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Original Article

Optimization and Validation of *in vitro* Proliferation of Rat's Peripheral Blood Mononuclear Cells (PBMNCs) Stimulated with Phytohemagglutinin-M (PHA-M) for Enhanced Immune Response

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ABSTRACT

Objectives: The main objective of this study is to develop a method for *in vitro* T cell proliferation from rat peripheral blood mononuclear cells. **Materials and Methods:** The study involved female adult Albino Wistar rats, acclimatized for a week and used mononuclear cells for T cell enrichment. The cells were cultured in 24 and 6-Well Plates (WP) in the presence of PHA-M and analyzed for cytokines, cell counts, DNA content and morphology characteristics. **Key Facts:** MNCs were isolated and quantified, which revealed moderate to high variability across samples. The study found that TNF-α concentrations increased with the addition of PHA-M in DMEM and SFM 24-WP, with a peak at 20 µL. The 24-WP showed higher baseline and stimulated TNF-α levels compared to the 6-WP. The addition of PHA-M to DMEM 24-WP increases IFN-γ concentration, particularly at 10 µL, while it slightly decreases at 20 µL. In SFM 24-WP, it increases significantly at 20 µL. DMEM 24 WP 10 µL PHA-M has lower IL-6 concentrations, while DMEM 24 WP 20 µL PHA-M has the highest concentration. SFM 24 WP 20 µL PHA-M has significantly higher IL-6 levels. PHA-M stimulation significantly increased DNA concentration in DMEM 24 WP, with the highest concentration at 10 µL. PHA-M stimulation increased cell count, particularly with 10 µL, indicating successful proliferation. However, average cell size decreased with 10 µL of PHA-M for both media types. **Conclusion:** DMEM 24-WP at 10 µL of PHA-M offers a promising setup for experiments due to its balance between cytokine production and effective proliferation.

Keywords: Cell count, Cytokine, DNA, Immune Response, Mononuclear cells, Proliferation, T-cells.

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INTRODUCTION

Mononuclear Cells (MNCs), a type of white blood cell, are characterized by a single round nucleus. They are smaller than neutrophils and eosinophils but are more numerous. MNCs are integral to the immune system, performing functions from killing infected cells to regulating immune responses, primarily through T cells. The ability of T cells to fight against bacteria or tumors makes them an ideal candidate for the therapeutic development of adoptive T cell therapy. Found in peripheral blood, MNCs include lymphocytes, monocytes and macrophages, collectively known as Peripheral Blood Mononuclear Cells (PBMCs). Monocytes can transform into macrophages or dendritic cells, contributing to PBMCs diversity. Dendritic cells, though few, are vital for adaptive immunity, highlighting the complexity of MNCs functions.[1] MNCs are vital components of the immune system, comprising lymphocytes, monocytes and dendritic cells, each playing distinct roles in maintaining immune homeostasis.

MNCs are pivotal in immune modulation, identifying pathogens and preventing autoimmune responses. They include T cells and B cells, essential for immune defense and antibody production, respectively. Monocytes, the largest MNCs, contribute to pathogen clearance by engulfing microbes and debris, supporting tissue health. Dendritic cells specialize in antigen presentation to T cells, crucial for initiating and regulating immune responses. T cells play a crucial role in initiating and sustaining immune responses, maintaining homeostasis and preserving immunological memory. Their receptors can recognize a wide range of antigens from pathogens, tumors and environmental sources, which allows them to maintain both immunological memory and self-tolerance. They are also significant in inflammatory and autoimmune diseases. Insights into their functions and mechanisms largely come from mouse models, which have propelled advancements in immune-based therapies and immunotherapies for humans. T lymphocytes

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originate from bone marrow progenitors that migrate to the thymus for maturation and selection before being exported to the periphery. In the periphery, T cells are categorized into subsets: naïve T cells that respond to new antigens, memory T cells that provide long-term immunity from past antigen encounters and regulatory T (T reg) cells that control immune responses. Immune responses are initiated when naïve T cells encounter antigens and costimulatory signals presented by Dendritic Cells (DCs), leading to the production of Interleukin 2 (IL-2), T cell proliferation and differentiation into effector cells that migrate to different sites to clear pathogens.[2-4]

Sources

Mononuclear Cells (MNCs) can be obtained from various tissues and biological fluids, including peripheral blood, bone marrow, umbilical cord blood, spleen, lymph nodes and adipose tissue. These sources provide diverse populations of MNCs with unique properties and potential applications in research and therapy. Techniques like density gradient centrifugation, immunomagnetic separation and surgical procedures can be used to isolate MNCs. MNCs are distinguished from other cell types by their unique properties and versatility in medical applications. They are rich in various stem cells, including hematopoietic and non-hematopoietic lineages, making them a valuable resource for therapeutic interventions. MNCs encompass an array of immature and mature cell types from myeloid, lymphoid and erythroid lineages, demonstrating their diverse cellular composition.[5]

