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Autologous Human Serum Supports Better Proliferation, Viability and Homogeneity of Peripheral Blood Mononuclear Cells over Fetal Bovine Serum

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Abstract

Xenogeneic serum is being used to expand stem cells for regenerative therapy. Xenoantigens present in these serums may be linked to xeno-contamination of cells during *in vitro* culture and transplant rejection resulting short term regenerative benefits. To prove the potentiality of autologous human serum over fetal bovine serum (FBS) this study was conducted to determine the effect of xenogenic serum and autologous human serum on proliferation, viability and spontaneous differentiation of peripheral blood mononuclear cells till day 4 under *in vitro* culture condition. Data received from the study has shown that autologous serum supports better growth kinetics and viability till day 4 compared to FBS. Meanwhile, autologous serum reduces spontaneous differentiation.
Keywords: Autologous serum, PBMC, Viability, Differentiation.

1. Introduction

In human body almost all the organ and tissue contain their lineage committed progenitor cells that involved in maintaining homeostasis by replenishing the lost cells with new cells during the process of turnover (da Silva Meirelles *et al.*, 2006; Fuchs and Chen, 2012). Different part of the body such as bone-marrow, adipose tissue, dental pulp contain mesenchymal stromal cells (MSCs) which are also involved in maintaining the homeostasis (Haque *et al.*, 2013). In some cases (e.g. myocardial infarction, stroke, spinal cord injuries) natural regenerative process alone cannot maintain homeostasis

or fails to repair a injured or diseased organ (Hatzistergos *et al.*, 2010). As a result stem cell based regenerative therapy received attention to treat degenerative diseases or repair an affected organ.

Among different types of stem cells MSCs are considered as a potential source of stem cell for regenerative therapy because of their multi-differentiation potential, immunomodulatory effects, trophic functions, vasculogenesis potential as well as large donor pool (Caplan and Correa, 2011; Haque *et al.*, 2013). In clinical trials for each regenerative therapy about 50-200 million MSCs are being used (Estrada *et al.*, 2013; Haque *et al.*, 2013). Due to the presence of very lower number of MSCs within the tissues it is impractical to isolate such a large number of MSCs from a single donor that makes *in vitro* expansion of MSCs prior transplantation an inevitable option (Pittenger *et al.*, 1999; Sun *et al.*, 2008).

Till now in most of the laboratories MSCs are expanded in xenogeneic serum especially fetal bovine serum (FBS) supplemented media. Usually FBS or other xenogeneic serums are contaminated with N-glycolylneuraminic acid (Neu5Gc) xenoantigen (Komoda *et al.*, 2010). During *in vitro* expansion MSCs receive Neu5Gc on their surface that cannot be removed by several washing (Spees *et al.*, 2004). MSCs contaminated with xenoantigen may cause hyper-immunogenicity (Sakamoto *et al.*, 2007) that may lead to acute rejection of transplanted cells.

As FBS contains xenoantigen, the peripheral blood mononuclear cells (PBMCs) should respond to them. Thus in this study we were focused on identifying the effect of FBS on proliferation, viability and spontaneous differentiation of PBMCs compared to atologous human serum.

2. Materials and Methods

2.1. Preparation of Serum from Whole Blood

With informed consent and ethical approval (DF RD1301/0012(L)) 50ml blood from each donor was collected. Blood was collected from non-smoker, non-alcoholic and non-addicted donors (n=4, **aged 21-32 years**). Donors were not suffering from any chronic and inflammatory diseases, not suffered from any inflammatory diseases in the last 4 weeks, not went through any major operation in the last 1 year, and not taken any immune therapy in the past as well. Total 50 ml of blood was collected in two steps: i) 20 ml blood was collected in syringe without any anti-coagulant for serum preparation, and ii) 30 ml blood was collected in green top vaccutainer tubes for PBMCs isolation. To prepare serum 20 ml blood was transferred to a 50 ml conical tube very carefully and left for half an hour. Blood was loosened by using sterile pipette and left for another half an hour to facilitate coagulation. Then the tube containing coagulated blood was centrifuged at 400g for 15 minutes. After that the yellow portion (crude serum) of the centrifuged blood was collected very carefully. Then it was centrifuged again at 3000rpm and supernatant (cell debris or insoluble particle free serum) was collected. This serum was heat treated at 59°C for 30 minutes. Freshly prepared serum was then used to prepare medium for PBMC.

