EFFECTS OF HUMAN PLATELET LYSATE PREPARATIONS ON THE PROLIFERATION OF BONE MARROW DERIVED MICE MESENCHYMAL STEM CELLS

Majid Hameed Mohammed

Faculty os science, Center of Scientific Research,University of Dohouk,Kurdistan,Iraq (Received31June 2016, Accepted 10 august2016)

Keywords: Stromal cells, Platelet lysate, lysate.

ABSTRACT

Human platelet lysates (HPL) have been described as appropriate cell culture supplement for cultures of mesenchymal stem cells (MSCs). The increase in demand for harmless and animal-free cultures of MSCs is related to the potential application of MSCs grow in human source supplements

In this study, human platelet lysates are derived from fresh and expired blood donor platelet concentrates. Mouse bone marrow-derived mesenchymal stem cells were cultured with one of three culture supplements; fetal bovine serum, lysates from fresh and expired prepared human platelet concentrates. The impacts of these platelet derived culture supplements on basic mesenchymal stem cell characteristics were evaluated. All cultures preserved the mesenchymal stem cells surface marker expression, growth carves and viability test. The result demonstrated that mesenchymal stem cells complemented with platelet lysates proliferated faster than conventional FBS cultured cells and increased the expression of the cells. In conclusion, the use of fresh and expired platelet units from blood banks to prepare platelet lysates for the culture of MSCs is appropriate as well as keep cells phenotype characteristics and also shortens culture time by increasing their growth.

INTRODUCTION

Mesenchymal stem cells considered great potential cellular tools used in regenerative medicine due to their remarkable ability to self-renew and differentiate into multiple tissues. They have been isolated from different sources including, but not limited to, bone marrow, adipose tissue, umbilical cord, skin, skeletal muscle, dental pulp and other dental tissues such as the apical papilla [1, 2, 3 and 4]. Because MSCs can be found, albeit in small numbers and necessitates there in vitro expansion, should they be used for therapeutic purposes [5, 6 and 7]. In this regard, the cells are cultured, under conventional conditions, in the presence of fetal bovine serum (FBS) should be replacement with other supplement new media

Until now fetal animal sera still use as medium supplements is widespread, despite the many disadvantages associated with that [8]. A major problem encountered is the risk of possible contamination with viruses, prions, bacteria, and mycoplasma. Additional problem in this culture supplement is cross species contamination, leading to the expansion of antibodies against FBS as noted in patients which were infused with MSCs cultured in the presence of FBS [9]. Additional scientific complications related with the use of FBS include batch-to-batch variability, fluctuating availability, unexpected cell growth characteristics, cytotoxicity of uncharacterized factors in the serum, and so forth [10]. The determination of appropriate culture conditions for optimal growth and suitable functional abilities of MSCs still remains a critical problem.

Numerous serum-free media have been used in experimental applications for the *in vitro* expansion of MSCs [11]. Human platelet lysate (HPL) containing media were recently described as possible substitutes for FBS-containing media for the expansion of MSCs for clinical use [12]. Indeed, HPL was described to stimulate MSC proliferation rate and maintain their differentiation potential and immunophenotypic characteristics [6-13]. Nevertheless, different scientist has used different approaches for platelet lysate preparation, leading to great variability amongst published work regarding which concentrations should be utilized for optimum results. In this study, the objectives were to describe and standardize method for the preparation and use of human fresh and expired platelets (FPL and EPL) in cultures, as well as describe the *in vitro* effects of FPL and EPL containing media on MSCs derived from mouse bone marrow.

MATERIALS AND METHODS

1- Preparation of human platelet lysates

Fresh and expired human donor platelets (10 bags, 5 per each of them) were provided by the blood bank of the Duhok-Kurdistan, Azadi Medical University Hospital. Platelet donor bags (one bag per donor) were moved under sterile situations into 250 ml centrifugation cups and centrifuged at 7319 rpm for 20 mins in order to remove platelet additive solution (PAS) The supernatant was aspirated and the platelets were washed with 0.9% NaCl. Platelets were resuspended in 15 ml 0.9% NaCl with a final cell count of ~1.5 x 10¹⁰ platelets/ml. The suspension was stored at - 20°C before lysate preparation by three freeze/thawing cycles. Aliquots of the platelet lysate were again stored at -20°C for use within 4 weeks. Before addition to serum-free culture media, aliquots were thawed and centrifuged at 8452 rpm for 10 mins and supernatants were taken. To identify the most efficient platelet activation method in order to achieve a maximum yield of growth factors [14].

