Original Research Article

The Egyptian Journal of Immunology
Volume 30 (2), 2023: 47–58.
www.Ejimmunology.org

In vitro expansion of human mesenchymal stem cells and hepatocytes using purified platelet derived growth factors, a potential therapeutic modality for liver fibrosis

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Abstract

Mesenchymal stem cells (MSCs) and hepatocytes are considered valuable candidates for cell-based therapy. The use of free zoonotic media, as purified platelets derived growth factors (L-GF) and human platelet lysate (hPL), instead of using fetal bovine serum (FBS) to support the growth and proliferation of these cells could be used as a promising therapeutic tool in hepatic regeneration. This study aimed to evaluate the usage of purified platelet derived growth factors and platelet lysate in both MSCs and hepatocyte cultures and to compare them with the usage of FBS. MSCs and hepatocytes were cultured in growth media supplemented with L-GF or hPL and compared them to their culture in growth media supplemented with FBS. Cells were subjected to population doubling (PD) and generation time (GT) calculations. The best result for MSCs was that obtained by using 10% hPL or 10% FBS with the highest cell count, highest viability and shortest incubation time needed to reach confluency compared to supplementation with 10%, 20% or 30% L-GF. As for hepatocyte culture, the use of 10% FBS for supplementation of media used for hepatic cell proliferation showed better performance regarding cell count, viability, and incubation time to reach confluency compared to the use of either hPL or L-GF. In conclusion, our study showed that 10% hPL had the best results in MSCs culture which suggests that hPL could be a better alternative for the development of xenofree stem cell culture that can be used for many clinical applications. On the other hand, 10% FBS showed the best results in hepatocyte culture.

Keywords: MSCs; FBS; hPL; hepatocytes; population doubling and generation time.

Date received: 11 September 2022; **accepted:** 01 February 2023

Introduction

Mesenchymal stem cells (MSCs) are defined as pluripotent cells capable of self-maintaining and proliferating extensively and whose progeny can differentiate into adipose tissues, bone, cartilage, tendon, and marrow stroma.[1] The usage of MSCs in clinical applications has generated an

increasing interest in them as potential therapeutic agents in recent years. The use of MSCs in such therapeutic strategies requires a large number of expanded cells and relies on their ability to maintain their multilineage differentiation potential.[2]

Hepatocytes could be an alternate treatment for liver-based metabolic conditions and acute liver failure.[3] This technique is less invasive, less radical, and potentially less expensive than liver transplantation. Cells can be injected by intravascular route whilst the recipient's liver stays intact, hence not jeopardizing future access to innovative therapies, including stem cells and/or gene therapy.[4]

MSCs and hepatocytes can be easily expanded many folds in-vitro. Recently in-vitro expansion of both has been mainly achieved in the presence of fetal bovine serum (FBS). However, for widespread clinical applications, contact with FBS must be minimized[5] as animal sera are ill-defined and pose a risk factor as a source of xenogenic antigens and possible transmitters of zoonotic infections. Their use is not recommended by European legislation.[6] Pooled human platelet lysate (hPL) is a hemoderivative containing a plethora of growth-promoting factors and can be used as a safe and efficient MSCs culture supplement for robust MSCs cultivation.[7]

The promotive effect of platelets in liver regeneration was first discovered in 2004.[8] Platelet-rich plasma transfusion was found to accelerate liver regeneration.[9] Liver regeneration is carried out by the proliferation of hepatocytes induced by intercellular interactions via many growth factors and cytokines leading to subsequent activation of downstream transcription cascades. This is associated with the transition of the quiescent hepatocytes into the cell cycle and progression beyond the restriction point in the G1 phase of the cycle.[10]

Cell therapy is an exciting but challenging frontier in hepatology, offering the potential for a range of new therapeutic interventions. This reinforces the need to develop strategies to improve liver regeneration.[11]

The present work was designed to assess the effect of platelets derived growth factors (L-GF) and hPL on the growth and proliferation of both MSCs and hepatocyte cell cultures and compare them to the usage of FBS. L-GF and hPL were used in different concentrations including 10%, 20% and 30% as a supplement for cell culture media in comparison with the used 10% FBS.

Materials and Methods

Media Preparation

-Media supplemented with 10% FBS: the media contained 100 μl (1%) penicillin/streptomycin (Biochrom, Berlin, Germany), 100 μl (1%) L-glutamine (lonza, Basile, Switzerland) and 1 ml (10%) FBS (hyclone laboratories inc., logan, Ut, USA), then completed to 10 ml with Dulbecco's Modified Eagle Medium (DMEM) with 1,0 g/L glucose (lonza, Visp, Switzerland) for stem cell culture or DMEM with 4.5g/L glucose for hepatic cell line culture.

-Media supplemented with 10% hPL: the media contained 100 ul (1%) penicillin/streptomycin, 100 μl (1%) L-glutamine and 1 ml platelet lysate, then completed to 10 ml with DMEM with 1,0 g/L glucose for stem cell culture or DMEM with 4.5g/L for hepatic cell line culture. For media supplemented with 20% and 30% hPL, 2ml and 3ml hPL were used respectively.

