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Tri-lineage Differentiation and Secretome Analylsis of FBS and LHPL Supplemented hUCT-MSCs - A Comparative Evaluation on ISCT Criteria

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With advancements in regenerative medicine based on human umbilical cord tissue derived mesenchymal stem cells (hUCT-MSCs) plays major role in clinical trials on tissue engineering and cell therapies to a variety of diseases like ischemia, diabetes, bone, cartilage and neurological disorders. Presently the gold standard supplement in culturing hUCT-MSC is foetal bovine serum (FBS), obtained in an unscrupulous way from the foetus of cow. Being a xenogeneic supplement, FBS increases the risk of zoonotic infection transmission and unfavourable immunological reactions in patients after transplantation. Also it is high on demand and with ethical issues. According to International Society for Cellular Therapy (ISCT) a supplement of culture media for hUCT-MSC must possess a few qualities like tri-lineage differentiation and must secrete certain cytokines with a zoonotic free environment and so called to find a human derived supplement alternate. Human platelet lysate (HPL) was considered a better supplement in culturing hUCT-MSCs but it can't be stored in normal temperature nor reconstituted in time as it needed freezing thawing cycles. New researches saying that lyophilized human platelet lysate (LHPL) can be an effective alternate as it can be stored and reconstituted without zoonotic infections. So we have formulated this study so as to evaluate the potency of LHPL in fulfilling the ISCT criteria on proliferation, tri-lineage differentiation and on secretomes as a supplement in culturing hUCT-MSCs.

Keywords: Alizarin red S; Lyophilized human platelet lysate; Mesenchymal stem cells; Oil Red O; Tri-lineage differentiation.

With technical evolution in clinical trials on tissue engineering and regenerative medicine, new cell based therapies are emerging every day to replace the dead or diseased cells for a lot of incurable diseases. Mahla et al., strongly believe the spectacular progress in cell based therapies, stem cell research and so hUCT-MSC's are potent candidates in most cell based therapies as they possess multipotency, high range of proliferation, homing capacity, immunomodulation and valuable secretomes.[1] Worldwide hUCT-MSC's are used to treat a variety of illness, such as autoimmune disorders, cancer, haematological disorders, metabolic disorders, physical trauma like wounds

and burns, and orthopaedic medical conditions, and so tissue engineering opened a new door for treating incurable diseases says Hoang et al.,[2]. The fact that these cells are presently being tested in numerous clinical trials for illnesses like diabetes, Parkinson's disease, Crohn's disease, osteoarthritis, liver failure, and acute kidney injury is not a surprise.

The media supplement we use in culturing hUCT-MSC is very important for the fine characterization of the MSC's. The gold standard supplement used until recently is FBS. It is a xenogeneic supplement obtained from the foetus of cow, says Lee et al.,[3] and so increases the risk of transmitting zoonotic infection and involve in deleterious immunological reactions. Its origin also raised a lot of ethical issues off late and so not available in the market as per need and is high in cost.

The above said issue pressed the ISCT to call for an invention as an alternate to FBS, a human based zoogenic free supplement, must be available in large quantity with relatively low in price without any ethical issues. A lot of supplements, both human, non-human and chemical based were tested till date for their characteristics in modulating hUCT-MSC for its optimum capacity. One of the supplement successfully tested was HPL. HPL was prepared from the expired human platelets from the blood bank and so originally considered as biological waste. Freshly prepared HPL is a very good alternate supplement for FBS as describes by Oeller et al., also it is cheap and easy to get and is a human substrate.[4] But we need to store HPL below -80° for a long time and can't reconstitute and use when needed as it includes freeze thaw cycles, states Yeh et al.[5]

A lot of researches in present days concentrating on LHPL, the lyophilized HPL, that is also obtained from expired human platelets. The lysate that obtained from the platelets will be lyophilized and so can be stored in large volume even in 4° and can also be reconstituted and used at time of need.[6] The LHPL as per the researchers, may be a good zoogenic free ready to use alternate for FBS.

According to ICST, a good culture media supplement must have certain standards with hUCT-MSC's like high range of proliferation with healthy and live cells, as applications in tissue engineering needs a range of $0.5 – 2.5 \times 10^6$ healthy cells/kg of body weight during transplantation as in trial by Erasmus.[7] Another required quality is tri-lineage differentiation, the supplement must help the hUCT-MSC's to get differentiated into adipocytes, chondrocytes and osteocytes with the addition of differentiating agents,[8] and they also must support the hUCT-MSC's to secrete certain cytokines that involves with therapeutic outcome.

