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OPTIMIZATION OF GLYCEROL DENSITY GRADIENT CENTRIFUGATION PROTOCOL FOR SEPRATAION OF PERIPHERAL BLOOD MONONUCLEAR CELLS

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Abstract:

Ficol is the commonly used gradient centrifugation medium for the separation of peripheral blood mononuclear cells (PBMCs) from the blood cells. After centrifugation based on the density of the blood cells will sediment from top to bottom in the tube. In this study, we have demonstrated alternative gradient medium for the separation of PBMCs which is easy to prepare and consumes less cost. In this study for the separation of PBMCs we performed one step density gradient centrifugation of diluted blood using glycerol as a gradient medium. Serial dilutions of glycerol are made using 1X PBS (10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%.). 4 ml of diluted blood was layered on 4mL of Glycerol medium and centrifuged at 4^0C for 45 min at 1000 rpm. We observed that 40% glycerol showed a clear separation of PBMCs. Quality check of separated PBMCs was performed in two methods we analyzed separated PBMCs in hematology analyzer which showed 74% lymphocytes, 24% monocytes and 2% of other cells. Separated cultured using RPMI-1640 medium and incubated overnight and 20 uL of cell suspension is dropped on slides to check for cells and these slides captured under microscope showed numerous numbers of cells. Hence, we conclude that 40% glycerol gives a pure and clear separation of PBMCs.

Keywords: Glycerol; PBMCs; Gradient centrifugation; Blood

Abbreviations: PBMC- Peripheral blood mononuclear cells; WBC- white blood cells; PBS- phosphate buffered saline

1. Introduction:

Blood is composed of four different types of cells, i.e. plasma, red blood cells, white blood cells, and platelets. peripheral blood mononuclear cells (PBMCs) are small white blood cells, size ranging from 7 to 8 micrometers in length. PBMCs are the only cells which can be transformed into proliferating cells in the human body. These cells originate from lymphatic tissue, which is present in throughout the body [1].

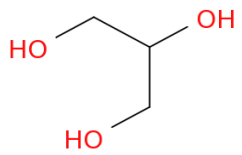
WBCs protect the body from diseases, invasion of foreign bodies, tumors and infections. WBCs are used for the immunological studies of histocompatibility antigens and cellular immunity, They are also used in cell cytotoxicity studies, for determining the proportion of T cells to B cells and for trying to separate Helper T cells and Suppressor T cells [1,2].

The separation of WBCs is carried out by density gradient centrifugation. Centrifugation is the most commonly used method for blood processing in which cells or molecules of the same size and density sediment as separate layers without any convection [3, 4, 5].

Separated blood components can subsequently use in their respective clinical and scientific applications and investigations. To separate and isolate different blood types aqueous density medium has been used for centrifugation [6, 7, 8].

1.1. Glycerol:

Glycerol is a simple polyol compound It is a colorless, odorless, viscous liquid that is sweet-tasting and non-toxic. Composed of Glycerol is an alcohol with three carbons, five hydrogens, and three hydroxyl (OH) groups. Commercially glycerol is obtained from plant and animal sources [9]. Molecular structure and molecular formula for glycerol is $C_3H_8O_8$.



1.2. Density gradient ultracentrifugation:

Density gradient centrifugation is the most common method for the isolation and purification of biomolecules and cellular structures. The separation is based on molecules in the suspension that are denser than the solvent will sediment at the bottom while the less dense molecules will float by layering liquids of decreased density [10,11].

1.3. Glycerol -gradient centrifugation:

This technique is used for characterization of subcellular particles. A centrifuge tube is filled with a glycerol gradient medium and the sample is layered on it. After centrifugation, more density molecules will settle in the bottom of the centrifuge tube and less dense molecules will float on top [12].

2. Materials and methods

2.1. Materials:

1X PBS Buffer, Glycerol (10, 15, 20, 25, 30, 35, 40, 45, 50%), Human blood in Heparinized green top vacutainer , Centrifuge tubes, Centrifuge

2.2. Method

2mL of Blood was collected in a sodium heparinized vacutainer by vine puncturing from normal healthy people. Collected blood is diluted using 1X PBS in 1:1 ratio. Different concentration of glycerol is prepared by diluting in 1X PBS. The dilutions are 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%. 4mL of Diluted blood was layered on each concentration of glycerol solution containing centrifuge tube Immediately centrifuge at 1000 rpm for 45 min in a cooling centrifuge (4°C) removed the tubes carefully from the centrifuge and observed the four layers top is reddish clear supernatant containing platelet and plasma, middle is opaque fluid containing the PBMC, bellow clear solution is glycerol and bottom is RBC. Transferred the opaque PBMC layer to another centrifuge tube Added 1x PBS to the PBMC suspension to obtain a final volume of 8 ml. Mixed well and centrifuged at 1000 rpm for 10 min in a cooling centrifuge (4°C) discard the supernatant. To the palate 1mL of 1X PBS is added and prepared sample to QC check.