2- Isolation of Mice Bone Marrow Stem Cells (MBSCs)

Mouse bone marrow stem cells (MBSCs) were obtained and processed as previously described [15]. MSCs were harvested from the bone marrow (BM) of the femurs and tibiae, Apply pools of 4-6 mice were sacrificed by cervical dislocation. Femurs and tibiae were dissected from the surrounding tissues. The extra growth plates were removed and the BM was collected by flushing with syringe through a 25 gauge needle containing 1 ml of growth medium MEM (US-Biological-USA) containing 100U/ml penicillin, 100 μ g/ml streptomycin and 10%FBS. Suspension cultures were grown in MEM medium supplemented with 10% FBS and antibiotics. After one passage the MSCs were harvested and transferred to expansion in mesenchymal stem cell screened FBS (MSCs-FBS), human fresh platelet rich concentrates (FPL) or expired

platelet rich concentrates (EPL). After two passages in expansion the MSCs were harvested and seeded in in 25 cm² sterile disposable polystyrene cell culture flask (Nunc Easyflasks, Sigma) for experiments. Morphology was evaluated after staining the MSCs with crystal violet and imaging them. Proliferation analysis was done by continuing the expansion in each media type and counting the cells after each passage.

3-Subculturing and harvesting of cells

When seeded cells had reached 80%-90% confluence, they were subcultured and cells were seeded for expansion in the three different expansion media (10% MSCs-FBS, 10% FPL or 10% EPL). Basal medium was prepared by adding 4 IU/ml of heparin (LEO Pharma A/S, Ballerup, Denmark) and 1% penicillin/streptomycin (Gibco, Grand Island, NY, USA) into MEM growth medium (US-Biological-USA)

4- Growth dynamics

Cells were seeded on 24-well microplates at the density of about 1.5×10^5 cells per well, cultivated for 7 days and calculated every day (3 wells each time) subsequently. The mean cell counts at each time point were then used to plot a growth curve, based on which the population doubling time (PDT) was calculated, with the following formula. PDT = 1 / [3.32 (log N- log N1) / (t2- t1)] where NH is the number of harvested cells at the end of the growth period, N1 is the number of seeded cells, t1 is the time at seeding, and t2 is the time elapsed between t1 and cell harvesting [16].

5-Cells Cryopreservation

After three passages, the growth cells were suspended in three different media containing 40% MEM, (50% MSC-FBS, 50% FPL and 50% EPL) respectively and 10% dimethyl sulfoxide (DMSO). Cells in logarithmic growth phase were counted with a hemocytometer and adjusted to a density of 3×10^6 viable cells per mL [17]. Aliquots of the cell suspension were transferred into sterile cryovials and labeled. The vials were sealed and kept at 4°C for 20-30 min to equilibrate DMSO, put into -30°C for one h, then into -80°C overnight, and finally transferred to liquid nitrogen for long-term storage.6 Vials taken from the liquid nitrogen were thawed in a 37°C water bath, then transferred to flasks with MEM containing (10% FBS, 10% FPL and 10% EPL) respectively and cultured at 37°C with 5% CO2. The medium was renewed after 24 h.

6- Immunocytochemistry analysis of BM-MSCs

The Immunocytochemistry was done according to the method of Ooi *et al* [18]. growth cells were tested at passage two mouse MSCs were seeded at a density 1×10^6 cells per coverslip and fixed with cold methanol: acetone (50:50 volum/volum) for 5 mins. The glass slides were then immersed in 1% hydrogen peroxide in absolute methanol for 30 mins. The phosphate puffer salain was then added to the glass slide for 15 mins. The glass slides were then air dried. The primary antibody of the following CD markers (CD44 and CD90,) were added, and then incubated for 30 mins. The cells were washed three time with PBS for 5 mins. Biotinylated secondary antibody approximately 1-3 drops was added to the coverslip for 30 mins. (Mouse, Rat, and Goat secondary antibodies added to specific primary antibody). Then the

cells were washed three time with PBS for 2 mins each. The mixture of streptavidin conjugated to horseradish peroxidase was incubated at room temperature for (30) mins before use, then added to cover the cells, and then the cells were washed with PBS three times. Each plate was received 1-2 drops of DAB solution and this step was made in darkness and then left for 10-20 mins. The cells were washed one time with distilled water. Two drops of Harris Haematoxylin stain were added and left for 5-10 seconds, and then washed with distilled water. Then left for five mins to dry. Drops of DPX were added to the slide, coverslip were adhered carefully, and finally the slide were inspected by inverted microscope for detection of MSCs.