In this present study the above said standards of LHPL supplemented hUCT-MSCs were evaluated in comparison with FBS supplemented hUCT-MSCs. The MSCs thus harvested were even better than the gold standard FBS in most of the parameters with an extended self-life and also were zoogenic and adverse immune reaction free.

MATERIALS AND METHODS

MATERIALS

- hUCT-MSC
- 20% FBS
- 10% LHPL
- Nuebauer chamber
- Adipogenic Differentiation Kit, STEMPRO, GIBCO, USA.
- Chondrogenic Differentiation Kit, STEMPRO, GIBCO, USA.
- Osteogenesis Differentiation Kit, STEMPRO, GIBCO, USA.
- Alizarin Red S – HI Media, India
- Oil Red O – Sigma, USA
- Safranin O - HI Media, India
- Inverted Microscope
- Cytokine arrays
- 7-Amino Actinomycin D
- BD FACSCanto II Flow Cytometer
- FlowJo Software (Tree Star)
- Level-3 10,000 clean room

METHODS

Collection of human umbilical cord

The total experimental design was 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 (IEC/C-P/18/2021). Umbilical cord samples were collected on caesarean deliveries to reduce

the potential risk of contamination with vaginal delivery after getting the mothers consent from Sri Lakshmi Narayana Institute of Medical Sciences in compliance with Helsinki Declaration (11). The cell line laboratorial works were carried out in AcaDiCell Innovations International Pvt. Ltd with level-3 10,000 clean room and industry standard equipment facility.

The umbilical cord samples were collected in collection tubes at 4ºC containing 1X PBS with Gentamycin, (Gibco, USA) and were taken to the laboratory Within 4 hours post collection. The samples were processed, divided into two groups and cultured in FBS and LHPL supplemented culture media for evaluation.

Preparation of lyophilized human platelet lysate

Blood types O and AB were pooled from the blood bank's expired platelets and were used as the basis for this investigation. All the steps in "Figure 1" were followed to carry out the LHPL preparation. Platelet concentrate (PC) obtained from the blood bank's platelet concentrate (PC) bags were pooled in 3:1 (O : AB) ratio and was kept at -80ºC for 24 hours. Then, a water bath of 37ºC was used to defrost the frozen PC. After three freeze-thaw cycles the platelets were centrifuged at 4000g for 15 minutes to get the lysate, then the lysate was sterile filtered by a 0.22 um PES syringe filter. Following filtration, the platelet lysate was lyophilized, gamma sterilized, and stored at 4 ºC for a short period or -20 ºC until use, recites Kerstin et al.,[9].

Parameter analysis

Analysis of Proliferation and Apoptosis of hUCT-MSCs

According to ICST, a good culture media supplement must aid the hUCT-MSCs to proliferate in high rate with healthy and live cells. The rate of proliferation of MSCs were analysed by counting them under the microscope in a Neubauer chamber.

Three culture groups were put into test in each group for the current parameter of this study, with 20%FBS and 10%LHPL supplements and cultured the MSCs in passage0 (P0 – Primary culture), P1 (Subcultures 1), P2 (Subcultures 2), and P3 (Subcultures 3). The cultured cells were harvested, diluted in buffer, loaded in a Nuebauer chamber and counted under an inverted microscope as Clausen et al.,[10] and the results were tabulated.

Percentage of apoptosis of cultured MSC's were analysed by 7-Amino Actinomycin D

(7-AAD) dye through flow cytometry. 7-AAD is a DNA stain that binds with the CG rich region in DNA. It is working on the principle of live cell exclusion technology as stated earlier by Zembruski et al., 2012, so live cells with intact plasma membrane will not allow the stain to permeate the membrane to bind with the DNA.[11] For analysis the P0 cells in 20%FBS and 10%LHPL were harvested, washed in buffer and centrifuged. The pelleted cells were suspended in staining buffer, added with staining solution (7-AAD) and incubated for 30 minutes in dark. The stained dead cells and non-stained live cells were counted by using FAC scan flow cytometer.

Trilineage differentiation

One of ISCT's minimal requirements for MSC's culture medial supplement is their capacity to differentiate the MSC's in-vitro into adipocytes, osteoblasts and chondroblasts in addition with differentiating media. Twelve culture groups were selected for this study. Six control groups of cultured cells without FBS and LHPL, three FBS test groups with MSC's cultured in FBS supplement and differentiated with Tri-lineage-inducing medium (for adipocytes, chondrocytes and osteocytes) and three LHPL test groups with MSC's cultured in LHPL supplement and differentiated with Tri-lineage-inducing medium (for adipocytes, chondrocytes and osteocytes).