-Media supplemented with 10% purified platelet derived growth factors: the media contained 100 μl (1%) penicillin/streptomycin, 100 μl (1%) L-glutamine and 1 ml of reconstituted L-GF, then completed to 10 ml with DMEM with 1.0 g/L glucose for stem cell culture or DMEM with 4.5 g/L for hepatic cell line culture. For media supplemented with 20% and 30% L-GF, 2ml and 3ml L-GF were used respectively.

All media preparations were filtered through 0.1 μm filter (lonza, Basile, Switzerland) before usage (media preparations containing platelet lysate were filtered twice due to the presence of fibrinogen in platelet lysate, this to filter out any tiny clots).

Preparation of human platelet lysate (hPL)

Platelet lysate was prepared by cycles of repeated platelets freezing and thawing.[12]

Preparation of purified platelets derived growth factors (L-GF)

Purified platelets derived growth factors (L-GF) were provided by one of the authors (Hossam M. Fahmy). L-GFs were prepared according to a patented method. [13,14,15] The platelets were isolated from individual whole blood donations using a platelet collection system based on apheresis technology (Haemonetics, MCS+9000 pheresis machine, USA).[16] Each individual unit of platelets was tested and found to be nonreactive for hepatitis B surface antigen (HBsAg), human immunodeficiency virus antibodies (HIV Abs I, and II), HIV p-24 antigen, hepatitis C virus (HCV) antibodies and antibodies to *Treponema pallidum* using a clinical chemistry and immunoassay analyzer (Architect, Abott, USA). Seronegative plasma was further examined by nucleic acid testing and exposed to viral/pathogen inactivation by ultraviolet-radiation and riboflavin treatment. The platelets were activated to release their L-GFs. Platelets were activated by lyophilized human thrombin produced by Cairo Medical Center Blood Bank. and ultra-filtrated to remove unwanted cellular elements and fibrinogen. The remaining GFs in fluid filtrate were dispensed by an aseptic technique in predetermined volumes and standardized to be equivalent to those coming from platelets found in 20 ml of whole blood, with a platelet count of 1 million per µl. The final step was lyophilization of such filtrate. The lyophilization technique was carried out in a freeze dryer (Tofflon, Shanghai, China). A total of 2.5-3.0 ml of purified growth factors solution (varies from batch to batch depending on initial platelets counts), were dispensed into sterile glass vials, and then covered with rubber stoppers in a semipermeable position. The lyophilization process followed the method adopted by Sisti et al. 2001[17] where the vialled material was frozen and held at -40°C for 6 hours at atmospheric pressure and subsequently the temperature decreased to -50°C for another 10 hours (super freezing) at a vacuum of 10m and maintained during the rest of the process. The primary drying time was 48 hours, after which the temperature was gradually increased to 35°C and maintained for a further 8-10 hours. The final product was subjected to aerobic and anerobic testing of micro-organisms prior to release. L-GFs were then supplied as lyophilized cake in a tightly sealed container and stored at 2–8°C.

MSCs Isolation

MSCs were extracted from umbilical cord blood (UCB) under complete aseptic conditions according to Elbaz et al. 2021. [18] Blood (50-100 ml) was collected from each cord. UCB was processed within 2 hours of collection. The collected UCB was tested for HCV Abs, HBsAg, and HIV Abs. Mononuclear cell fraction was isolated by density gradient separation method. This was done by centrifuging of tube containing ficoll and the collected UCB in a ratio of 1:1.5 at 880 xg for 30 min at 20°C. The mononuclear cell layer was collected and washed with PBS. The supernatant was discarded, and the pellet was dislodged, washed with PBS, and then resuspended in 10 ml 10% FBS media. Cells were counted using an automated cell counter (TC20 automated cell counter, Bio-Rad laboratories, USA). After that, they were seeded into cell culture flasks using 10% FBS media. Cells were incubated in Co_2 incubator at 37°C in an atmosphere of 90% relative humidity and 5% CO_2. The first medium change was done 2 weeks after seeding, and then the medium was changed twice weekly. When cells reached 90-95 % confluency, they were detached with 5ml of 0.25% trypsin. Trypsin containing detached cells was immediately neutralized by adding media supplemented with 10% FBS to the flask. Cells were transferred into a 50 ml test tube, and then centrifuged at 800 xg for 7 min at 20°C. The pellet was resuspended in a fresh medium, and cells were counted and tested for viability.