7- Statistical analysis

GraphPad® version 5 and Microsoft Office Excel 2007 were used for all data analysis. Two-way ANOVA was used where applicable and Student's t-test was used to confirm statistical significance. P<0.05 was considered statistically significant

RESULTS

1- Morphology

Morphology was examined visually and imaged in an inverted microscope at 10 x magnifications during expansion in growth medium supplemented with MSCs-FBS, FPL or EPL at 10% concentration respectively. After 1 weeks of culture, an adherent and stable cell layer was obtained from BM-derived MSCs with all media. In fact, all the cells shown same morphological changes initially from time 0 were found to be round and glistening. After 7 days the cells were changed to spindle-shaped cells like fibroblast (Figure 1)

2- Population doubling assay

MSCs cultured in either FPL or EPL supplemented media showed a higher growth rate than MSCs cultured in MSC-FBS (n=2 for each expansion media at each passage). The maximum difference was observed at P4-P5 with mean difference of 3.221 ± 0.6212 CPD (p<0.05, Figure 2) between cells expanded in MSC-FBS or HPL containing media.

3- Indirect Immunoperoxidase characterization of MSCs

The morphological appearance of the monolayer cultures at the second passage by immunocytochemistry staining analysis, for the MSCs isolated in both FBS and HPL. Immunophenotypic evaluation demonstrated that mouse BM-MSCs uniformly positive for CD44 and CD90 (Figure 3), All immunoreactivity staining cells were performed with marker stained strongly brown.

4- Cryopreservation

MSCs in three different media recovered from storage in liquid nitrogen one month and after 6 months. Cells grew to confluence within 7 days post one month freezing while no confluence noticed post 6 months freezing (Table 1).

Table 1: Comparison of the viability and confluence of MSCs fibroblast cells before freezing and after

Freezing time	Cell viability and confluence	No. of days to reach confluent
Before freezing	+++	5
1 month	+	7-10
6 months	+/-	-

(+++): Excellent growth, $<10^6$ viable cells, <80% confluence (+): Medium growth, 10^3 viable cell, 20-40% confluence

(+/-): Little growth, 10-10² viable cells

(-): No growth, no confluence



Figure: 1 Images of MSCs after day 0 (A-C), 3 (D-F) and 7 days (G-I) in culture with MSC-FBS, FPL or EPL supplemented media. Cells grown in FPL or EPL exhibit spherical growth patterns (E-F) and faster growth compared to MSC-FBS grown cells (H-I). Images were taken in an inverted microscope at 10x magnification. Images are representative of three experiments.



Figure 2: Cumulative population doublings of MSCs after culture in FPL,EPL and FBS

Population doubling assay was performed at the end of every passage for a total of six passages (P1–P6). MSC cultured in either FPL or EPL consistently had higher numbers of population doublings at the end of every passage compared to MSC cultured in FBS.



Figure 3: Immunophenotypic analysis of mouse BM-MSCs at the second passage of culturing on coverslips revealed by light microscope (X10). (A and B): the most of adherent MSCs were positive response for CD44 andCD90 marker respectively were stained with brown color stain DAB.

DISCUSSION

In this study, the results of three different expansion media (10% MSC-FBS, 10% FPL and 10% EPL) on MSC basic appearances were compared. The characteristics analyzed were proliferation and immunohistochemistry. Mesenchymal stem cells from three mice were expanded for six passages in each media and then used in experiments evaluating the basic characteristics.

After growth in platelet lysates and FBS supplemented media, all cells displayed the same characteristic morphology of MSCs, long, spindle-shaped cells resembling fibroblasts and doesn't exhibit any different in morphology and characteristics of expansion cells throughout the study, This result might have been different with [19] where noted that the spherical growth pattern of HPL supplemented cultures was accompanied by circular areas free of cell growth before cultures reached confluence. We found that culturing MSCs in both FPL and EPL-supplemented medium significantly increased proliferation rate by 2-fold compared with FBS-cultured MSC in passage six because platelet lysates are extremely rich in various growth factors necessary for MSC growth, with even higher concentrations than in FBS[19]. Scientists noted that the mature cells in HPL supplemented media proliferate faster and reach the limit of population doublings earlier, there is the possibility that these cells will cease growth sooner than cells grown in media supplemented with FBS [20]. Moreover, the actual proliferation rate after long term culture in FBS media compared to HPL supplemented media can be expected to be very similar since a plateau phase is observed after a few passages in cultures with platelet lysates.