Adipogenic Differentiation

The hUCT-MSCs that were cultured with á-MEM with either FBS or LHPL supplement were harvested at the end of P3. After that, 4000 cells/cm^2 were seeded at 37°C with 5% CO_2 in a humidified environment on 6-well plates coated with 0.1% gelatin. Adipogenic differentiation was induced in both test cultures using an adipogenesis-inducing medium (AIM -Adipogenesis Differentiation Kit, STEMPRO, Gibco, USA), and was replaced every third day, both control groups were cultured without AIM. After 14 days the cells were stained with Oil Red O (Sigma, USA), fixed with 4% paraformaldehyde and observed under the microscope. The differentiation of MSC's into adipocytes were confirmed by the accumulation of lipid droplets stained with Oil Red O as affirmed by Augustyniak et al.,[12].

Chondrogenic Differentiation

The hUCT-MSCs that were cultured with á-MEM with either FBS or LHPL supplement were harvested at the end of P3. 5µl culture droplet containing 0.1x106 cells per droplet were seeded onto 6 well plates coated with 0.1% gelatin to generate a micro mass culture. The plates were then incubated for two hours at 37°C and 5% CO_2. A chondrogenesis-inducing medium (CIM - STEMPRO, chondrogenesis differentiation kit, GIBCO, USA) was added with both test cultures with a change on every third day, both control groups were cultured without CIM. After 14 days the cells were harvested and stained with Safranin O (HiMedia, India) after fixed in 4% paraformaldehyde following Deedwania et al.,[13]. The differentiation of MSCs into chondrocytes were confirmed with the deposition of Glycosaminoglycans inside the cells and that was analysed by using bright-field microscopy.

Osteogenic Differentiation

The hUCT-MSCs that were cultured with á-MEM with either FBS or LHPL supplement were harvested at the end of P3. Subsequently, 4000 cells/cm^2 were seeded at 37°C with 5% CO_2 in a humidified atmosphere on 6-well plates coated with 0.1% gelatin. Following that, an osteogenesis-inducing medium (OIM - STEMPRO, Osteogenesis differentiation Kit, Gibco, USA) was added to both test cultures, inducing them to undergo osteogenesis, both control groups were cultured without OIM. The media was changed for every 3rd day for 21 days. After 21 days the cells were fixed using 4% paraformaldehyde and stained with Alizarin red S (HI Media, India). The differentiation of MSCs into osteocytes were confirmed as stated by Gou et al., by observing the mineral deposition under bright field microscopy.[14]

Secretome analysis

The cytokines and other growth factors secreted by the hUCT-MSCs were very important for the therapeutic effectiveness of hUCT-MSCs during cell seeding in tissue engineering. A good culture media supplement is very important in inducing the necessary cytokine or growth factor secretion by the MSCs.

Three study groups were considered for this parameters. A control group with expected array map cytokines and growth factors, FBS array test group and LHPL array test group "Figure-5". The spent culture media after the second passage of hUCT-MSCs cultured with á-MEM without FBS and LHPL (control) and á-MEM with either FBS or LHPL supplement was collected and centrifuged. The culture supernatants of FBS and LHPL groups and control cytokines or growth factors were