Flowcytometric Analysis

Cells were washed twice in PBS buffer supplemented with 0.5 % FBS and then suspended at a concentration of 3×10^6 cells in 1 ml PBS. Fluorescein isothiocyanate (FITC) mouse anti-human CD44, FITC mouse anti-human CD45, FITC mouse anti-human HLA-DR, FITC mouse anti-human CD105 coupled with PE mouse anti-human CD90, FITC mouse anti-

human CD34 coupled with PE mouse anti-human CD73 (Thermo Fisher Scientific, USA) were added to flow cytometry tubes (the monoclonal antibodies were added according to manufacturer's instructions). Cells were incubated for 20 min at 4°C. After that, cells were washed twice with PBS, and then resuspended in 500 µl PBS. The final analysis was done using a flow cytometer (Beckman Coulter Epics XL-MCL, USA).

Cell Proliferation and Expansion

Isolated MSCs and hepatocytes (hepatic cell line BNL 1ME A.7R.1; ATCC TIB-75, USA) were inoculated in T-25 flasks in 10 ml media supplemented with 10% FBS and incubated at 37°C with 5% CO2 and 90% relative humidity. Note that low glucose DMEM was used in stem cell culture while high glucose DMEM used in hepatics cell line culture. Media was changed every other day till reaching 90-95% confluency. Media was aspirated from the flasks. A volume of 1.5 ml of trypsin was added, and the flask incubated at 37°C for 7 min. Flasks were checked under an inverted microscope to ensure cell detachment. The cell suspension was aspirated from the flask, transferred to a centrifuge tube, and centrifuged at 800 xg for 7 min at 20°C. The supernatant was discarded, and the cell pellet washed twice by adding 3 ml PBS and centrifuged at 800 xg for 7 min at 20°C. The supernatant was discarded, and the cells were resuspended in media and counted using an automated cell counter (TC20 automated cell counter, Bio-Rad laboratories, USA).

MSC and Hepatic culture experimental setup

Under aseptic conditions, MSCs and hepatic cell line were individually propagated into 25 cm^2 culture flasks. The count was adjusted to a density of 5000 cells/cm^2. For each of the MSC and hepatic cell line, cells were cultured as shown in Figure 1 and Table 1.

-Experiment 1

In this experiment, MSC and hepatic cell line were cultured into 4 flasks containing media supplemented with 10% FBS, 4 flasks containing media supplemented with 10% hPL and 4 flasks containing media supplemented with 10% L-GF.

-Experiment 2

In order to confirm results from the first experiment and to assess cell growth after supplementation with higher concentrations of hPL and L-GF (20% and 30%), a second experiment was done. This included: 2 flasks containing media supplemented with 10% FBS, 2 flasks containing media supplemented with 10% hPL, 2 flasks containing media supplemented with 20% hPL, 2 flasks containing media supplemented with 30% hPL, 2 flasks containing media supplemented with 10% L-GF, 2 flasks containing media supplemented with 20% L-GF and 2 flasks containing media supplemented with 30% L-GF.

Cell culture in different media preparations

A count of 5000 cells/cm^2 into 25 cm^2 culture flasks were inoculated in 10 ml of corresponding media (as shown in Table 1) in T-25 flasks and incubated at 37°C with 5% CO_2. Media was changed every other day till reaching 90-95% confluency. The confluency was assessed visually under an inverted microscope (CKX53, Olympus, Japan). Media was then aspirated from the flasks; 1.5 ml of Trypsin was added, and the flasks were incubated at 37°C for 7 min. Flasks were then checked under the inverted microscope to ensure cell detachment. Cell suspensions were aspirated from the flasks, transferred to centrifuge tubes, and centrifuged at 800 xg for 7 min at 20°C. Supernatants were discarded and the cell pellets washed twice by adding 3 ml PBS and centrifuged at 800 xg for 7 min at 20°C. Supernatants were discarded, and cells resuspended in 5 ml of media and counted using an automated cell counter (TC20 automated cell counter, Bio-Rad laboratories, USA).

Calculation of cell population doubling and generation time

-Population doubling (PD): Cells were subjected to population doubling rate calculation. It is defined as the ratio of the number of cells obtained to the number of cells seeded and calculated by the equation: PD = [log (N2)-log (N1)]/log 2. Where N1 is the first cell count and N2 the cell count after reaching 100% confluency.

-Generation time (GT): GT is defined as the average time a cell population takes to double in number. GT=T Log2/(Log Xe – Log Xb), where T is the incubation time in any unit; Xb the cell number at the beginning of the incubation time, and Xe the cell number at the end of the incubation time.

Statistical analysis

Data were statistically analyzed using the IBM SPSS statistics (V. 26.0, IBM Corp., USA, 2019). Data are expressed as mean±SD for quantitative parametric measures. Student t test was used for comparison between two independent group means for parametric data. Analysis of variance (ANOVA) was used for comparison between more than 2 groups for parametric data. A *p* value ≤ 0.05 was considered significant.