2.3. Quality Control checks for Separated PBMCs

2.3.1. Culture of Isolated PBMCs

500uL of Isolated PBMCs are cultured using RPMI-1640 culture mediums with 300 UL of PHA and incubated overnight at 37^0 C and 5% CO_2.

2.3.2. PBMC counts using hematology analyzer

500uL of Isolated PBMCs are analyzed using hematology analyzer (MS-H630)

3. Result:

The separation of lymphocytes showed accurate in 40% of glycerol solution shown in Image- I and II

Isolated cells are counted using haeme analyzers it showed 74/% lymphocytes, 24% monocytes and 2% of other cells.

Isolated PBMCs are cultured using RPMI1640 and incubated overnight at 5% CO2 at 37^0 C.

Cultured Cells are dropped on clean slides and viewed under a microscope, it showed the more viable cells (shown in Image III)

Image I

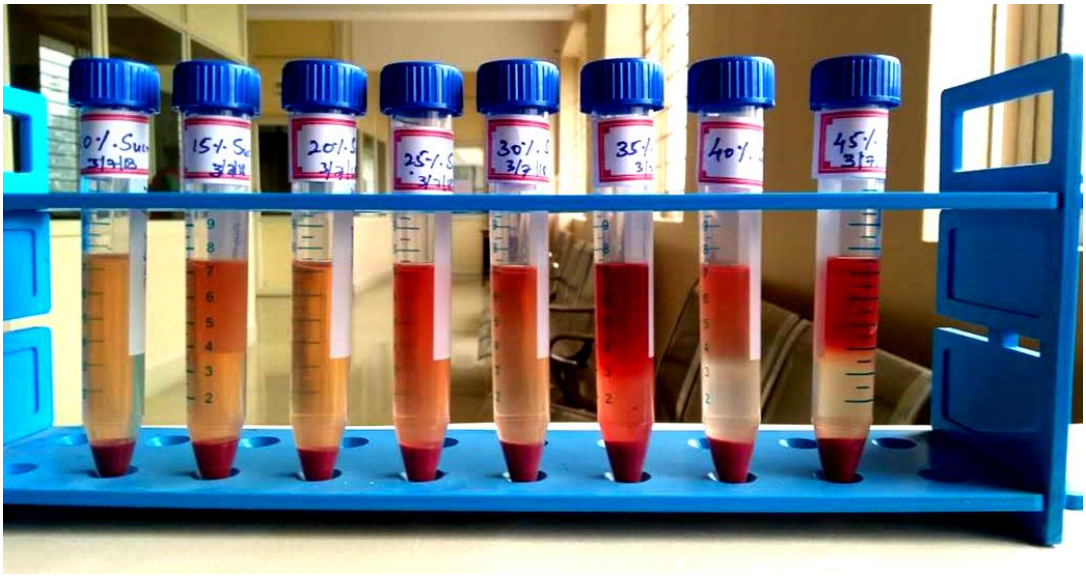


Image I: Glycerol gradient centrifugation of blood samples using 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%. Glycerol solution, respectively.

Image II

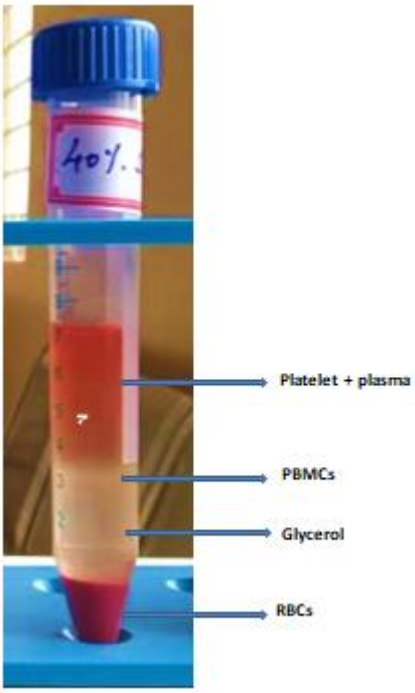


Image II: PBMCs are separated 40% Glycerol solution separated blood cells

Image III

Image III: PBMCs Cultured Cells were dropped and viewed under a microscope (10X), it showed the more viable cells in two different fields.

4. Discussion:

The available ficol gradient centrifugation method for separation of PBMCs is accurate and highly efficient but it consumes more cost. The proposed method is simple and more economic. Therefore, this method is recommended even in low-technology laboratories for high-throughput sample preparation suitable for various immunological assays.

5. Conclusion:

Based on above results we conclude that 40% glycerol is suitable for separation of blood cells or isolation of PBMCs by using less cost glycerol. The Resulted PBMC pellet can be used for any immunological and cytotoxicity assays and this method we can store lymphocytes for longer duration of time for any hematological studies or for related scientific research.

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