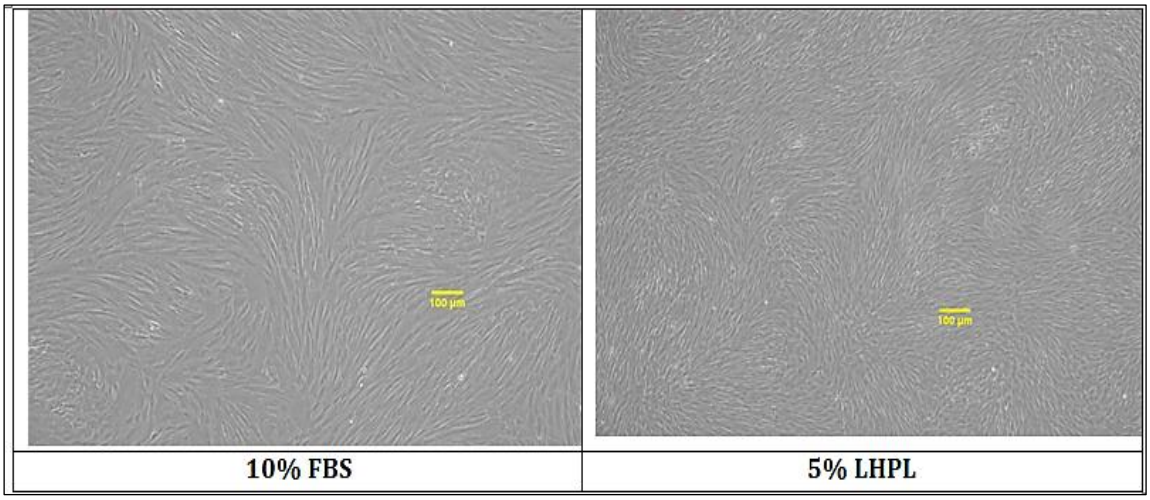

Figure 2: Showing the Plastic Surface Adherence and Fibroblast like Morphology (left to right) of Explant Cultures obtained from hUCT at P2 with FBS & LHPL Supplementation. (4X Magnification)

The cell yield and cell viability of all the 6 groups were analysed by staining the subculture cells by trypan blue and counting the live and dead cells in a Neubauer chamber. The values were tabulated and the mean values were calculated. A bar chart was created based on the mean values to compare the cell viability and cell yield between FBS and LHPL supplemented hUCT-MSCs - "Figure 3". Cell yield and cell viability between LHPL and FBS supplement shown more viable cell yield in LHPL supplemented hUCT-MSC.

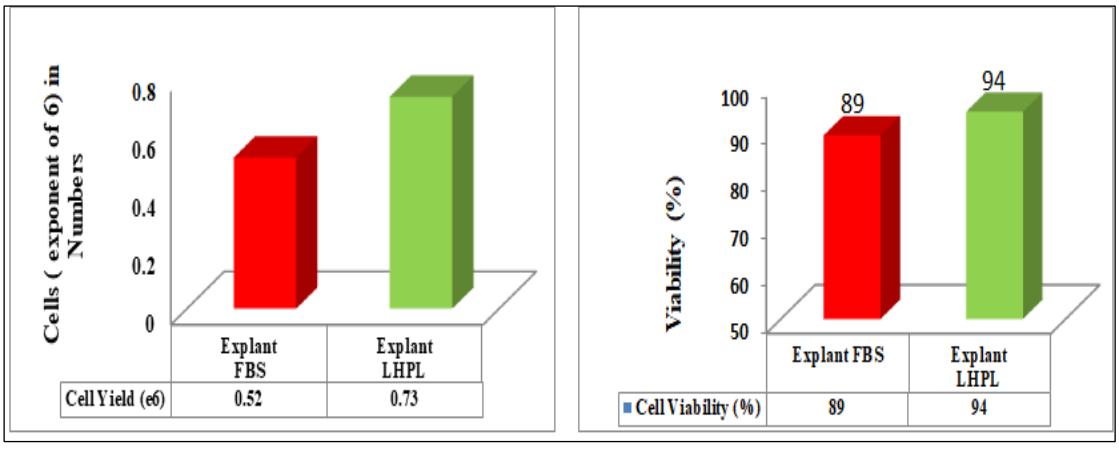

Figure 3: Comparison of Cell Yields in Exponents (e6), A and Percentage of Viability (B) from Explant FBS and LHPL Cultures Supplemented with 20% FBS and 10% LHPL using Trypan Blue Dye Exclusion at P0

The FBS supplemented culture yielded a mean of 0.52e6 explant cells per yield and LHPL supplemented culture yielded a mean of 0.73e6 explant cells per yield. The mean percentage of cell viability was found to be 84% with LHPL supplement and 89% with FBS supplement. The above findings demonstrated that the mean cell yield and viability of MSCs cultured with LHPL supplement met the ISCT criteria and even over-powering the results of FBS supplement.

