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Research Article

A Modified Protocol for Isolation and Mitogenic Stimulation of Rat Peripheral Blood Mononuclear Cells

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Abstract

Peripheral blood mononuclear cells (PBMCs) are key components of the immune system and serve as a practical *in vitro* model in many research programs. Despite the widespread applications, popular standard methods for isolating and stimulating PBMCs in rats (rPBMCs) have remained limited. In this study, rPBMCs were isolated from rat peripheral blood using a modified Ficoll density gradient protocol, followed by a short-term adherence step to remove monocytes. A complete blood count (CBC) analysis and Giemsa staining confirmed that the isolated population consisted of approximately 98% lymphocytes, indicating high purity of the isolated cells. The cells were subsequently exposed to different concentrations of mitogens, concanavalin A (ConA; 1.25–10 µg/mL) and phytohemagglutinin (PHA; 1–2%) for 3 and 5 days. Cell proliferation and viability were assessed by MTT assay, and TNF-α secretion was quantified by ELISA. ConA treatment resulted in a concentration-dependent increase in rPBMCs proliferation, with the highest viability rate observed at 1.25–5 µg/mL on day 3. At a concentration of 5 µg/mL, ConA significantly enhanced rPBMCs viability. This elevated level remained constant between days 3 and 5, indicating a sustained cellular response. By comparison, the stimulatory effect of PHA proved either weaker or delayed, as it reached its peak only on day 5. Furthermore, TNF-α analysis revealed that ConA markedly enhanced cytokine secretion, whereas PHA triggered only a modest increase. These findings demonstrate that ConA is a more potent mitogen than PHA in rat PBMCs, promoting both proliferation and cytokine release in a dose-dependent manner. The optimized protocols for isolation and stimulation of rPBMCs presented here, providing a reliable framework for future *in vitro* studies on immune function and immunomodulation in rat models.

***Keywords*:** Rat peripheral blood mononuclear cells, Ficoll density gradient protocol, Concanavalin A, Phytohemagglutinin, Cell viability, TNF-α secretion

Introduction

Peripheral blood mononuclear cells (PBMCs) are a critical component of the immune system. PBMCs constitute a heterogeneous population of immune cells that include lymphocytes (T cells, B cells, and natural killer cells), monocytes, and dendritic cells. They are identified by their single and round-shaped nucleus (Kleiveland, 2015). PBMCs are considered as a valuable *in vitro* model for studying immunological processes. The extraction of PBMCs from blood is essential for investigating the effects of different stimuli on immune cell function. Murine models (especially rats) are the primary preclinical models for studying human diseases and drug evaluation due to ethical and practical advantages. A key difference between rat and human blood is found in their white blood cells, specifically the inverse ratio of lymphocytes to neutrophils (Fasanmade & Jusko, 1995; Kohn & Barthold, 2013). The standard method for isolating PBMCs, established in 1968, uses density gradient centrifugation with Ficoll-Paque medium (1.077 g/cm³) (Boyum, 1968). It has been shown that the lymphocytes in rodents have a slightly higher average density than in human (Mizobe et al., 1982; Walter et al., 2023). Consistent with this, GE Healthcare recommends a 1.084 g/cm³ Ficoll gradient for rodents, which is higher than the 1.077 g/cm³, used for human PBMCs isolation (Healthcare, 2010). Nevertheless, some published protocols continue to use the human standard Ficoll (1.077 g/cm³) for rodent blood (Song et al., 2021; Torres Crigna et al., 2020). Optimization of this critical step is essential for ensuring reproducible

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and physiologically relevant PBMCs preparations in experimental rat models.

Mitogenic stimulation of PBMCs serves as a classical approach for assessing immune cell functionality and responsiveness *in vitro*. Lectins such as concanavalin A (ConA) and phytohemagglutinin (PHA) are plant-derived carbohydrate-binding proteins. They play a crucial role in modulating immune cell activation through their ability to recognize and cross-link glycan structures on the surface of lymphocytes (Bojar et al., 2022). ConA is isolated from *Canavalia ensiformis*, and PHA is a complex lectin mixture extracted from *Phaseolus vulgaris*. ConA binds primarily to α-D-mannosyl residues, while PHA binds to structures containing N-acetyl-D-glucosamine (Bojar et al., 2022). These specific interactions initiate intracellular signaling cascades by activating enzymes such as adenylate cyclase, which catalyze the production of secondary messengers. These messengers transmit activation signals to the lymphocyte nucleus, ultimately leading to cell proliferation, cytokine secretion, and the induction of an immune response under *in vitro* conditions (Wattrang et al., 2012). Although these agents are well established in human immunological assays (Jiao et al., 2019; Lijnen et al., 1997; O'flynn et al., 1986; Pandit et al., 2016), comparative data on their efficacy and optimal dosing in rat PBMCs remain limited. Understanding these differences is crucial, as rat PBMCs are increasingly used to model immunopathological conditions, test drug safety, and evaluate novel immunotherapeutics.