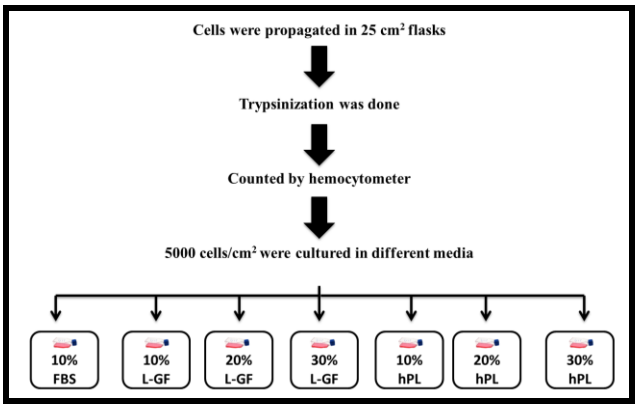

Figure 1. Schematic diagram showing steps for MSC/Hepatic culture in different media mixtures. FBS: fetal bovine serum; L-GF: platelets derived growth factors; hPL: human platelet lysate.

Table 1. Design of study experiments.

Media supplemented with	First experiment	Second experiment	Total number
	Number of flasks		
10% FBS	4	2	6
10% hPL	4	2	6
20% hPL	-	2	2
30% hPL	-	2	2
10% L-GF	4	2	6
20% L-GF	-	2	2
30% L-GF	-	2	2

FBS: fetal bovine serum; L-GF: platelets derived growth factors; hPL: human platelet lysate.

Results

Mesenchymal stem cell culture results: Comparison between proliferation of MSCs culture regarding cell count after reaching 90-95% confluency, cell viability, incubation time needed for confluency, population doubling, and generation time are shown in (Table 2). A statistically significant difference was found between the seven media preparations regarding cell count after reaching 90-95% confluency with the highest count being in 30% hPL and lowest count in 30% L-GF ($p< 0.001$) (Figure 2). A statistically significant difference was found between the seven media preparations regarding cell viability after culture with the highest viability being in 10% hPL and lowest in 10% L-GF ($p< 0.001$) (Figure 3). A statistically significant difference was found between the seven media preparations regarding incubation time needed to reach confluency with the lowest time being in 10% FBS and highest in both 20% L-GF and 30%L-GF ($p<0.05$) (Figure 4). A statistically significant difference was found between the seven media preparations regarding PD with the highest being in 30% hPL and lowest in 30% L-GF ($p< 0.001$) (Figure 5). A statistically significant difference was found between the seven media preparations regarding GT with the highest being in 30% L-GF and lowest in 10% hPL ($p< 0.001$) (Figure 6).

Table 2. Comparison between proliferation of mesenchymal stem cells (MSCs) in different media preparations.

MSCs	10% FBS	10% L-GF	20% L-GF	30% L-GF	10% hPL	20% hPL	30% hPL
Cell count/mL	2.01×10^6 ± 3.68×10^5	5.79×10^5* ± 2.79×10^5	4.78×10^5* ±6.36×104	2.13×10^5* ± 2.54×10^5	2.20×10^6 ± 2.33×10^5	2.07×10^6 ±2.40×10^5	2.22×10^6 ± 1.34×10^5
Viability (%)	93.3% ± 8.8%	45.7% * ±26.8%	48.5% * ± 9.2%	54% * ± 12.7%	94.7% ± 1.5%	93% ± 1.4%	92.5% ± 2.1%
Incubation time (Days)	9.17 ± 1.47	15.33** ± 7.23	20.00** ± 0.00	20.00** ± 0.00	9.33 ± 2.07	13.00** ± 0.00	13.00** ± 0.00
PD	3.98 ± 0.26	2.06 * ± 0.76	1.93 * ± 0.19	1.78 * ± 0.18	4.13 ± 0.15	4.05 ± 0.16	4.15 ± 0.09
GT	2.30 ± 0.34	7.68 * ± 2.36	10.45 * ± 1.06	11.30 * ± 1.13	2.27 ± 0.56	3.21 ± 0.15	3.14 ± 0.06

Data are presented as mean ± SD. FBS: Fetal bovine serum; L-GF: lyophilized purified platelet derived growth factors; hPL: platelet lysate; PD: population doubling; GT: Generation time. *$p < 0.001$ compared to 10% FBS, **$p < 0.05$ compared to 10% FBS.

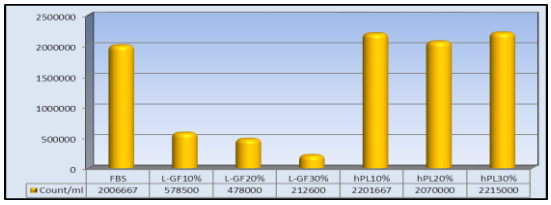

Figure 2. Bar chart for MSCs cell count after culture. Highest count was reached with media supplemented with 30% hPL and lowest with 30% L-GF.

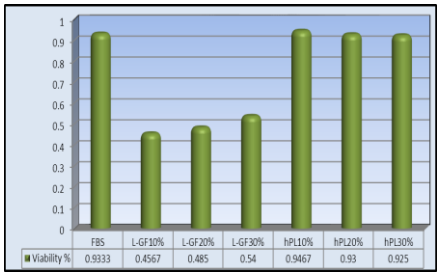

Figure 3. Bar chart for MSCs viability after culture. The highest viability was in 10% hPL and lowest in 10% L-GF.