T cells originate from hematopoietic stem cells in the bone marrow and undergo maturation and differentiation in the thymus gland. The thymus is crucial for T cell development, where precursor cells mature into various T cell subtypes. After maturation, T cells migrate to peripheral lymphoid organs, including lymph nodes, spleen and mucosal tissues, where they encounter specific antigens. Although a small fraction resides in peripheral blood, the majority of T cells are distributed throughout these lymphoid tissues to execute immune functions.[3]

Therapeutic Applications

T lymphocytes are one of the major immune components which play an important role in immunological responses and infection defence. They produce pro- and anti-inflammatory cytokines, making them viable for potential immunotherapeutic applications. Recent research has demonstrated promising therapeutic applications of autologous PBMCs in treating spinal cord injuries, with studies in animal models showing improved tissue integrity and partial restoration of spinal axon conduction. In regenerative medicine, PBMCs are valuable due to their ease of isolation and have shown potential in treating conditions like limb ischemia and myocardial infarction. Mononuclear cells, including lymphocytes and monocytes, play critical roles in immune responses against infections and autoimmune diseases and hence, driving research towards developing new treatments. Studies on PBMCs have also contributed significantly to understanding hematological malignancies like leukemia and lymphoma, aiding in the development of targeted therapies. In vaccine development, antigen-presenting cells such as dendritic cells are pivotal for studying immune responses and improving vaccine strategies. Additionally, mononuclear cells are crucial in transplant immunology, where they influence immune responses to transplanted tissues and organs, contributing to efforts to enhance transplant outcomes and reduce rejection rates.[6]

In this pioneering study, we aim to optimize and validate methods for the proliferation of T cells using PHA-M from rat PBMNCs. To date, there has been no published literature addressing the optimization of T cell proliferation *in vitro* from animal models, making this research a novel and significant contribution to the field. Our goal is to develop reliable and efficient methods to induce T-cell proliferation from MNCs. This advancement holds considerable potential for the treatment of various diseases, particularly those characterized by low tumor burden. The successful optimization of T-cell proliferation could lead to improved therapeutic strategies and enhance our understanding of immune responses in animal models, paving the way for future clinical applications.

MATERIALS AND METHODS

All the reagents and materials used in this study were sourced from Gibco, Sigma and Hi-Media, purchased from authenticated vendors in Bangalore, India. The equipment and facilities utilized for this research are located in the tissue culture laboratory within the Department of Pharmacology at KCP, Bangalore.

Experimental Design

The study involved female adult Albino Wistar rats, weighing 150-200 g, acclimatised for 1 week under standard conditions and provided with a standard diet as per CPCSEA guidelines.

Isolation of MNCs

As per the modified protocol mentioned by Lefort CT *et al.*, (2010),[7] 1.5 mL of blood was withdrawn from the rat through a cardiac puncture under anesthesia and transferred into an EDTA tube. The blood was then diluted with an equal volume of normal saline. In a 15-mL centrifugation tube, density gradient media (HiSep, Hi-Media) was placed at the bottom of the tube. The diluted blood was carefully added on top of the gradient media using a sterile pipette to avoid mixing, maintaining a HiSep to blood ratio of 1:3. The tubes were centrifuged at 1000 rpm for 20 min. After centrifugation, the upper layer containing plasma and platelets was removed using a sterile pipette, leaving the MNCs (buffy coat) at the interface. The MNC layer was gently aspirated and transferred to a new tube. The harvested cells were washed with normal saline twice by centrifuging at 1500 rpm for 10 min. After the final centrifugation, the supernatant was removed and

the cells were resuspended in two cell culture medias:[1] Dulbecco's Modified Eagle's Medium (DMEM) and[2] Serum Free Medium (SFM).

Cell counting calculation

In a separate tube, 10 µL of MNC suspension was mixed with 90 µL of crystal violet, creating a 10-fold Dilution Factor (d_f). From this mixture, 10 µL of the stained cell suspension was then further diluted with 90 µL of crystal violet, resulting in an additional 10-fold dilution factor. This brings the total dilution factor to 100-fold. Finally, 10 µL of this 100-fold diluted stained cell suspension was loaded onto a hemocytometer for cell counting. The cells were observed under upright microscope and calculated total cell count using the following formula; Total Cell Count (TCC) in million cells per mL=average number of cells counted using hemocytometer x cell suspension total volume (a) in mLxdfx104.

Culture of T Lymphocytes

Method 1

$1x10^6$ cells were cultured in 24-well plates with either 10% FBS in 1 mL of DMEM containing 1% antibiotic or SFM containing optimizer (approx. 26 µL in 1 mL SFM), glutamine (approx. 10 µL in 1 mL SFM) and 1% antibiotic respectively. 10 and 20 µL of PHA-M was added to each well plate and were incubated in a CO_2 incubator at 37°C for 2 days.