Therefore, the present study aimed to (i) optimize a reproducible protocol for isolating high-purity rPBMCs from rat peripheral blood, (ii) compare their proliferative and cytokine responses to two common mitogens of ConA and PHA, and (iii) identify the effective and safe concentration ranges for each stimulant (Figure 1).

Materials and Methods

Blood Sample Collection

Peripheral blood was obtained from healthy adult Wistar rats (*Rattus norvegicus*). All animal procedures were conducted in accordance with relevant guidelines and regulations and were approved by the Animal Ethics Committee of Ferdowsi University of Mashhad (Approval No. IR.UM.REC.1401.271). Rats were deeply anesthetized *via* intramuscular injection of a ketamine (70 mg/kg, Bremer Pharma GmbH, Warburg, Germany) and xylazine (5 mg/kg, Alfasan, Woerden, The Netherlands) cocktail. Upon confirmation of surgical anesthesia, whole blood was procured by terminal cardiac puncture using a sterile technique. The collected blood was immediately transferred into K2-EDTA vacuum collection tubes (Xinel) to prevent coagulation and preserve cellular integrity.

Peripheral Blood Mononuclear Cell Isolation

rPBMCs were isolated using an optimized density gradient centrifugation protocol, designed to maximize yield and purity. Briefly, EDTA-anticoagulated whole blood was diluted at a 1:1 (v/v) ratio with sterile phosphate-buffered saline (PBS), supplemented with 1-2% fetal bovine serum (FBS). The diluted blood was then carefully layered over Ficoll-Hypaque solution (1.077 g/cm³, Lymphodex, Inno-Train), using a 3:1 ratio of blood to density gradient medium. Samples were centrifuged at 600 *g* for 30 minutes at 4°C without brake to preserve the gradient interface. Following centrifugation, the buffy coat layer, containing mononuclear cells, was carefully collected with a sterile pipette. To remove residual platelets, Ficoll, and plasma proteins, the cells were washed twice with PBS containing 10% FBS. Each wash was performed by centrifugation at 500 *g* and 400 *g* for 10 minutes, respectively.

To further enrich for lymphocytes, a critical purification step was employed. The final cell pellet was resuspended in Roswell Park Memorial Institute (RPMI) 1640 medium (Gibco, Scotland), supplemented with 10% FBS, 100 U/mL penicillin, and 100 µg/mL streptomycin. The cell suspension was then incubated in a sterile cell culture plate for 2 hours at 37°C in a humidified 5% CO_2 atmosphere. This monocyte depletion step enriches for lymphocytes in the non-adherent fraction, which was carefully collected for subsequent experiments. Viable cell numbers were quantified using a Neubauer hemocytometer with Trypan blue staining. Cell viability consistently exceeded 98%. Following isolation, the purity and number of the lymphocyte population were assessed using an Auto Hematology Analyzer (BC-2800, China).

Giemsa Staining and Morphological Analysis of rPBMCs

To determine the cellular composition of the isolated rPBMCs fraction, Giemsa staining was performed. Smear preparations were fixed with absolute methanol for 5 minutes and stained with diluted Giemsa solution for 20 minutes, and washed with distilled water. The slides were examined under a light microscope at 1000× magnification using oil immersion. Cells were classified based on nuclear morphology and cytoplasmic characteristics.