Parameter Analysis - Immuno Phenotypic Analysis - Flow Cytometry

Flow cytometric examination of UC-MSC at P0 using conjugated antibodies against the hematopoietic markers shown the expression of marker like, CD90, CD105, CD13, and HLA DR over the surface of hUCT-MSC supplemented with both LHPL and FBS "Figure 4".

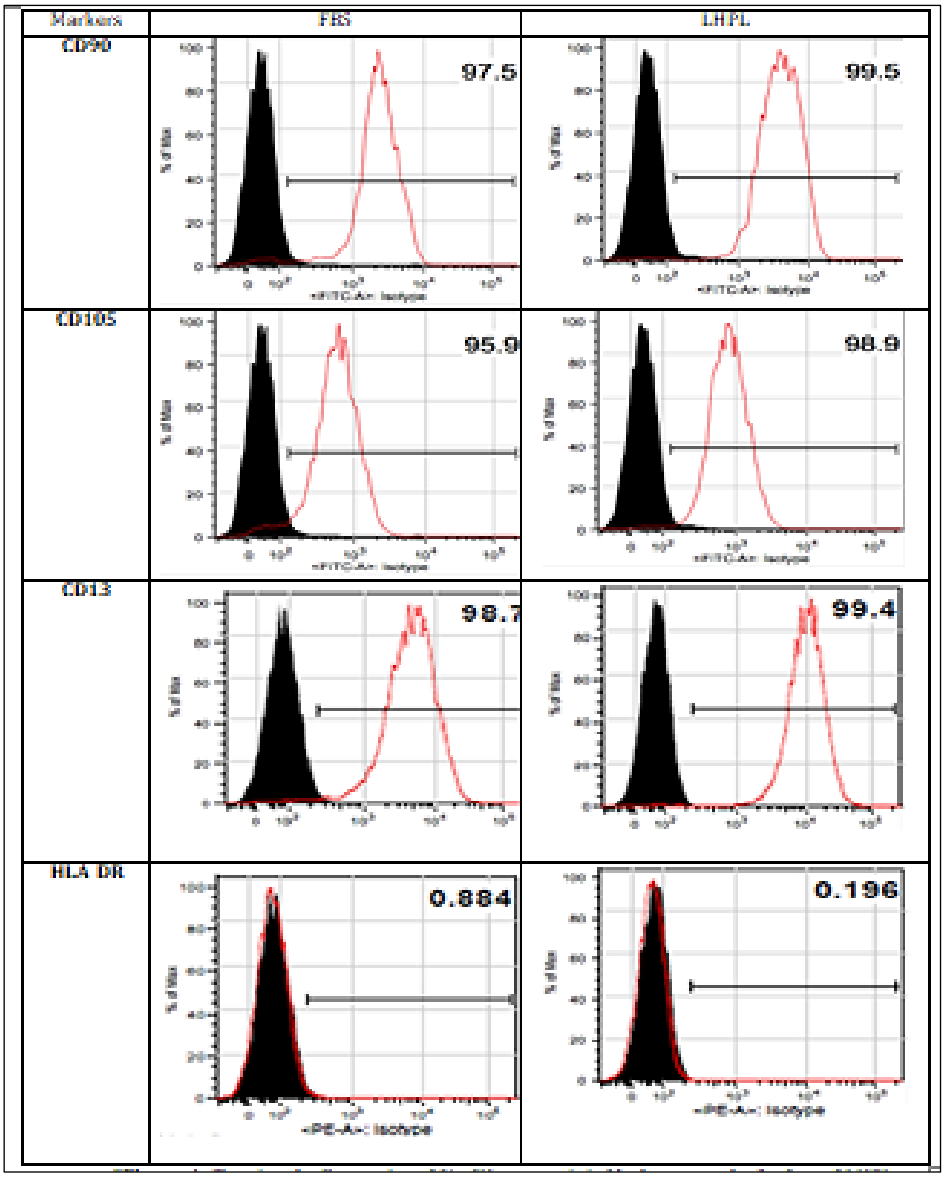

Figure 4: Showing the Expression of % of Hematopoietic Markers over the Surface of LHPL or FBS Supplemented hUCT-MSC by Flowcytometry

On comparison, the expression of positive markers such as CD90 (99.5%), CD105 (98.9%) and CD13 (99.4%) were more in LHPL supplemented hUCT-MSCs than in than in FBS supplemented hUCT-MSCs - "Table 1".