Method 2

$2x10^6$ cells were cultured in 6-well plates with either 10% FBS in 2 mL of DMEM containing 1% antibiotic or SFM containing optimizer (approx. 52 µL in 2 mL SFM), glutamine (approx. 20 µL in 2 mL SFM) and 1% antibiotic. 20 µL of PHA-M to the well plate and were incubated in a CO_2 incubator at 37°C for 2 days.

After 48 hr, the supernatant was carefully collected without disturbing the cells. The collected supernatant was centrifuged at 1500 rpm for 10 min to remove any remaining cells and debris and the clarified supernatant was collected as T-cell-conditioned medium and checked for the presence of cytokines. The remaining T-cell pellets were resuspended in fresh saline for further analysis of their counts, DNA and morphology (by examining blast cells) and these parameters were compared to those of both methods treated with PHA-M.

Quantification of Cytokines from T cell conditioned medium using ELISA

The study by Jha DK *et al.* (2024)[8] evaluated the concentrations of TNF-α, IFN-γ and IL-6 using ELISA according to the manufacturer's instructions. The protocol is briefly described as follows:[1] To the pre-coated wells of the respective cytokines, 100 uL of standards, samples and blank was added and kept for incubation at Room Temperature (RT) for 2 hr for the formation of antigen-antibody complexes. The wells were washed with the appropriate buffer to remove the unbound antigens and antibodies. Enzyme linked antibody was added to the complex and was incubated for an hour at RT. It was then followed by another round of washing step to remove unbound enzyme linked antibodies. 100 uL of the respective substrate was added and kept in dark for 30 min. Finally, the reaction was stopped using a stop solution. The absorbance was measured at 450 nm using a microplate reader. The concentrations of TNF-α, IFN-γ and IL-6 in unknown samples were determined by extrapolating their absorbance values from the standard calibration graph.

DNA Isolation and Quantification

Isolating DNA from T-cell involves several key steps; lysing T-cells in appropriate volume of lysis buffer (1xTES Lysis buffer; 1 M Tris-HCl (pH 8.0), 0.5 M EDTA (pH 8.0) and 10% SDS solution), followed by addition of RNase A and Proteinase K and incubating the mixture at 56°C for 1-3 hr. To the lysed sample, an appropriate volume of phenol: chloroform: isoamyl alcohol (25:24:1) was added and incubated in ice-cold condition for 10 min. It is then followed by centrifugation at 12000 rpm for 15 min. The resulting upper aqueous layer (containing DNA) obtained after centrifugation is then precipitated using 70-80% ethanol. The DNA pellet is then air-dried. The DNA pellet is resuspended in TE buffer or sterile deionized water and stored at -20°C or 4°C for short-term use.

DNA Concentration Calculation

The DNA concentration is calculated using the absorbance ratio at 260/280 nm (A260/A280) multiplied by 50 and the dilution factor. The dilution factor was calculated based on the volume of the sample added to the total volume (e.g. 3 mL of d.H_2O, 5 µL of sample, d_f=600).

Morphology Study

A small volume of the cell suspension (10 µL) was transferred onto a microscopy slide. The cells were smeared evenly and air-dried. These cells were stained with crystal violet solution and were observed under a light microscope at various magnifications (e.g., 10X and 40X). Morphological characteristics such as cell size, shape and nuclear-to-cytoplasmic ratio were noted. The morphology of T cells was compared under different conditions (e.g., with or without PHA-M stimulation, 10 or 20 µL PHA-M, 24 or 6 well plates and DMEM or SFM).

RESULTS AND DISCUSSION

Quantification of MNCs

MNCs were successfully isolated and quantified from peripheral blood (blood sample volume: 1.5 mL) using crystal violet staining, revealing high variability across samples, i.e., an average MNC count of 9.5±6.3×10^6 cells per 1 mL cell suspension.

Consistency in all steps, from blood collection to cell isolation, is crucial to minimising variability for future experiments.

Figure 1: The Process of isolating and visualising mononuclear cells from rat blood involves a step-by-step process: Blood was withdrawn through a cardiac puncture under anaesthesia and then layered over HiSep density gradient media in a centrifuge tube. The blood components were separated based on their densities, resulting in a plasma layer, a whitish layer containing mononuclear cells (lymphocytes and monocytes) and a dark red layer containing RBC. The resulting smear was then examined under a microscope at 10x magnification and captured images of the isolated cells, ensuring the integrity of the layers and the viability and counts of the isolated cells.

Quantification of Cytokines (TNF-α, IFN-γ and IL-6)

Table 1 shows the concentration of TNF-α under various experimental conditions. The concentration of TNF-α in control is approx. 9.7 pg/mL, which serves as the baseline for DMEM in a 24-WP, but there is an increase in TNF-α concentration with the addition of PHA-M in DMEM at 10 and 20 µL (p<0.001), with a peak at 20 µL. There is a substantial increase in TNF-α concentration with the addition of PHA-M in SFM 24-WP, especially at 20 µL. There is a decrease in TNF-α concentration with the addition of 20 µL PHA-M in DMEM 6-WP but a significant increase in concentration of TNF-α with the addition of 20 µL PHA-M in SEM 6WP compared to DMEM 6-WP. The 24-well plates tend to show higher baseline and stimulated TNF-α levels compared to the 6-well plates in both media types.