2.2. Media Preparation and Culture

PBMC culture media were prepared by using RPMI-1640 (GIBCO, 11875-093) supplemented with 10% (v/v) FBS (USA defined, Hyclone) or freshly prepared autologous serum and 0.5% (v/v) Penicillin-Streptomycin (10000 units/ml, GIBCO, 15140-122). Basal medium without any supplementation was also used for culture of PBMC. The complete media were sterilized by filtration through a 0.22µm membrane filter. Freshly prepared media were used for culture and rest stored at 4°C for further use.

2.3. PBMC Isolation

With maintaining all the aseptic conditions 30 ml blood was collected from healthy donors in green top vaccutainer (sodium heparin) tubes. Tubes were inverted 5 times to mix the blood with anti-coagulant properly. 10 ml blood was transferred to a 50 ml centrifuge tubes and equal volume of D-PBS was added. Diluted blood (20 ml) was added on top of the Ficoll-Paque Plus (GE Healthcare, 17-1440-03) solution (15 ml) very carefully in another 50ml centrifuge tube. Then it was centrifuged at 400g for 30-40 minutes with brake off. Buffy coat containing PBMCs was appeared in between the plasma and ficoll. PBMCs were collected by pipetting very carefully to avoid ficoll contamination. Then PBMCs were washed twice with D-PBS (without Ca^{++} and Mg^{++}, GIBCO, 14190-250) by centrifuging at 200g for 10 minutes. Supernatant was discarded and the pellet was flicked and mixed

with basal medium and cell number was counted using trypan blue dye exclusion method. After counting PBMCs were seeded at a concentration of 0.55×10^6 cells/ml in basal, FBS supplemented and autologous serum supplemented media. Plates were then left at 37°C and 5% CO_2 in humidified chambers.

2.4. Growth Kinetics

At every 24 hours interval cultured PBMCs were counted by using trypan blue dye exclusion method. The counting of cells was maintained till day 4 and growth curve has been prepared by using the collected data.

2.5. Cell Viability Assay

Cell viability was measured by using prestoblue (Invitrogen, A-13261) at every 24 hours interval till day 4. By using producer's protocol viability of PBMCs cultured in different medium were analysed and corrected absorbance was used to present data for this assay.

2.6. Cytospin Slides Preparation, Fixation and Storage

Cytospin slides were prepared for differential count of PBMCs at every 24 hours interval. In brief, cells with media were collected in 15 ml centrifuge tubes. After that tubes were centrifuged at 250g for 10 minutes. Supernatant were discarded and cell pellets were suspended in 2% FBS containing D-PBS. The cell concentration was maintained less than 0.5×10^6 cells/ml. Slides were pre-labelled and set in cytospin clip along with cytofunnel. Then clips were placed in appropriate slots in the cytospin. Cytofunnels were loaded with 500µl of cell suspension and closed. Lid of the cytospin were placed properly and spined at 1000 rpm for 3 minutes. After that filters were removed carefully without contacting the smear on slides. Slides were dried overnight in desiccation chamber. Then the slides were fixed in absolute methanol (Sigma-Aldrich, 322415-1L) for 5 minutes. Slides were dried in room temperature for 10-15 minutes. After that slides were stained using Giemsa stain (Sigma 32884) for differential count or wrapped in aluminium foil and stored at -80°C for further uses.

2.7. Differential Count of Cultured PBMCs

Differential count of cultured PBMCs was done by staining the cytospin slides with Giemsa stain (Sigma 32884). To stain the slides first of all slides were fixed in absolute methanol by dipping the film briefly (two dips) in a Coplin jar containing absolute methanol. Then slides were left for air dry. After that slides were stained with diluted Giemsa stain (1:20, vol/vol) for 20 min. Then the slides were washed by briefly dipping the slide in and out of a Coplin jar of buffered water (one or two dips). Then they were left in a vertical position for air dry. Dry stained slides were used for differential count.