Table 1: Showing the Comparison between the Expression of Hematopoietic Markers over the Surface of LHPL or FBS Supplemented hUCT-MSC by Flow Cytometry

Surface Marker (%)	CD 90	CD 105	CD 13	HLA DR
FBS	97.5	95.9	98.7	0.884
LHPL	99.5	98.9	99.4	0.196

A bar chart was drawn to ease the comparative statistical analysis, on the percentage of expression of hematopoietic markers over the MSC's supplemented with FBS and LHPL and the values were also marked over "Figure 5".

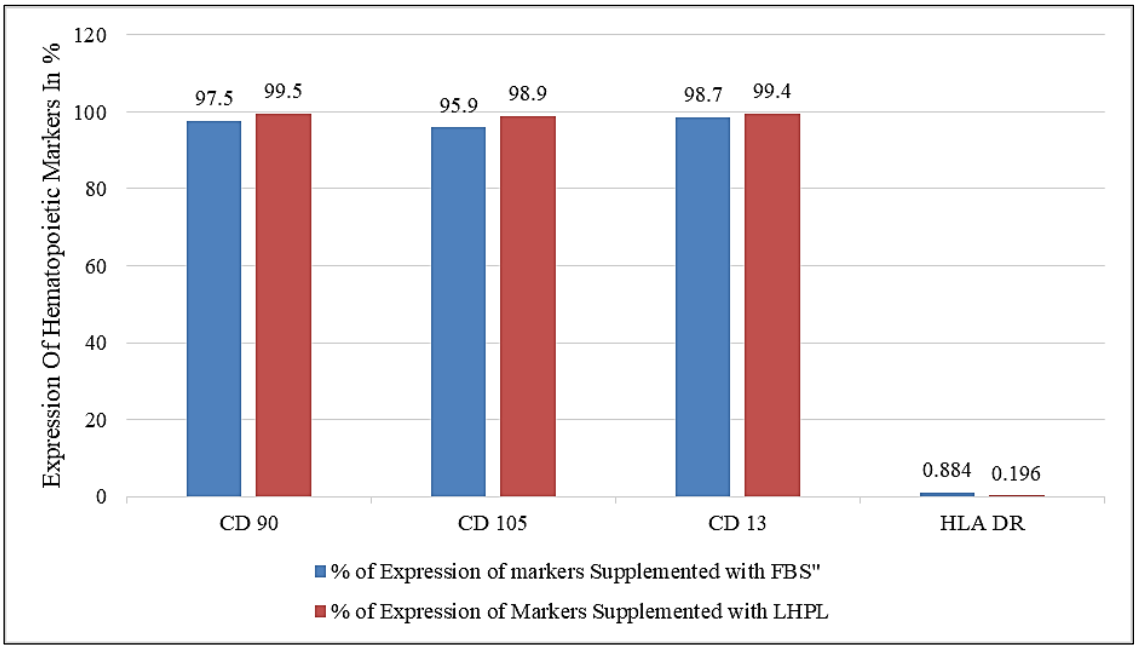

Figure 5: Showing the Comparison between the Expression of Hematopoietic Markers over the Surface of LHPL or FBS Supplemented hUCT-MSC by Flow Cytometry

That clears that the hUCT-MSC's cultured with both FBS and LHPL has immunomodulatory effect but the hUCT-MSC cultured with LHPL has a clear upper hand and is very much important to maintain the immune homeostasis.