The differential responses in TNF-α concentration between DMEM and SFM could be attributed to the presence or absence of serum components that might interact with PHA-M. The increase in TNF-α with PHA-M in SFM suggests a possible enhancement of immune activation in a serum-free environment, which could be important for studies focused on serum-free conditions. The variability between 24-well and 6-well plates could be due to differences in cell density, surface area and culture conditions that affect cell signalling and cytokine production.

Quantification of Cytokine: IFN-γ

Table 1 depicts the concentration of IFN-γ (pg/mL) under different conditions, which is crucial for optimizing experimental setups in immunological studies. The concentration of IFN-γ increases with the addition of PHA-M, especially at 10 µL (p<0.001) and drops or slightly decreases with the addition of PHA-M at 20 µL in DMEM 24-WP. There is a notable increase in IFN-γ concentration with the addition of PHA-M in SFM 24-WP, especially at 20 µL compared to 10 µL, but it remains the same or no change if compared to baseline. There is an increase in IFN-γ concentration with the addition of 20 µL PHA-M in DMEM 6-WP and SFM 6-WP compared to 24-WP in both media types. The differential responses in IFN-γ concentrations between DMEM and SFM could be attributed to the presence or absence of serum components that might interact with PHA-M. The increase in IFN-γ with PHA-M in SFM suggests a possible enhancement of immune activation in a serum-free environment, which could be important for studies focused on serum-free conditions.

Quantification of Cytokine: IL-6

Table 1 illustrates the concentration of IL6 under different conditions. The study reveals that the DMEM 24 WP Control and DMEM 24 WP 10 µL PHA-M show lower IL-6 concentrations. DMEM 24 WP 20 µL PHA-M exhibits the highest IL-6 concentration. SFM 24 WP Control and SFM 24 WP 10 µL PHA-M have moderate IL-6 levels. SFM 24 WP 20 µL PHA-M shows significantly higher IL-6 levels than its control and lower dose counterparts. DMEM 6 WP Control has a minimal IL-6 concentration, whereas DMEM 6 WP 20 µL PHA-M has an elevated IL-6 level. SFM 6 WP Control shows a slight negative

Table 1: Estimation of Cytokines from different cultured conditions.

Sl. No.	Cultured Condition/Estimation parameters	Estimation of Cytokines from T-cell conditioned medium (pg/mL)		
		TNF-α	IFN- γ	IL-6
1	DMEM, 24 Well Plates_Control	9.707±0.2	4.066±0.025	0.0092±0.001^{ns}
2	DMEM, 24 Well Plates_ 10 µL PHA-M	17.42±0.5***	8.715±0.135***	0.6532±0.05***
3	DMEM, 24 Well Plates_ 20 µL PHA-M	19.77±0.2	4.252±0.03	3.893±0.05
4	SFM, 24 Well Plates_Control	14.25±0.45	10.11±0.095	3.511±0.055
5	SFM, 24 Well Plates_ 10 µL PHA-M	15.19±0.015	7.610±0.045	1.916±0.025***
6	SFM, 24 Well Plates_ 20 µL PHA-M	17.69±0.23	9.39±0.135	3.935±0.1
7	DMEM, 6 Well Plates_Control	24.12±0.5	4.066±0.025	0.1667±0.045^{ns}
8	DMEM, 6 Well Plates_20 µL PHA-M	14.72±0.225	7.544±0.04***	2.555±0.09
9	SFM, 6 Well Plates_Control	38.56±0.18	19.67±0.18	11.89±0.009
10	SFM, 6 Well Plates_20 µL PHA-M	27.53±0.5	11.97±0.025	7.054±0.005

Values are expressed as Mean ± SEM (n=3), ***p<0.001 compared to their respective group.

IL-6 concentration, while SFM 6 WP 20 µL PHA-M has increased IL-6 levels. The addition of PHA-M significantly increases IL-6 production, especially at the 20 µL concentration. SFM conditions show higher IL-6 levels than DMEM under the same conditions.

The increase in IL-6 levels in PHA-activated cells is primarily due to the activation and proliferation of T-cells, leading to the production of cytokines. IL-6 is a key cytokine involved in the immune response, playing roles in inflammation, immune regulation and haematopoiesis. Upon activation, T-cells and other immune cells produce a cascade of cytokines, including IL-6, which promotes B-cell differentiation, T-cell survival and the acute phase response. IL-6 also plays a crucial role in T-cell differentiation into Th17 cells, which may be upregulated by PHA activation.