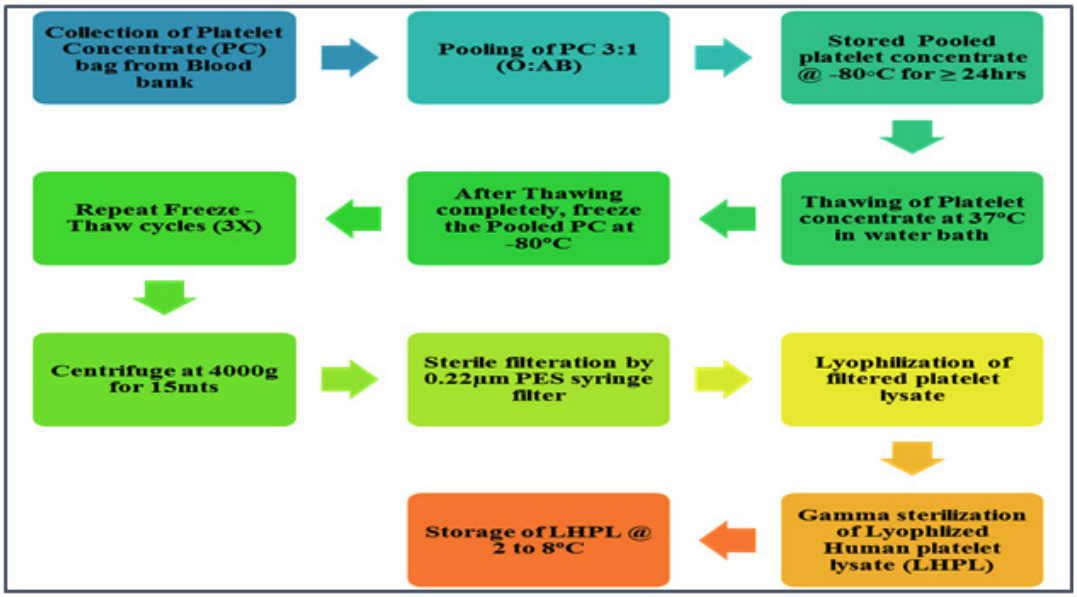

Fig. 1. Schematic diagram of the preparation of lyophilized human Platelet lysate (LHPL) in culturing hUCT-MSC

spotted in duplicate vertically on a microarray membrane precoated with specific antibodies (Figure - 5). Following Garcia et al., the microarray membranes were then incubated for 24 hours and analysed for the presence of required cytokines and growth factors.[15] The spot signals caused by the cytokines and growth factors were compared with control and between FBS and LHPL array maps.

RESULTS

Analysis of Proliferation and Apoptosis of hUCT-MSC's

Proliferation – while comparing the proliferation of cells in P0, P1, P2 and P3 supplemented with FBS and LHPL, the proliferation rate was much higher in cultures supplemented with LHPL in Primary culture and also with all the three subcultures "Figure 2".

The mean proliferation rate of P0, P1, P2 and P3 were taken and a bar chart was drawn to show the comparison "Figure 3". A trend line also was drawn to analyse the pattern of proliferation of cells in LHPL culture supplement. A clear increase in number of hUCT-MSCs from P0 to P3 were observed without any decline in LHPL supplemented media whereas there is a decline in the rate of proliferation with the P3 subculture supplemented with FBS.

Apoptosis – this parameter analyses the viable and healthy cells cultures in the primary culture as percentage of viable cells are more important than rate of proliferation in terms of tissue engineering. Though both FBS and LHPL supplemented cultures had dead cells, the percentage of dead cells are much less (4.05%) in LHPL supplemented culture when compared with FBS (6.98%) supplemented culture. Flow cytometric analysis also shown a much less percentage of dead cells grown in LHPL supplemented culture "Figure 4".

Tri-lineage differentiation analysis

One of ISCT's essential minimal requirements for cultured MSCs, is their capacity for tri-lineage differentiation. Tri-lineage differentiation of this study shown an effective differentiation of both test group hUCT-MSCs into adipocytes, osteocytes or chondrocytes depends on the differentiation media utilized. When analysing the cells of FBS and LHPL control groups, no notable trileneage differentiation was found. But comparison between test groups, the FBS and LHPL groups shown difference in the level of tri-lineage differentiation "Figure 5".

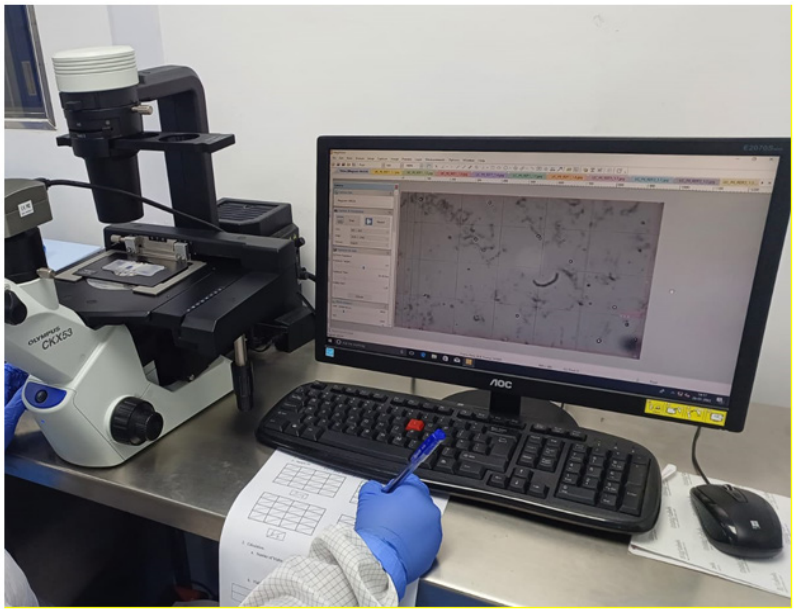

Fig. 2. Showing the process of counting the cells in Nuebauer chamber

Differentaion of hUCT-MSCs into adipocytes with Oil Red O was more or less equal between both test groups. But with other two cell lines, the differentiaion into chondrocytes with Safranin O staining and osteocytes with Alizarin red S staining clearly indicated, more differentiation with LHPL supplemented group than FBS supplementation.