2.8. Data Analysis

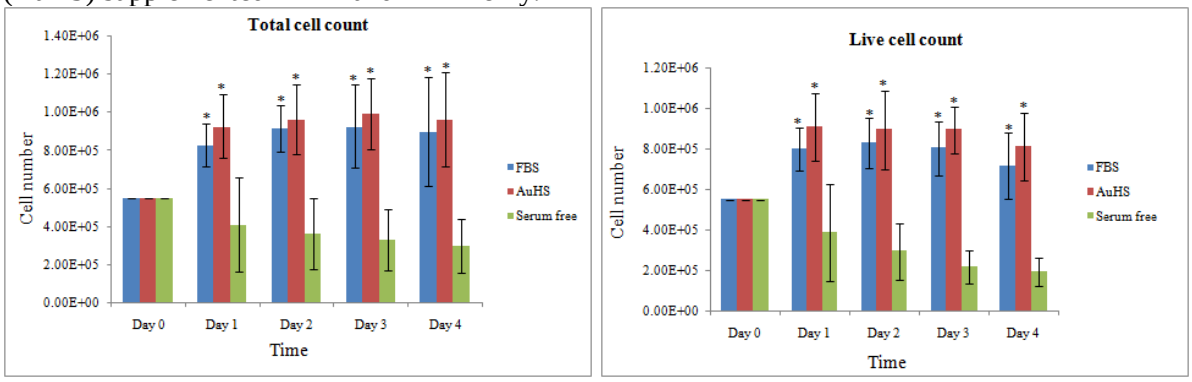
Triplicate was maintained for each experiment. Results are presented as mean ± SD. Data were analyzed using ANOVA and Bonferroni post hoc with values of $p < 0.05$ considered significant. All data were analyzed using SPSS.

3. Results and Discussion

3.1. Growth Kinetics of PBMC

Total cell count and live cell count of PBMCs were done by using trypan blue dye exclusion method, and they were significantly ($p<0.05$) higher in FBS and autologous human serum supplemented media when compared to serum free medium (Figure 1). No significant difference in growth kinetics of PBMCs cultured in FBS and autologous serum supplemented was observed. However, higher proliferation of PBMCs was observed in medium supplemented with autologous human serum than FBS supplemented medium till day 4. This result shows that autologous human serum support better proliferation of PBMCs than FBS.

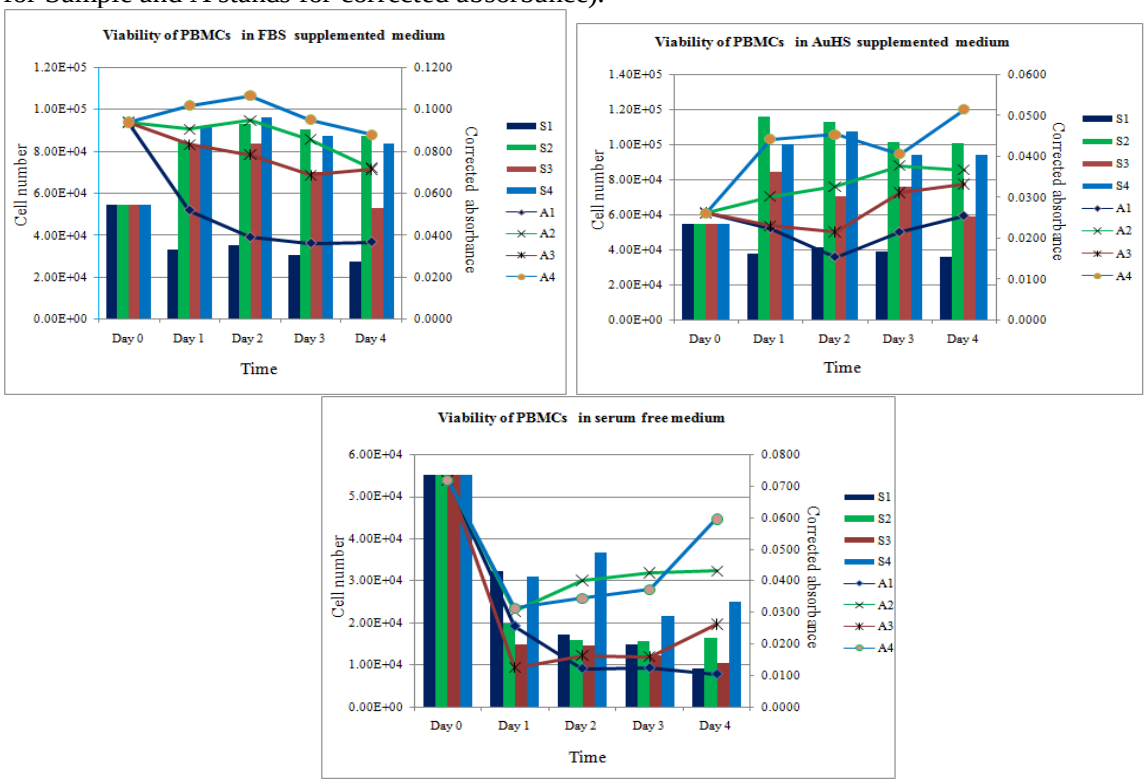
Figure-1. Growth kinetics of PBMCs cultured in FBS supplemented RPMI, autologous human serum (AuHS) supplemented RPMI and RPMI only.