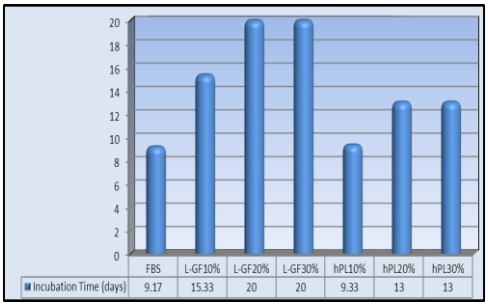

Figure 4. Bar chart for MSCs incubation time after culture. The lowest time was in 10% FBS and highest in both 20% L-GF and 30% L-GF.

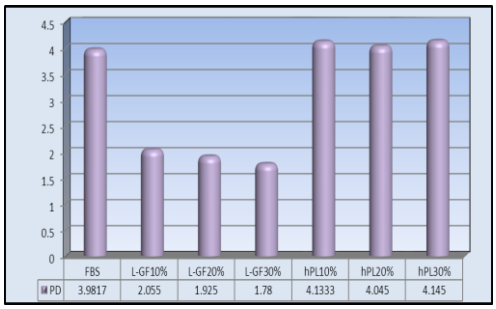

Figure 5. Bar chart for MSCs population doubling after culture. The highest was in 30% hPL and lowest in 30% L-GF.

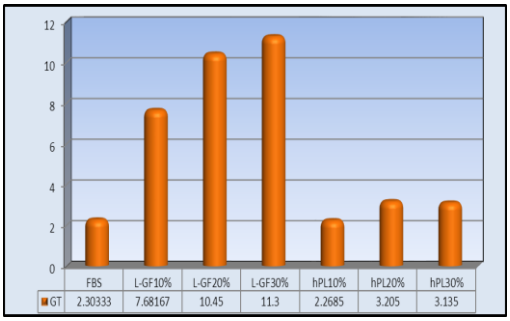

Figure 6. Bar chart for MSCs generation time after culture. The highest was in 30% L-GF and lowest in 10% hPL.

Morphological changes were noticed after culture in 10% L-GF (Figure 7 and Figure 8). To confirm that MSCs did not differentiate into any other cell type, flowcytometry analysis was performed using markers for stem cells identifications. The cells showed high expression levels of adhesion marker (CD44), typical mesenchymal markers (CD90 and CD73), endoglin receptor (CD105), and dual expression of CD105/90. However, they were negative for hematopoietic lineage marker (CD34), Leukocyte common antigen (CD45), and human leukocyte antigen class II (HLA-DR) expression [14] (Table 3).

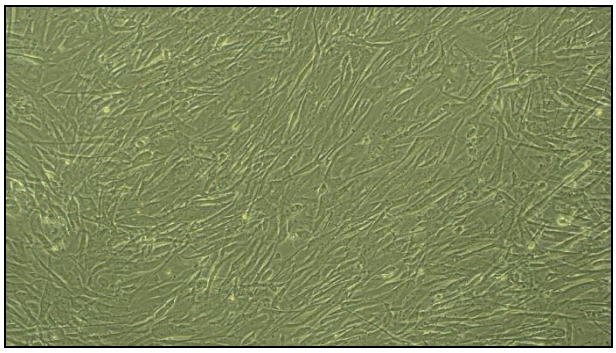

Figure 7. MSCs cultured in 10% FBS showing elongated adherent spindle shaped cells with about 90 % confluency.

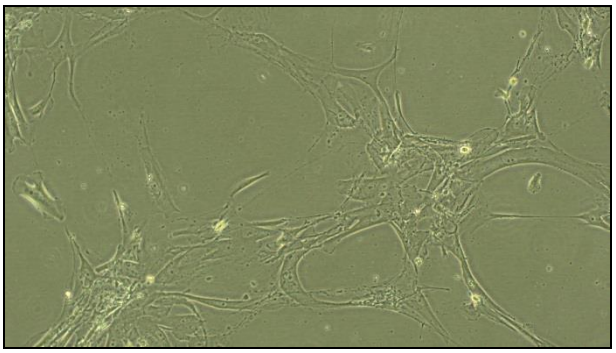

Figure 8. MSCs cultured in 10% L-GF, showing the adherent spindle shaped cells, became more elongated than usual with more spaces in-between.

Table 3. Percentage of immunophenotype markers expression in MSCs as determined by flowcytometry analysis.