Secretome analysis

Analysis of difference in cytokine upregulation and secretion of growth factor in MSCs harvested media supplemented with FBS and LHPL was done by this parameter. The image of the spot signals caused by the cytokines were read between the control and FBS, LHPL supplemented groups on the membrane array and the results were tabulated. The results among the tested 12 cytokine and growth factor secretion between both media supplements were expressed differently with clear advantage towards LHPL as more secretomes were found with LHPL "Figure 6" "Table 1".

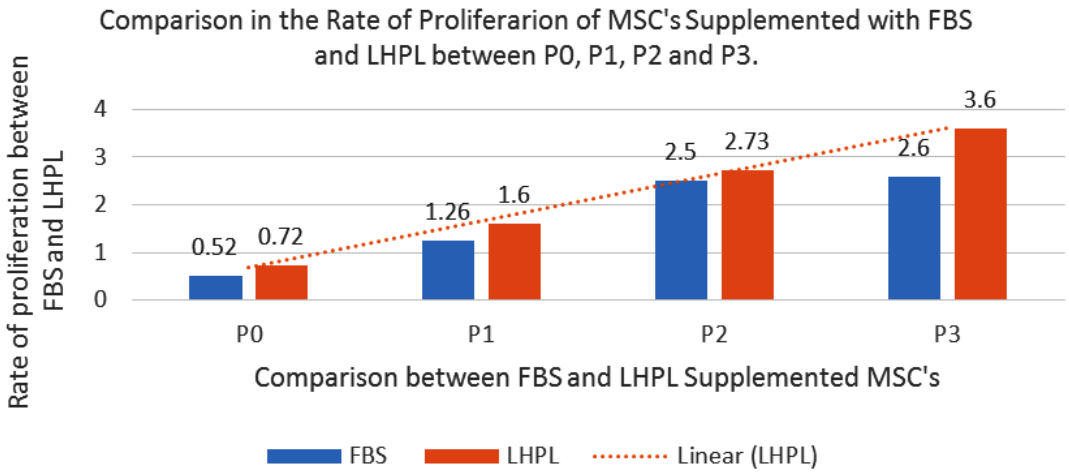

Fig. 3. Showing the Comparison in the Rate of Proliferarion of MSC's Supplemented with FBS and LHPL between P0, P1, P2 and P3

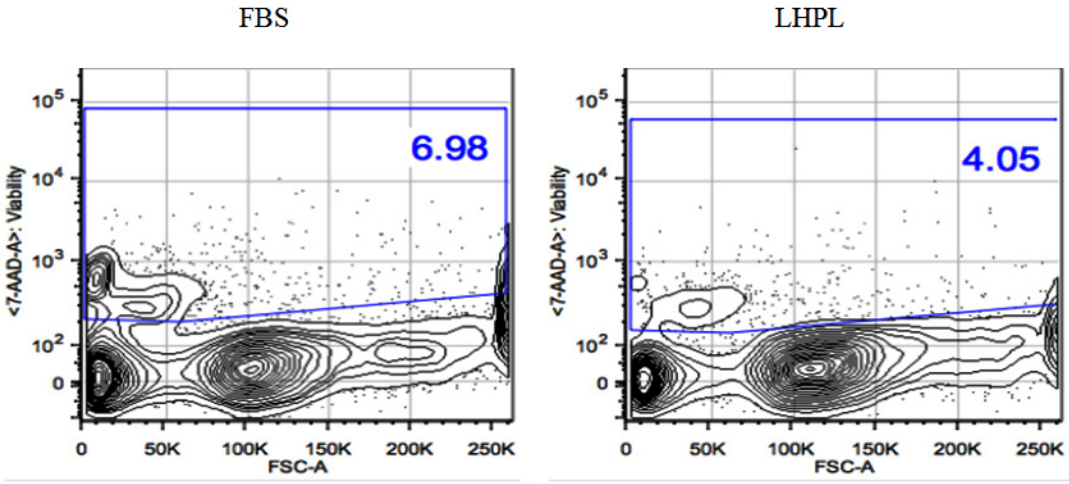

Fig. 4. Showing the analysis of % of Apoptosis among the cultured MSC's supplemented with FBS and LHPL by FAC Scan Flow cytometry

DISCUSSION

The advancements in tissue engineering technology enabling scientist world-wide to engineer MSC's from various sources on clinical trials in the treatment of a variety of diseases including incurable diseases. The universal accepted candidate for nearly all cell line therapies are the hUCT-MSCs for their high potential telomerase activity, as short telomerase are said to be the agents for biological aging, claims Arellano et al.,[16] as in prevention of biological aging and immune naivety. FBS was the all-time gold standard supplement in the expansion of the hUCT-MSCs.