Discussion

With more clinical trials focusing on using MSCs to treat various diseases, the need to increase MSC output has gained more attention. While bone marrow MSCs has pioneered the development of MSC-based therapies, the obvious increase in the use of hUCT-MSCs can be attributed to their apparent advantages. The hUCT-MSCs are desirable candidates for nearly all cell therapy studies because they are naïve and exhibit high telomerase activity, a sign of a significant proliferative ability. FBS has historically been used to culture-expand hUCT-MSCs as stated by Jochems (21). It is accepted, nonetheless, that FBS might not be the best supplement for growth. Concerns in the utilization of FBS include batch-to-batch variability and ethical questions surrounding the collection of blood via cardiac puncture from unborn calves. Moreover, the use of FBS has two further downsides. First, there is a chance that this xenogeneic growth supplement will cause human immune reactions. Second, disease transmission via zoonotic means can possibly occur (22).The US FDA recommends using allogeneic or other non-xenogeneic growth supplements instead of FBS as a source of the cytokines and growth factors required for MSCs migration and culture expansion. Several FBS substitutes have been developed through many years of study, including cord blood serum, human platelets, as well as platelet lysate produced from human blood. Because a lot of platelets are wasted and not used, we considered Guiotto's idea that expired platelets might be utilized for cell culture (23). Previous researcher Azouna et al., proved that freshly prepared HPL can fully replace FBS in culturing hUCT-MSCs (24). In this present study we used lyophilized HPL (LHPL) as a media supplement in culturing hUCT-MSCs. The LHPL can be stored and can be reconstituted when needed. According to our findings, neither plastic adherence nor the cell shape was changed by applying LHPL, and the hUCT-MSC growth and viability of MSCs were increased than unaffected. This suggests that LHPL can be a highly effective substitute for FBS in hUCT-MSC explant cultures.The ISCT's recommendation for MSC characterization focusing on the expression some surface markers like CD90, CD105 and CD13 and the lack of other surface markers like HLA DR. Likewise our work proves the expression immune modulatory markers like CD90, CD105 and CD13 better than FBS supplemented culture. The expression of HLA DR, that elicit the immune response was much reduced in LHPL grown MSC than in FBS grown

MSC that proves the LHPL grown MSC will not involve with eliciting the immune response of the recipient during cell therapy, these cell markers proves the multipotent (25) haematopoietic ability of the MSCs grown in both supplements. This present study highlights the advantages of using LHPL as a substitute for FBS in culture media to expand human MSCs, in terms of maintaining the MSCs' plastic surface adherence, rate of proliferation, cell yield and cell viability which are very important in clinical setting as we need potent and viable cells 3-8×10^6 cells/kg body weight during transplantation of these cells in human and as per this present study results cell yield and viability is for more better with LHPL than FBS. Immune modulatory effect of MSC's plays a crucial role in differentiation of cells in clinical procedures. The present study proved that LHPL supplementd cells are more immune-modulatory than the present days gold standard FBS supplement. Added with that LHPL can be obtained from a human biological waste and so easy availability in large quantity, cost effective and can be stored and reconstituted when needed in clinical procedure without any major limitations. Our work investigated and validated the potential use of LHPL, and its ability to promote the culture development of hUCT-MSCs compared to that of FBS, a comparably less expensive FBS substitute. In clinical practice, lyophilizing the platelet lysate could increase its usefulness for a longer period of time with a positive note on immune-modulation.

Conclusion

Our findings suggest that LHPL may have long-term usefulness in situations where storage and reconstruction of LHPL is possible on demand and is cheap. MSC culture can also be carried out more successfully using the reconstituted LHPL with better result than FBS supplement. Based on our findings, it is possible to increase the usage of expired platelets, first by creating lysates and subsequently by lyophilizing those lysates. Such lyophilized platelet lysates may provide a non-xenogeneic, less expensive growth supplement with a longer shelf life promoting the best MSC culture.

Abbreviations

LHPL: lyophilized human platelet lysate, FBS: Foetal bovine serum, UCT: Umbilical cord tissue, MSCs : Mesenchymal stem cells, hUCT: Human umbilical cord tissue, PBS: Phosphate buffered saline, P0: Passage 0 – Primary cell culture,P1: Passage 1 – 1st subculture,P2: Passage 2 – 2nd subculture,P3: Passage3 – 3rd subculture, ISCT: International Society for Cellular Therapy, EDTA: Ethylene Diamine Tetra Acetic Acid ,CD: Cluster Differentiation, HLA-DR: human leukocyte antigen – D receptor, FITC: Fluorescein Iso Thio Cyanate, US-FDA: United States Food and Drug Administration.

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Author Contributions

Designing the work – All Authors - Dr. Najwa Abdur Rashid, Dr. Mary Anne W.Cordero, Dr. Mary Antony Praba, Dr. Kavitha Ganesh, Dr. Raja el Hasnaoul-Saadani, Dr. Venkataramaniah and Dr. Angeline Julius. Execution – Authors - Dr. Najwa Abdur Rashid, Dr. Mary Anne W.Cordero, Dr. Kavitha Ganesh, Dr. Raja el Hasnaoul-Saadani,Manuscript preparation – Authors Dr. Mary Antony Praba, Dr. Venkataramaniah.

Conflict of Interest

The authors declare no conflict of interest.

Ethics Approval

Institutional Human Ethical Committee, Committee for the Purpose of Control and Supervision of Experiments on Human, Sri Lakshmi Narayana Institute of Medical Sciences - IEC/C-P/18/2021.

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