Immunophenotype marker	MSCs
CD73	97.30 %
CD44	97.00 %
CD90	96.00 %
CD105	90.5 %
CD105/90	89.12%
CD34	5.33 %
CD45	1.18 %
HLA-DR	0.66 %

Hepatic cell culture results: Results of hepatic cell culture regarding cell count after reaching 90-95% confluency, cell viability, incubation time needed for confluency, population doubling, and generation time are shown in Table 4. A statistically significant difference was found between the seven media preparations regarding cell count after reaching 90-95% confluency with the highest count being in 10% FBS and the lowest count in 20% L-GF ($p< 0.001$) (Figure 9). A statistically significant difference was found between the seven media preparations regarding cell viability after culture with the highest viability being in 10% FBS and the lowest in 20% L-GF ($p< 0.001$) (Figure 10). A statistically significant difference was found between the seven media preparations regarding incubation time needed to reach confluency, with the lowest time being in 20%L-GF, 30%L-GF, 20% hPL and 30% hPL while the highest time was in 10% L-GF ($p< 0.001$) (Figure 11). A statistically significant difference was found between the seven media preparations regarding PD, with the highest being in 10% FBS and the

lowest in 20% L-GF (p< 0.001) (Figure 12). A statistically significant difference was found between the seven media preparations regarding GT, with the highest being in 20% L-GF% and the lowest in 10% FBS (p< 0.001) (Figure 13).

Table 4. Comparison of different media in hepatic cell culture.

Hepatic cell	10% FBS	10% L-GF	20% L-GF	30% L-GF	10% hPL	20% hPL	30% hPL
Count	2.38×10^{6} $\pm2.12\times10^{5}$	2.48×10^{5}* $\pm4.79\times10^{4}$	1.50×10^{5}* $\pm1.41\times10^{4}$	2.25×10^{5}* $\pm2.12\times10^{4}$	6.42×10^{5}* $\pm1.12\times10^{5}$	5.05×10^{5}* $\pm2.12\times10^{4}$	5.83×10^{5}* $\pm5.23\times10^{4}$
Viability (%)	96.7% ± 1.2%	34.8%* ± 4.3%	27.5%* ± 3.5%	31.0%* ± 2.8%	51.3%* ± 9.4%	43.0%* ± 4.2%	42.0%* ± 4.2%
Incubation time (Days)	14.00 ± 0.00	20.00* ± 0.00	11.00 ± 0.00	11.00 ± 0.00	20.00* ± 0.00	11.00 ± 0.00	11.00 ± 0.00
PD	4.24 ± 0.13	0.97* ± 0.29	0.26* ± 0.14	0.85* ± 0.13	2.34* ± 0.27	2.02* ± 0.06	2.22* ± 0.13
GT	3.30 ± 0.10	22.72* ± 8.48	49.10* ± 25.74	13.20* ± 2.12	8.63* ± 1.07	5.45* ± 0.21	5.00* ± 0.28

Data are presented as mean ± SD. FBS: Fetal bovine serum; L-GF: lyophilized purified platelet derived growth factors; hPL: platelet lysate; PD: population doubling; GT: Generation time. *p < 0.001 compared to 10% FBS.

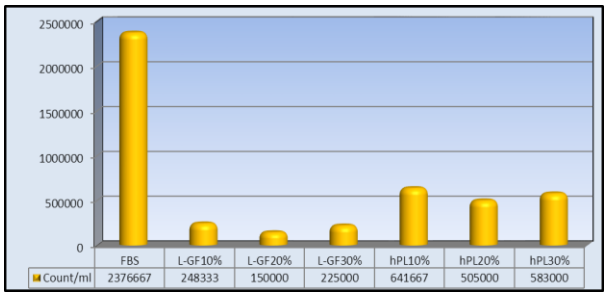

Figure 9. Bar chart for hepatocyte call count after culture. The highest count was in 10% FBS and the lowest count in 20% L-GF.

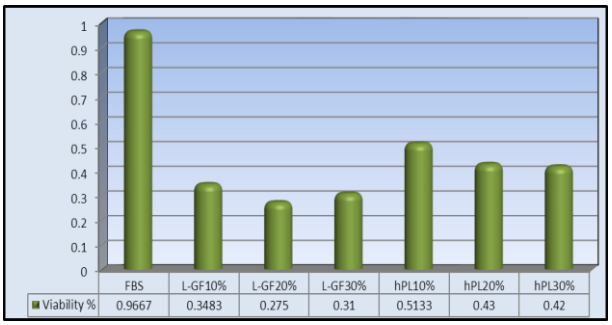

Figure 10. Bar chart for hepatocyte viability after culture. The highest viability was in 10% FBS and the lowest in 20% L-GF.

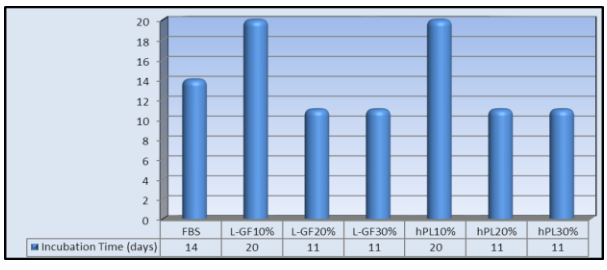

Figure 11. Bar chart for hepatocyte incubation time after culture. The lowest time was in 20%L-GF, 30%L-GF, 20% hPL and 30% hPL while the highest time in 10% L-GF.