It is important to note that, the most serious clinical issues with FBS is the ethical consideration in the extraction of FBS and the possibility of adverse effects from the contamination of viruses or prions following the injection of MSCs grown in FBS, says Pilgrim et al.,[17] as it is a non-human supplement. It may also cause adverse responses from bovine xenoproteins. FBS proteins have been found to be incorporated by MSCs that caused antibody production post-injection and significantly lowered the therapeutic potential of MSCs, because the newly formed antibodies were said to target the injected MSCs, explained by Heiskanen et al.,[18]. Adhering to the increase in the clinical trials on tissue engineering and regenerative medicine, finding a new and improved alternate for FBS is the need for today's research according to ISCT.

Recent researches like Sadrifar et al., focussed on the finding of a new human based supplement for culturing MSCs, including frozen human plasma and HPL and postulated that FBS combined with fresh frozen plasma or HPL was

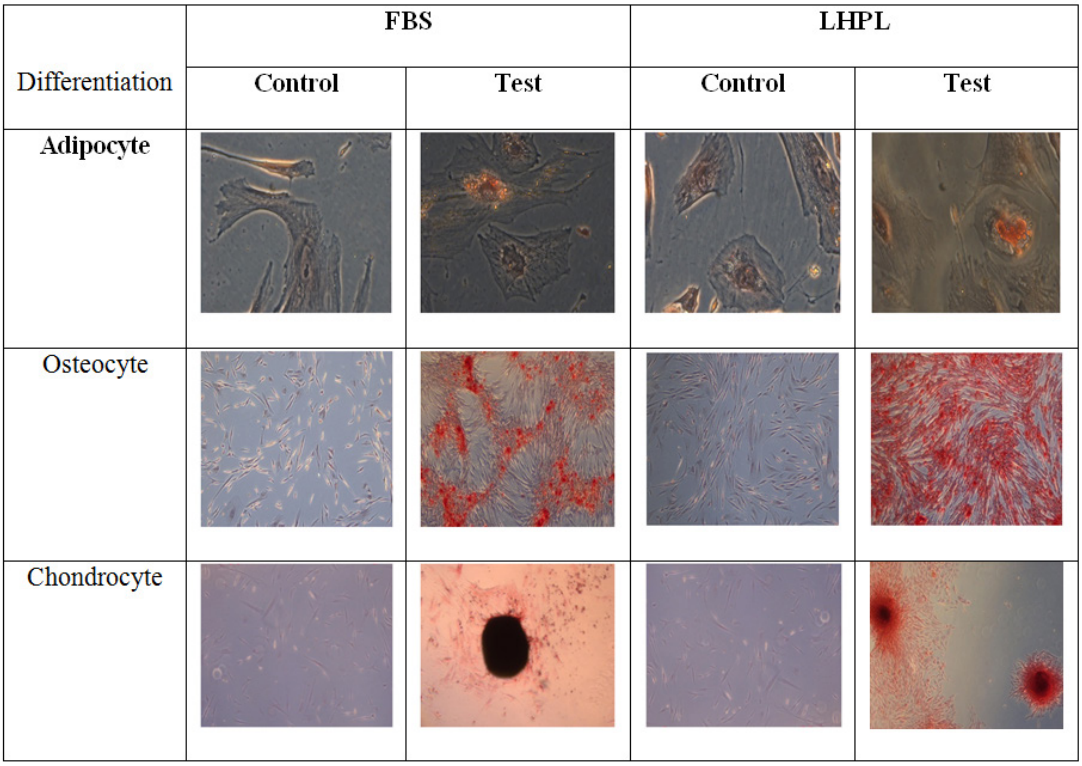

Fig. 5. Tri-lineage differentiation potential to form Adipocytes, osteocytes, and chondrocytes, stained by oil red O, alizarin red S and safranin O, respectively at Passage 3.
Figures of differentiated Adipocytes given in 40X – Inverted Microscopy.
Figures of differentiated Osteocytes given in 10X – Inverted Microscopy.
Figures of differentiated Chondrocytes given in 4X – Bright field Microscopy

more effective than FBS alone.[19] 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 MSC's' phenotype, their ability to differentiate, decreasing the culture period, and accelerating growth, LHPL-containing media does not differ much from FBS-supplemented media. But on in-depth analysis of this current study, the percentage of proliferation was more in LHPL supplemented culture as stated by the studies of Asrianti et al.,[20] and the cell death was less with LHPL primary culture than with FBS.