Quantification of DNA from T-cells pellets

Table 2 illustrates the quantification of DNA from T-cell pellets under various culture conditions. DMEM 24 WP: PHA-M stimulation significantly increased DNA concentration, with the highest at 10 µL PHA-M (p<0.001). The 20 µL PHA-M conditions showed a lower DNA concentration compared to the 10 µL PHA-M condition, suggesting an optimal PHA-M concentration around 10 µL for these conditions. Other groups of both media types found no significant difference between the two PHA-M concentrations. The highest proliferation, as indicated by DNA concentration, is observed with 10 µL of PHA-M in DMEM 24-well plates. An optimal concentration of PHA-M needs to be identified, as too high a concentration (PHA-M 20 µL) may not significantly increase DNA concentration compared to PHA-M 10 µL or may even reduce it.

Distinct Morphological Characteristics of Activated Lymphocytes

Figure 2: Method 1: Cultured 1x10^6 cells in 24-well plates with DMEM with and without stimulation of PHA-M. Figure 2A: Shows Mononuclear cells without stimulation of PHA in DMEM.

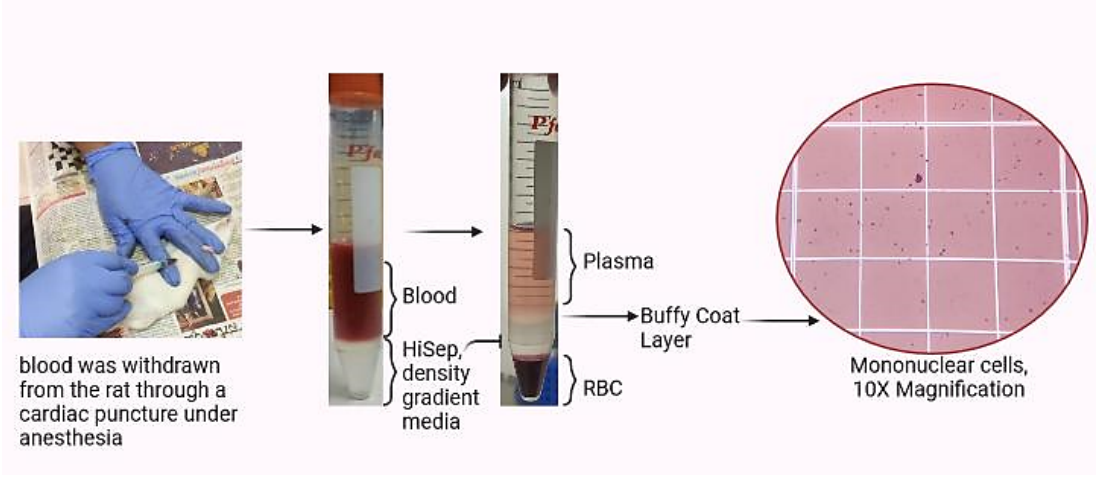

Figure 1: Quantification of MNCs. The Process of isolating and visualising mononuclear cells from rat blood involves a step-by-step process.

Table 2: Estimation of DNA from different cultured conditions.

Sl. No.	Cultured Condition/Estimation parameters	Estimation of DNA from T-cell pellets. Quantification of DNA (mg/mL)
1	DMEM, 24 Well Plates_Control	55.56±1.015
2	DMEM, 24 Well Plates_10 µL PHA-M	99.52±1.0***
3	DMEM, 24 Well Plates_20 µL PHA-M	45.68±1.0
4	SFM, 24 Well Plates_Control	64.08±1.41
5	SFM, 24 Well Plates_10 µL PHA-M	61.95±1.0
6	SFM, 24 Well Plates_20 µL PHA-M	61.74±1.14
7	DMEM, 6 Well Plates_Control	63.33±1.44
8	DMEM, 6 Well Plates_20 µL PHA-M	65.0±1.0
9	SFM, 6 Well Plates_Control	63.22±1.0
10	SFM, 6 Well Plates_20 µL PHA-M	63.47±1.0

Values are expressed as Mean ± SEM (n=3), ***p<0.001 compared to DMEM 24WP control, rest other groups were observed non-significant, ^{ns}p>0.05 with their respective group.

Table 3: T-Cells Proliferation results under different conditions.

Sl. No.	Cultured Condition	Count	Total Area	Average Size	% Area
1	DMEM, 24 Well Plates_Control	11495	1780029	154.852	56.586
2	DMEM, 24 Well Plates_ 10 µL PHA-M	26715	2252074	84.3	71.592
3	DMEM, 24 Well Plates_ 20 µL PHA-M	12887	1382428	107.273	74.269
4	SFM, 24 Well Plates_Control	15109	1498725	99.194	47.643
5	SFM, 24 Well Plates_ 10 µL PHA-M	19076	831363	43.582	26.428
6	SFM, 24 Well Plates_ 20 µL PHA-M	14862	1512671	101.781	48.087
7	DMEM, 6 Well Plates_20µL PHA-M	30240	1950933	64.515	62.018
8	SFM, 6 Well Plates_20 µL PHA-M	37433	1792559	47.887	56.984

DMEM, 24WP_10 µL PHA-M, DMEM, 6 WP_20µL PHA-M, and SFM, 6 WP_20 µL PHA-M was shown significant cell count compared to other groups.