Our study clearly showed that the presence of FPL or EPL is necessary for MSC growth to substitute the standard expansion medium containing the FBS. The addition of FPL or EPL in expansion media did not modify the immunophenotype of MSCs, irrespectively of the amounts used and of the presence or absence of FBS. These cells display a typical feature of MSCs bearing CD44 and CD90 [21].

Cryopreservation, a method to preserve cells, is as important as cell culture method. The application of cryopreserved mouse MSCs in cell therapy also requires preservation of their differentiation and proliferation ability. Thus, it is essential to investigate whether the freezing process influenced or not by using three different media FBS, FPL and EPL and their proliferation ability. The study showed excellent cell adhesion rate, proliferation capability, with appreciable recovery after thawing of up to 90%. Furthermore, these cells did not lose their differentiation capacity after cryopreservation. After storage for 1 month, cryopreserved cells had a fairly high degree of viability. Most of the thawed cells can attach surface of culture flasks, similar to primary cultured cells. In addition to characteristics described above, another defining feature that thawed MSCs still kept was their differentiation potential or their ability into fibroblast when compared with that of primary cultured cells.

These observations clearly showed cryopreserved MSCs could be stored and maintained high degrees of viability and differentiation potential.

CONCLUSION

The possibility to use of HPL in supplemented media may be offered some benefits like faster growth of MSC and improved differentiation as compared to the traditional use of FBS. Meanwhile no difference between expired and fresh HPL could be observed, expired platelet units at blood banks and transfusion centers are an attractive choice for MSC culture, instead of being discarded as is the general practice today

```
تاثير استخدام الصفائح الدموية البشرية المحضره على نمو وانتشار الخلايا الجذعية المعزوله من تتاير استخدام الفئران
```

ماجد حميد محمد

كلية العلوم، مركز الابحاث العلميه، جامعة دهوك كردستان-العراق

الخلاصه

لقد وصفت الصفائح الدمويه البشريه على انها وسط مغذي ومكمل لنمو الخلابا الجذعيه ونتيجة الى زيادة الطلب عاى الخلايا الجذعيه بدء التفكير في استخدام مصادر مغذيه غير حيوانيه وغير ضاره ومعزوله من البشر يمكن ان تستخدم كوسط مغذي يساعد في نمو الخلايا الجذعيه. في هذه الدراسه تم استخدام ثلاث اوساط زر عيه مغذيه و هي الصفائح الدمويه المشتقه من دم طازج ودم قديم وكذلك المصل الجنيني للابقار وملاحظة تاثير ها في نمو وتضاعف الخلايا الجذعيه المشتقه من نخاع العظم للفئران. قيم التاثير اعتمادا في خصائص الخلايا الجذعيه، ومنحني النمو ومستقبلات الخلايا الجذعيه على الغشاء. اظهرت النتائج ان الخلايا التي نمت في وسط الصفائح الدمويه المشتقه دم طازج ودم قديم على الغشاء. النهرت النتائج ان الخلايا التي نمت في وسط الصفائح الدمويه المشتقه دم طازج ودم قديم قد نمت اسرع من وسط المصل الجنيني للابقار كما لم يظهر هناك فرق في النمو بين الصفائح الدمويه المشتقه من الدم الطازج والدم القديم

REFERENCES

1. Young HE, Steele TA, Bray RA, Hudson J, Floyd JA, Hawkins K. Human reserve pluripotent mesenchymal stem cells are present in the connective tissues of skeletal muscle and dermis derived from fetal, adult, geriatric donors. Anatomical Record, 2001; 264: 51–62.

2. Zuk PA, Zhu M, Mizuno H, Huang J, Futrell JW Katz AJ..Multilineage cells from human adipose tissue. Tissue Engineering, 2001; 7:211–228.

3. Arai F, Ohneda O, Miyamoto T, Zhang XQ, Suda T. Mesenchymal stem cells in perichondrium express activated leukocyte cell adhesion molecule and participate in bone marrow formation. Journal of Experimental Medicine, 2002; 19(5):1549–1563

4. Sonoyama W, Liu Y, Yamaza T, Tuan RS, Wang S, Shi S, Huang GT. Characterization of the apical papilla and its residing stem cells from human immature permanent teeth: a pilot study. Journal of Endodontics, 2008; 34(2):166-71.