3.2. Viability of PBMCs

Viability of PBMCs cultured in FBS supplemented medium, autologous human serum supplemented medium and serum free medium was measured using prestoblue. Results of viability have been presented in corrected absorbance (Figure 2). From the viability data it has been seen that corrected absorbance of PBMCs cultured in FBS did not increase with the increase in the cell number. However, corrected absorbance of PBMCs in FBS supplemented medium decreased with the decrease in the cell number. PBMCs cultured in autologous human serum supplemented medium have been shown an increase in their corrected absorbance with the increase in their cell number. Positive correlation between cell number and corrected absorbance has also been seen for the PBMCs cultured in serum free medium only. These viability data represents that autologous serum supplemented medium is more physiological to human PBMCs than FBS supplemented medium.

Figure-2. *Viability of PBMCs cultured in different media.* A) viability of PBMCs in FBS supplemented RPMI medium, B) viability of PBMCs in autologous human serum (AuHS) supplemented RPMI medium, C) viability of PBMCs in RPMI medium only (in these figures S stands for Sample and A stands for corrected absorbance).



3.3. Differential Count of PBMCs

Differential count of PBMCs cultured in FBS supplemented medium, autologous human serum supplemented medium and serum free RPMI medium only was done using Giemsa stain (Table 1). Higher percentages of neutrophils and monocytes were seen in PBMCs cultured in FBS supplanted medium till day four compared to PBMCs cultured in autologous human serum supplemented medium. As the life span of neutrophiles is very short (usually few hours) the nutrophiles found in the cultured PBMCs till day four denote that they were generated from the precursor cells. In addition, higher number of differentiated cells in FBS supplemented medium denotes that FBS has immunogenic properties.

Table-1. Differential count of PBMCs cultured in FBS supplemented medium, autologous human serum supplemented medium and serum free medium only.

	FBS				AuHS				RPMI			
	Lymphocyte	Monocyte	Neutrophil	Eosinophil	Lymphocyte	Monocyte	Neutrophil	Eosinophil	Lymphocyte	Monocyte	Neutrophil	Eosinophil
Day 1	77.02%	11.82%	11.15%	0	72.19%	17.22%	10.60%	0.00%	82.55%	14.15%	2.36%	0.94%
Day 2	83.43%	9.14%	6.29%	1.14%	90.80%	5.12%	3.77%	0.27%	-	-	-	-
Day 3	87.24%	9.57%	2.73%	0.46%	91.25%	4.96%	3.21%	0.58%	83.33%	13.89%	0	2.89%
Day 4	83.21%	12.04%	4.20%	0.54%	93.79%	4.24%	1.67%	0.30%	80%	20%	0	0

-Not measured

4. Conclusion

This is an ongoing project and this article presents all the preliminary data. Still we are processing more samples and we have also planned to do cytokine profiling of supernatant collected from different culture to come to a conclusion. However, all the preliminary data indicate that autologous human serum is more physiological compared to FBS. Moreover, FBS has also shown immunogenic properties. As use of autologous human serum for therapy is impractical we should try to find a proper replacement of FBS for therapeutic uses. But it is impractical to use FBS replacement from human source for the entire *in vitro* and *in vivo* experimental process related to therapy. Thus, the use of FBS should be confined in bench while the use of human serum or other replacement of FBS from human source should be ensured for therapeutic purposes.

5. Acknowledgment

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6. Author Disclosure Statement

No competing financial interests exist.

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