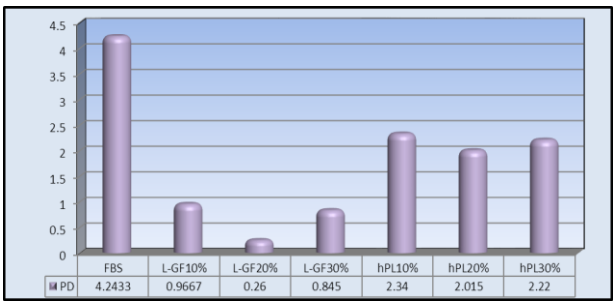

Figure 12. Bar chart for hepatocyte population doubling after culture. The highest was in 10% FBS and the lowest in 20% L-GF.

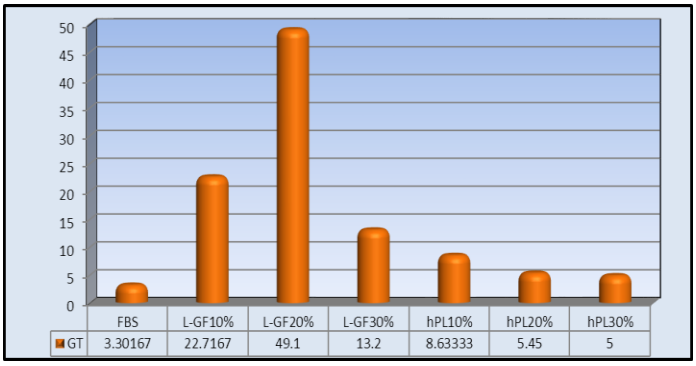

Figure 13. Bar chart for hepatocyte generation time after culture. The highest was in 20% L-GF% and the lowest in 10% FBS.

Discussion

The present study aimed to evaluate the performance of L-GF and hPL as an alternative to FBS in media supplementation in both stem cell and hepatocyte cell culture and compared it to the usage of FBS. FBS, usually used at a concentration of 10%.[19] Thus, in the present study a concentration of 10% L-GF/hPL was used in media supplementation in the experiment "1". Later in the experiment "2", higher concentrations (20% and 30%) were used in media supplementation.

Concerning MSCs culture, results of the current study revealed that both supplementation with 10% FBS and 10% hPL showed the best results in MSCs culture regarding the highest cell count, highest viability, and shortest incubation time needed to reach confluency compared to supplementation with 10%, 20% or 30% L-GF. Meanwhile, supplementation with 20% and 30% hPL showed comparable results to 10% FBS regarding cell count and viability while more time was needed for cells to reach confluency (13 days for supplementation with 30% hPL versus 9 days with 10% FBS). Several studies have shown that hPL can replace FBS in stem cell culture. The study by *Doucet et al. 2005* [20] was among the first to show that hPL made from fresh platelet concentrates could replace FBS as a growth medium supplement for human stem cell propagation.

Our study is consistent with those reported by Barro et al. 2019,[21] who evaluated the use of 10% hPL in stem cell culture in comparison to 10% FBS and reported better performance of the former with higher viability and higher cell count compared to the latter. Nevertheless, Barro et al. 2019[21] highlighted that the most important issue facing the routine use of hPL in cell culture would be its consistency. Batch-to-batch consistency in hPL batches seems to require the pooling of 40 to 50 platelet donations. Pooling would minimize inherent donor-to-donor variability due to the impact of age or sex, such as the content in growth factors and other cell growth-promoting molecules, which are important components for growth medium supplementation. However, pooling would increase the risk of contamination by viruses that are not tested for. A systemic review by Guiotto et al. 2020[22] recommended the usage of hPL as a replacement of FBS. This supports our present study findings that hPL can replace FBS with comparable count, viability, incubation time needed to reach confluency, PD and GT.

In the current study, supplementation with 20% and 30% hPL showed comparable results to 10% FBS regarding cell count and viability, while more time was needed for cells to reach confluency (13 days for supplementation with 30% hPL versus 9 days with 10% FBS). Similar results were reported by *Tancharoen et al. 2019* [23] who assessed the use of 10% and 20% of hPL in stem cell culture and reported no difference between the performance of 10% FBS and 10% hPL regarding cell viability, morphology, cell surface markers, and proliferation capacity. However, the authors reported that cells cultured in media supplemented with 20% hPL

showed decreased proliferation capacity. Thus, data of the previously mentioned studies in addition to these of the present study open important perspectives for the development of xenofree cell culture conditions for medical applications of expanded stem cells.

The present study also assessed the use of L-GF in supplementation of media used for MSCs culture in concentrations of 10%, 20% and 30%. Our results showed that the performance of FBS and hPL was superior to L-GF (in the fore-mentioned concentrations), with significantly lower cell count and viability and with significantly longer incubation time needed to reach confluency.