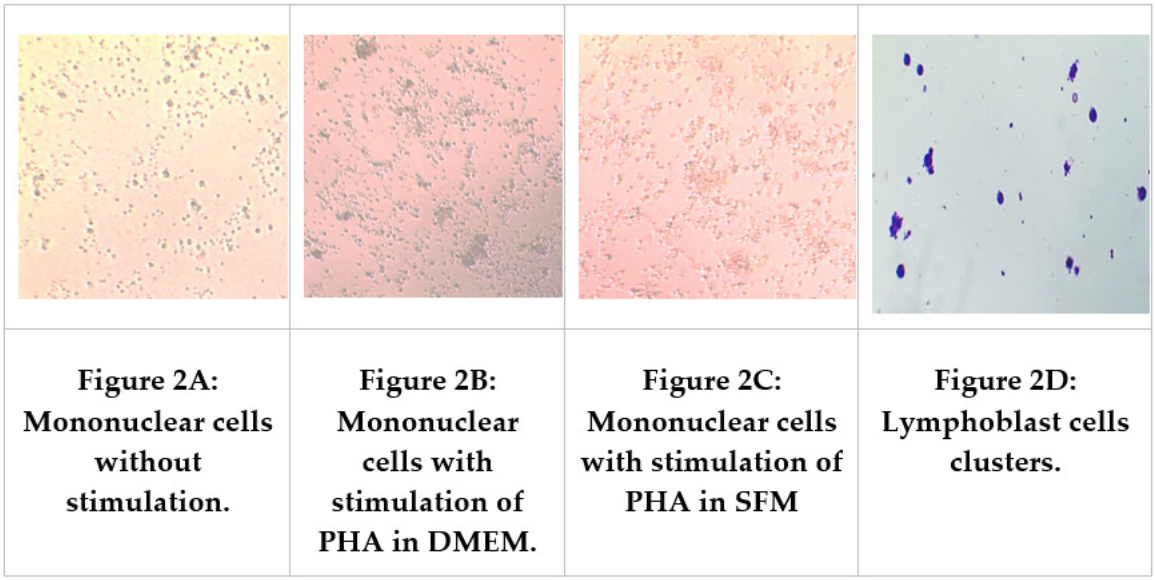

Figure 2A: Mononuclear cells without stimulation.

Figure 2B: Mononuclear cells with stimulation of PHA in DMEM.

Figure 2C: Mononuclear cells with stimulation of PHA in SFM

Figure 2D: Lymphoblast cells clusters.

Figure 2: Method 1: Cultured 1x10^6 cells in 24-well plates with and without stimulation of PHA-M.

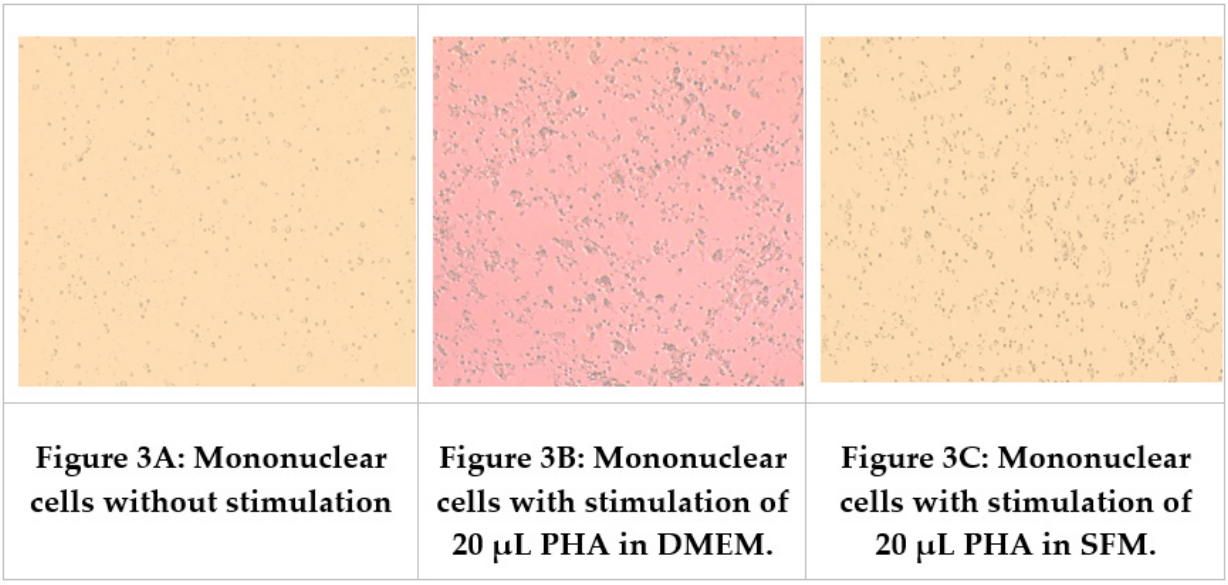

Figure 3A: Mononuclear cells without stimulation

Figure 3B: Mononuclear cells with stimulation of 20 µL PHA in DMEM.