5. Ball LM, Bernardo ME, Roelofs H. Co-transplantation of ex-vivo expanded mesenchymal stem cells accelerates lymphocyte recovery and may reduce the risk of graft failure in haploidentical hematopoietic stem cell transplantation. Blood, 2007;110:2764–2767.

6. Blande IS, Bassaneze V, Lavini-Ramos C, Fae KC, Kalil J, Miyakawa AA, Schettert IT, Krieger JE. Adipose tissue mesenchymal stem cell expansion in animal serum-free medium supplemented with autologous human platelet lysate. Transfusion, 2009;49:2680–2685.

7. Bernardo ME, Ball LM, Cometa AM. Co-infusion of ex vivo expanded parental mesenchymal stromal cells prevents life-threatening acute GvHD, but does not reduce the risk of graft failure in pediatric patients undergoing allogeneic umbilical cord *blood* transplantation. Bone Marrow Transplant, 2011;46 (2):200-207.

8. Tekkatte C, Gunasingh GP, Cherian KM, Sankaranarayanan K. Humanized Stem Cell Culture Techniques: The Animal Serum Controversy. stem cells international, 2011; 2011: 504723.

9. Selvaggi TA, Walker RE, Fleisher TA. Development of antibodies to fetal calf serum with arthus-like reactions in human immunodeficiency virus-infected patients given syngeneic lymphocyte infusions. Blood, 1997; 89 (3):776–779.

10. Shahdadfar A, Fronsdal K, Haug T. In vitro expansion of human mesenchymal stem cells: choice of serum is a determinant of cell proliferation, differentiation, gene expression, and transcriptosome stability. Stem Cells., 2005; 23:1357–1366.

11. Jung S, Sen A Rosenberg. Identification of growth and attachment factors for the serum-free isolation and expansion of human mesenchymal stromal cells. Cytotherapy, 2010; 12:637–657.

12. Doucet C, Ernou I, Zhang Y, Llense JR, Begot L, Holy X Lataillade JJ. Platelet lysates promote mesenchymal stem cell expansion: a safety substitute for animal serum in cell-based therapy applications. Journal Cell Physiology, 2005; 205: 228–236.

13. Schallmoser K, Strunk D. Preparation of pooled human platelet lysate (pHPL) as an efficient supplement for animal serum-free human stem cell cultures. Journal of Visualized Experiments, 2009; 32: 1523.

14. Lee JY, Nam H, Park YJ, Lee SJ, Chung CP. The effects of platelet-rich plasma derived from human umbilical cord blood on the osteogenic differentiation of human dental stem cells. In Vitro Cellular & Developmental Biology-Animal, 2011; 47(2):157-64.

15. Friedenstein, AJ. Chailakhjan R K, Lalykina KS. The development of fibroblast colonies in monolayer cultures of guinea pig bone marrow and spleen cells. Cell Tissue Kinet, 1970; 3:393-403.

16. Bieback K, Hecker A, Kocaomer A, Lannert H, Schallmoser K, Strunk D, Kluter H. Human alternatives to fetal bovine serum for the expansion of mesenchymal stromal cells from bone marrow. Stem Cells, 2009, 27(9), 2331-2341.

17. Hay RI. Cell line preservation and characterization. In: Animal Cell Culture: A Practical Approach (2nd ed). Freshney. 1992; R.I. pp. (104–135), Oxford University Press,ISBN 0199637962,Oxford

18. Ooi YY, Rahmat Z, Jose S, Ramasamy R, Vidyadaran, SH.Immunophenotype and differentiation capacity of bone marrow-derived mesenchymal stem cells from CBA/Ca, ICR and Balb/c mice. World Journal. Stem Cells, 2013; 5: 34-42.

19. Gottipamula S, Sharma A, Krishnamurthy S, Majumdar A, Sen Seetharam RN. Human platelet lysate is an alternative to fetal bovine serum for large-scale expansion of bone marrow-derived mesenchymal stromal cells. Biotechnology Letters, 2012; 34:1367–1374.

20. Baik MD, Lim MD, Kang MD, Ahn MD. Lee MD, Kim MD. Effects of Platelet Lysate Preparations on the Proliferation of HaCaT Cells. Transfusion Medicine, 2014; 34:43-50

21. Inamadar AA, Inamadar AC. Culture conditions for growth of clinical grade human tissue derived mesenchymal stem cells: comparative study between commercial se- rum-free media and human product suspplemented media. Journal of Tissue Engineering and Regenerative Medicine, 2013; 2:10.