While HPL was traditionally been produced by repeatedly freezing and thawing pooled platelets, a novel technique that involves calcium gluconate treatment of the pooled platelets followed by mechanical extraction of the growth factors offers a quicker and more straightforward solution. The aforementioned techniques are more concerned with releasing growth factors and cytokines than with the product's long-term usefulness. However the long-term efficacy of goods made from human blood like HPL has become a problem because that involves a few freeze and thaw cycles and preservation in -80° as explained by Sharon et al.,[21] that has become a barrier to their widespread, in-time and long-time usage. Our work investigated the potential use of LHPL, the lyophilized human platelet lysate and its ability to promote the culture development of hUCT-MSCs compared to that of FBS, a comparably less expensive FBS substitute. In practice, lyophilizing the platelet lysate could increase its usefulness for a longer period of time without any freezing and thawing, can be stored just in 4°C as declared by Notodihardjo et al., and thus proved by this study.[22]

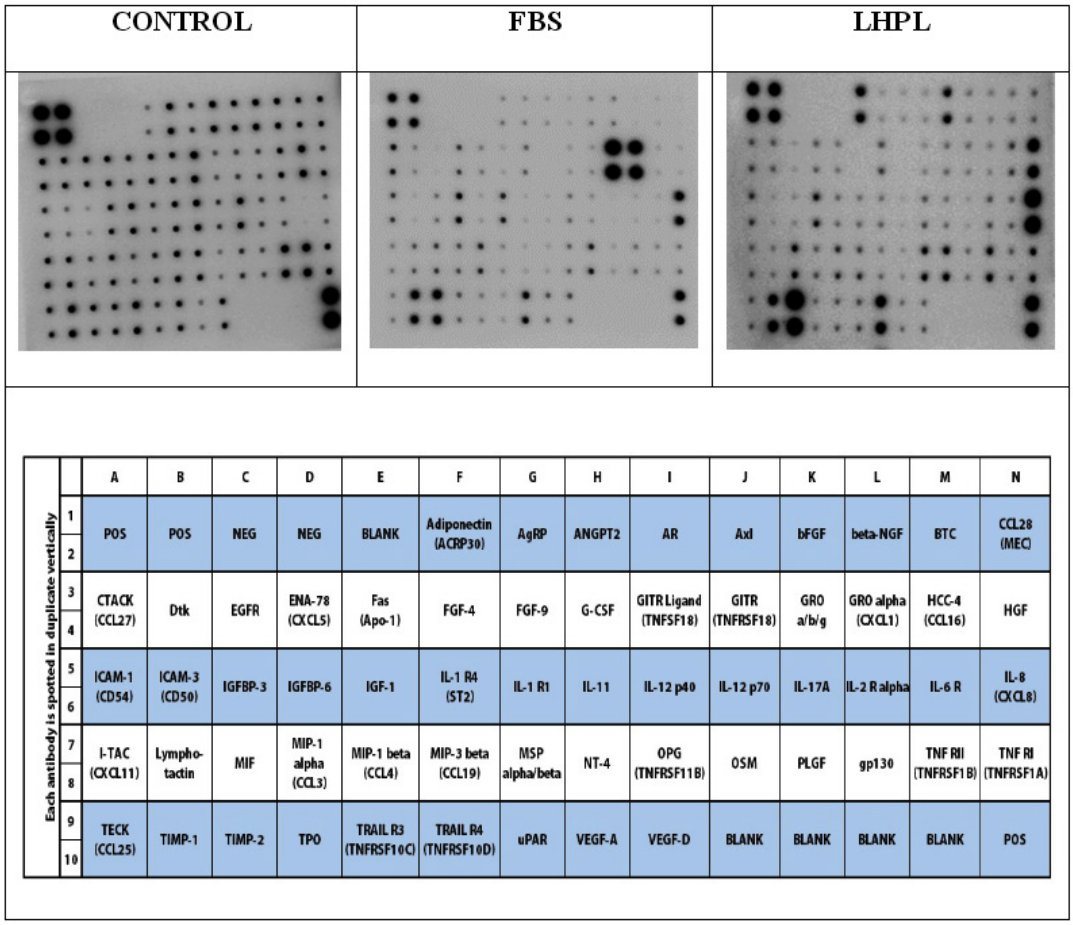

Fig. 6. MSC secretome analysis supplemented with FBS/LHPL in comparison to the control

Table 1. Qualitative Expression of Growth factors in hUCT-MSC spend media, supplemented with FBS & LHPL