Figure 3C: Mononuclear cells with stimulation of 20 µL PHA in SFM.

Figure 3: Method 2: Cultured 2x10^6 cells in 6-well plates with DMEM and SFM with and without stimulation of PHA-M.

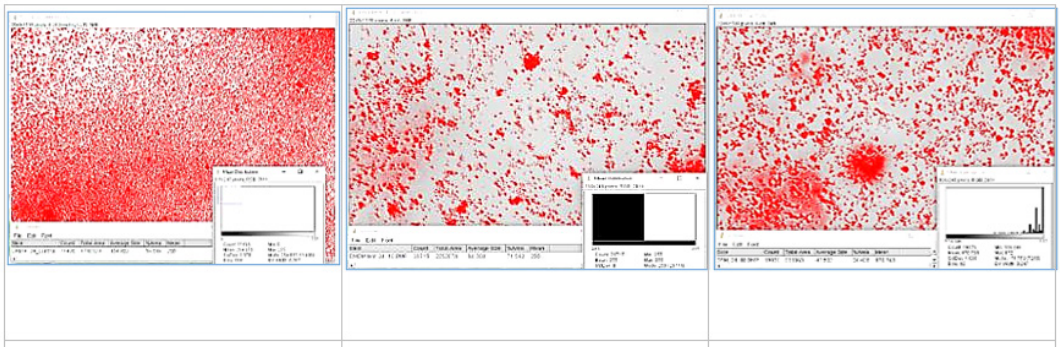

Figure 4A: DMEM, 24 well Plates_Control.	Figure 4B: DMEM, 24 well Plates_10 µL PHA-M.	Figure 4C: SFM, 24 well Plates_10 µL PHA-M.

Figure 4: Method 1: Cultured 1x10^6 cells in 24-well plates with DMEM with and without stimulation of PHA-M.

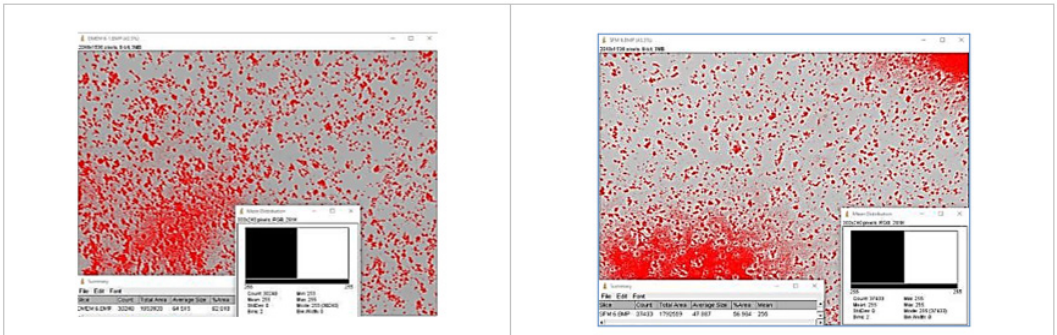

Figure 5A: DMEM, 6 Well Plates_20 µL PHA-M.	Figure 5B: SFM, 6 Well Plates_20 µL PHA-M.

Figure 5: Method 2: Cultured 2x10^6 cells in 6-well plates with DMEM and SFM with and without stimulation of PHA-M.

Figure 2B: Shows Mononuclear cells with stimulation of PHA-M in DMEM, Figure 2C: Cultured 1x10^6 cells in 24-well plates with SFM stimulation of PHA-M and Lymphoblast cells clusters (Figure 2D).

Figure 3A to 3C): Method 2: Cultured 2x10^6 cells in 6-well plates with DMEM and SFM with and without stimulation of PHA-M shows Mononuclear cells without stimulation of PHA in DMEM, Mononuclear cells with stimulation of 20 µL PHA in DMEM, Mononuclear cells with stimulation of 20 µL PHA in SFM.

Comparative studies of lymphocyte cell count, total area, average size and distribution using ImageJ software.

Figure 4: Method 1: Cultured 1x10^6 cells in 24-well plates with DMEM with and without stimulation of PHA-M Figure 4A DMEM, 24 well Plates_Control, Figure 4B DMEM, 24 well Plates_10 µL PHA-M, Figure 4C SFM 24 well Plates_10 µL PHA-M.

Figure 5: Method 2: Cultured 2x10^6 cells in 6-well plates with DMEM and SFM with and without stimulation of PHA-M Figure 5A DMEM, 6 Well Plates_20 µL PHA-M, Figure 5B SFM, 6 Well Plates_20 µL PHA-M.