The use of growth factors as a substitute to FBS in stem cell culture has not been assessed before. However, other studies assessed the use of growth factors in proliferation of other cell types. Effects of growth factors on proliferation and differentiation in human lens epithelial cells was studied by *Ibaraki et al .1995* [24] who stated that the use of growth factors enhanced the proliferative potency and differentiation of human lens epithelial cells in culture. Also, population doubling was significantly higher with epidermal growth factor, and insulin-like growth factor-I compared to 20% FBS media. *Nelson et al. 2011* [25] also found that human neural progenitor cells cultured in media supplemented with 100 ng/ml platelets derived growth factors showed significantly increased growth rates.

In the current study, morphological changes were observed for cells grown in media supplemented with L-GF compared to regular MSCs morphology for cells grown in media supplemented with either FBS or in hPL. Flowcytometry was used to confirm that the cultured cells did not differentiate into another cell line. Immunophenotypic analysis of cell surface markers (CD44, CD90, CD73, CD105, dual expression of CD105/90, CD34, CD45, and class II HLA-DR) revealed that the cells were stem cells. Two studies reported morphological changes for cells grown in media supplemented with hPL. Barro et al., 2019[21] reported the presence of morphological changes in bone marrow–derived MSCs when cultured in media supplemented with hPL, where stem cells showed more elongated spindle-shaped morphology compared to cells cultured in media supplemented by FBS. However, the authors assessed cell surface markers and reported that cells were positive for CD105, CD73 and CD90 and negative for CD45, CD34 and HLA-DR, thus confirming the characteristics of stem cells. The other study was conducted by Schallmoser et al., 2007[26] who reported morphological changes for MSCs grown in hPL, performed immunophenotypic analysis of the cells and reported that the morphological changes did not translate into significant immunophenotypic differences. The authors stated that flowcytometry revealed that MSCs were positive for the presence of HLA-AB, CD13, CD29, CD73, CD90, CD105, CD146, and BS-1 and negative for the presence of HLA-DR, CD5, CD10, CD14, CD31, CD34, CD45, and CD56 regardless of whether the MSCs had been generated with hPL or FBS.

Liver diseases can be caused by viral infection, metabolic disorder, alcohol consumption, carcinoma, or injury, chronically progressing to end-stage liver disease or rapidly resulting in acute liver failure. In either situation, liver transplantation is most often sought for life saving, which is, however, significantly limited by severe shortage of organ donors. Until this time, tremendous multi-disciplinary efforts have been dedicated to liver regenerative medicine, aiming at providing transplantable cells, microtissues, or bioengineered whole liver.[41]

In the present study, the use of 10% FBS for supplementation of media used for hepatic cell proliferation showed better performance regarding cell count, viability, and incubation time to reach confluency compared to the use of either hPL or L-GF (in concentrations of 10%, 20% or 30%) for media supplementation. To date, this study might be the first to evaluate the effect of media supplementation with L-GF in culture of a hepatic cell line. However, *Hesler et al. 2019* [27] evaluated the use of hPL in media supplementation for culture of HepG2 hepatic cell line. The authors assessed hPL in two concentrations (6% and 10%) in comparison to 10% FBS and reported no significant difference was observed between the three supplementations regarding cell morphology, viability, or proliferative capability.

The failure of the purified L-GF in proliferating both MSCs and hepatocytes in our study could be due to the high concentration of these factors. *Graziani et al., 2006* [28] showed that increased concentrations of platelet rich plasma (PRP) resulted in a reduction in proliferation and a suboptimal effect on osteoblastic function. *Weibrich et al., 2004* [29] also reported that higher concentrations have a paradoxically inhibitory effect. So, our results may be due to the inhibitory effects of the growth factors on the cells proliferation.

Of note, one major limitation of our study was that we did not try lower concentrations of L-GF for cell proliferation. Therefore, for future studies, we recommend the use of lower concentrations (5 - 7.5%) in proliferating the cells.

In conclusion, the present study showed that concerning media supplementation for *in vitro* cell culture, 10% hPL had the best results in umbilical cord blood MSCs culture which suggest that hPL might be a better alternative for the development of xenofree stem cell culture and for medical application in regenerative therapy. On the other hand, 10% FBS still showing the best results in hepatocyte culture.

Author Contributions

AY, designed and approved the whole research protocol. HMF, contributed to the protocol design, revised laboratory work, and provide the growth factors and platelets lysate. DR and ME, monitored and processed the laboratory work, interpreted the data, and critically revised the manuscript. SE and DAS, approved the final version of the manuscript. HME, carried out the laboratory work, performed statistical analysis and drafted the manuscript.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Funding

The author(s) denies receipt of any financial support for the research, authorship, and/or publication of this article.

Ethical approval

The study protocol was reviewed and approved by the Research Ethical Committee at Faculty of Medicine, Ain Shams University and Theodor Bilharz Research Institute (TBRI).

Informed consent

A signed informed consent was obtained from all subjects donated blood samples before included in the study.

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