No	Name of cytokines	In FBS	In LHPL	Nature	Function
1	*Adiponectin (ACRP30)	X	√	Beneficial	Increases insulin release from pancreas as well as fatty acid breakdown.
2	*AXL	X	√	Beneficial	Suppressing TLR inflammatory signalling, natural killer cells, and limited expression of pro-inflammatory cytokines.
3	GRO a/b/g	√	X	Not	In the presence of P-selectin, it functions as an arrest chemokine for monocyte.
4	*GRO alpha (CXCL1)	√	X	Beneficial	Plays a crucial part in the host immune response
5	*HGF	X	√	Beneficial	Regulates cell growth, motility, and morphogenesis.
6	IGFBP - 6	√	√	Not	Inhibit IGF2-induced proliferation, migration, and survival of cells
7	IL – 8 (CXCL8)	√	√	Not	Attract and activate Neutrophils.
8	*OPG (TNFRS11B)	X	√	Beneficial	Suppresses osteoclast differentiation in endothelial cells and VSMCs.
9	OSM	X	√	Not	Involved in homeostasis and chronic inflammatory reactions.
10	*gp130	X	√	Beneficial	Maintains bone formation and prevents the formation of osteoclast.
11	TIMP - 1	√	√	Not	Regulates extracellular matrix (ECM), wound healing, and pregnancy.
12	*TIMP - 2	√	√	Beneficial	MMPs are both activated and inhibited by TIMP2.

LHPL also is a great supplement in trilineage differentiation of hUCT-MSCs as stated by Marino et al., when compared to other culture supplement alternates like iron-fortified calf serum.[23] Its efficacy is equal or even better than FBS regarding. When MSCs were grown with LHPL, their ability to differentiate into adipocytes, chondroblasts, and osteoblasts was not only preserved but also more osteogenic foci seemed to originate from these cells than those cultured with FBS. In-depth comparison of this present study revealed an equal differentiation of hUCT-MSCs into adipocytes in both supplements and more foci formation and differentiation in osteogenic and chondrogenic differentiation of hUCT-MSCs.

There were also some notable variations in cytokine production between MSCs expanded in media supplemented with LHPL and FBS that suggested the functional distinctions that need to be taken into account for particular clinical applications in agreement to the work done by Azouna et al., comparing the efficacy of HPL and FBS as supplements.[24]

On spot analysis over the cytokine microarray membrane, the presence of cytokines and growth factors ACRP30, AXL, HGF, IGFBP-6, IL-8, OPG, OSM, gp130, TIMP-1, TIMP-2 were predominant in LHPL in comparison to FBS that helps in the secretion of growth factors like GRO a/b/c, GRO alpha, IGFBP-6, IL-8, TIMP-1, TIMP-2. Among them ACRP30, AXL, GRO alpha, HGF, OPG, gp130, TIMP-2 are highly beneficial for human tissue engineering applications, that denotes the importance of LHPL as a supplement in culturing hUCT-MSCs. "Table 1" elaborates on the significance of these factors in culture.

CONCLUSION

This present study reveals the potency of LHPL that prepared from expired human blood platelets, a considered biological waste in culturing hUCT-MSCs, it's ability in supporting the secretion of expected beneficial cytokines and growth factors and it's long life in normal temperature with reconstitution ability without freezing and thawing. This study pave a way to stop sacrificing the calf foetus for FBS and increasing the availability of culture media supplement in no time with low cost from a biological waste. As it is human in origin it is non-zoonotic, has high potency in inducing the proliferation of hUCT-MSCs with very less cell death. The most important characteristic of LHPL as a culture media supplement is its potency to induce the hUCT-MSCs to secrete a good number of helpful growth factors, more than what was induced by FBS according the present study. All the above said qualities are proof for a better culture media supplement like LHPL in low cost with ready availability.

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Conflict of interest

The author(s) do not have any conflict of interest.

Data availability statement

This statement does not apply to this article.

Ethics statement

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.

Informed consent statement

This study did not involve human participants, and therefore, informed consent was not required

Clinical trial registration

This research does not involve any clinical trials.

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Not Applicable.

Author contributions

Conceptualization - Dr. Najwa Abdur Rashid, Dr. Mary Antony Praba, Dr. Mary Anne W.Cordero, Dr. Kavitha Ganesh, Dr. Raja El Hasnaoui-Saadani and Dr. Venkataramaniah; Investigation, Data curation & Validation – Authors - Dr. Najwa Abdur Rashid, Dr. Mary Antony Praba, Dr. Mary Anne W.Cordero, Dr. Kavitha Ganesh, Dr. Raja El Hasnaoui-Saadani; Drafting, review & editing – Authors Dr. Mary Antony Praba, Dr. Venkataramaniah

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