Table 3 summarizes the proliferation results under different conditions. The cell count increased with PHA-M stimulation, especially with 10 µL, indicating successful proliferation. However, there is a notable decrease in the average cell size with 10 µL PHA-M for both media types. There is a significant increase in cell count with PHA-M stimulation in DMEM 6-WP compared to the 24 well plates, indicating that larger wells may support more proliferation. PHA-M stimulation in the SFM 6-WP condition shows the highest cell count among all, indicating a strong proliferative response in larger wells with SFM. There is a clear increase in cell count with PHA-M stimulation in both DMEM and SFM across different well sizes, confirming the proliferative effect of PHA-M. The average cell size tends to decrease with PHA-M stimulation, which might be due

to higher cell division rates leading to smaller average cell sizes. Both DMEM and SFM support cell proliferation with PHA-M, but the effect is more pronounced in larger wells (6-well plates). For maximal proliferation, using larger wells (6-well plates) with SFM and PHA-M appears optimal based on cell count data.

CONCLUSION

The study successfully demonstrated that PHA-M stimulation significantly influences cytokine production, DNA concentration and cell proliferation in MNCs isolated from rats' peripheral blood. The concentration of TNF-α increased with PHA-M stimulation, peaking at 20 µL, with 24-well plates showing higher baseline and stimulated TNF-α levels compared to 6-well plates. IFN-γ concentrations were highest at 10 µL in DMEM 24-well plates, while in SFM, the highest concentration occurred at 20 µL. IL-6 production increased with PHA-M, with the highest levels seen in DMEM 24-well plates at 20 µL and SFM at 20 µL. The DNA concentration peaked at 10 µL PHA-M in DMEM, indicating that this dose was optimal for DNA synthesis and proliferation. Larger wells (6-well plates) supported higher cell proliferation, particularly in SFM, with a higher PHA-M dose leading to greater cell counts, although average cell size decreased with higher doses. These findings suggest that DMEM 24-WP at 10 µL of PHA-M is proving to have the most effective results, particularly with respect to IFN-γ concentration, DNA synthesis and cell proliferation.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ABBREVIATIONS

MNCs: Mononuclear Cells; **Wp:** Well Plate; **SFM:** Serum-Free Medium; **DMEM:** Dulbecco's Modified Eagle Medium; **CPCSEA:** Committee for the Purpose of Control and Supervision of Experiments on Animals (India); **PHA-M:** Phytohemagglutinin-M; **HiSep Media:** A density gradient medium for cell separation; **EDTA:** Ethylenediaminetetraacetic Acid (anticoagulant); **FBS:** Fetal Bovine Serum; **RT:** Room Temperature; **ELISA:** Enzyme-Linked Immunosorbent Assay; **TNF-α:** Tumor Necrosis Factor Alpha; **IFN-γ:** Interferon Gamma; **IL-6:** Interleukin-6; **IAEC:** Institutional Animal Ethics Committee.

AUTHORS' CONTRIBUTION

Dr. Deepak Kumar Jha: Conceptualization, design and development of work.
Saroj Sah: Data Collection and Processing.
Mehdi Fathima: Contributed to the development and refinement.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

The protocol was approved by IAEC, Karnataka College of Pharmacy, Bengaluru-560064 and Sl. No. KCP-IAEC/14/23-24/10/28/03/24.

SUMMARY

The study aimed to develop methods to induce T-cell proliferation from Mononuclear Cells (MNCs) in animal models, which could enhance immune responses and therapeutic strategies. The study involved female adult Albino Wistar rats and used Mononuclear Cells (MNCs) cell suspension for T lymphocyte isolation, culture and proliferation analysis. The cells were cultured in 24-well and 6-well plates and analyzed for counts, DNA and morphology. The concentrations of TNF-α, IFN-γ and IL-6 were evaluated using a sandwich immunoassay ELISA assay. The study found high variability in MNCs isolated from peripheral blood using crystal violet staining. The addition of PHA-M generally resulted in an increase in TNF-A concentration at 24 well plates, but a slight decrease at 6 well plates. In SFM, the addition of PHA-M resulted in a substantial increase in TNF-A concentration, particularly notable in the 24 well plates addition with 20 µL of PHA-M. The increase in IFN-γ and PHA-M in SFM suggests a possible enhancement of immune activation. The study also revealed that the addition of PHA-M significantly increases IL-6 production, especially at a 20 µL PHA-M concentration. The increase in IL-6 levels in PHA-M activated cells is primarily due to the activation and proliferation of T-cells, leading to the production of IL-6. Larger wells generally support better proliferation and an optimal concentration of 20 µL PHA-M may not significantly increase DNA concentration compared to 10 µL PHA-M. The combination of DMEM 24-Well plates and PHA-M yields promising results in IFN-γ concentration, DNA synthesis and cell proliferation, balancing cytokine production and effective proliferation.

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