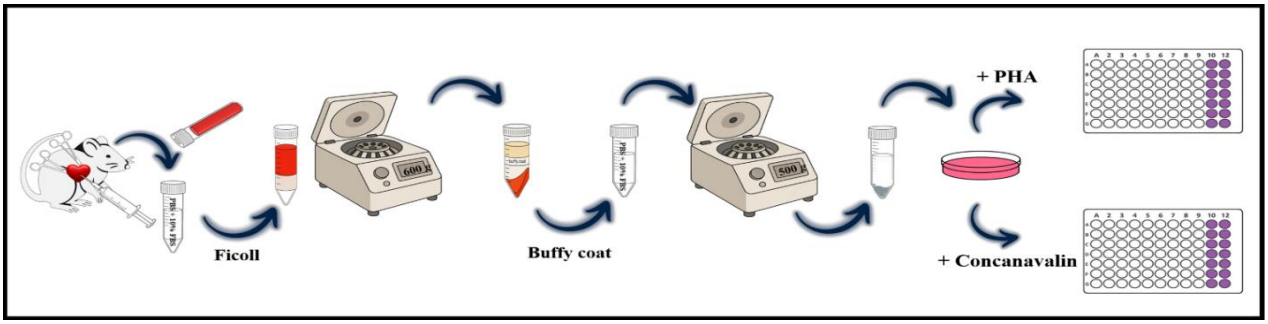

Figure 1. A schematic illustration of the experimental methodology and materials for the isolation, stimulation, and analysis of rat PBMCs. Whole blood was collected from rat, layered over Ficoll, and centrifuged to separate the buffy coat. White blood cells were extracted, washed, and cultured *in vitro*. Cells were stimulated with ConA or PHA mitogens for further evaluation.

Comparative Analysis of Mitogens Efficacy on rPBMCs

To directly compare the mitogenic potency of two PBMC stimulators, rPBMCs were subjected to parallel stimulation with either Concanavalin A (ConA, Sigma-Aldrich, USA) or PHA (PHA-M, Gibco). Cells were seeded in 6-well plates at a density of 1×10^6 cells per well in complete RPMI 1640 medium.

The experimental groups were defined as follows: test groups were treated with a serial dilution of ConA (1.25, 2.5, 5, and 10 µg/mL), while the comparison groups were treated with PHA at a corresponding concentration of 1 and 2% (v/v). An unstimulated control (medium alone) was included to establish the baseline proliferation index. All conditions were assayed in triplicate. Cells were maintained in culture for 3 and 5 days.

Cell Viability Assay

Cell proliferation was quantified at both time points using the MTT assay. Following the standard protocol, MTT solution was added to each well and incubated for 4 hours at 37°C. The resulting formazan crystals were solubilized in DMSO, and absorbance was measured at 545 nm with an ELISA reader (Awareness Technology, Inc., USA). The proliferative response for each mitogen, across all concentrations and time points, was reported as a viability percentage relative to the unstimulated control.

ELISA for Determination of TNF-α Production

To further characterize the immunostimulatory function of the mitogens, TNF-α secretion was measured. Supernatants were harvested from rPBMC cultures after 5 days incubation, specifically from groups treated with 2.5 µg/mL and 5 µg/mL ConA, 2% PHA, and the unstimulated control. TNF-α levels were determined using a commercial enzyme-linked immunosorbent assay (ELISA) kit (Karmania Pars Gene, Iran) according to the manufacturer's instructions. Briefly, undiluted supernatant samples and standards were added to the antibody-coated microplate. After incubation and a series of washes, the wells were incubated with detection antibody and HRP-Avidin conjugate. The reaction was developed with a substrate solution and stopped, and the optical density was measured at 450 nm with an ELISA reader. The sample concentrations were calculated from the standard curve.

Statistical Analysis

Statistical analyses were performed using GraphPad Prism software (version 8; GraphPad Software Inc., CA, USA). A two-way analysis of variance (ANOVA) and multiple comparisons was applied. Data are presented as the mean ± SEM of three independent experiments. A *p*-value of less than 0.05 was considered statistically significant.

Results

The Isolation Procedure Resulted in a Highly Enriched rPBMCs Fraction

Following the isolation and monocyte-depletion steps, the lymphocyte concentration was quantified using two independent methods. Manual cell counting with a Neubauer hemocytometer established a concentration of approximately 1.63×10^6 cells per milliliter. This result was consistent with the data obtained by an automated complete blood count (CBC) analysis, which yielded a count of 1.7×10^6 cells per milliliter. More than 5 million rPBMCs were recovered from 1 ml of blood.

Microscopic evaluation of Giemsa-stained smears revealed that the isolated rPBMCs were highly enriched with lymphocytes. Approximately 98% of the cells displayed lymphocytic morphology, characterized by a round nucleus and scant cytoplasm, whereas only 2% of the cells exhibited neutrophilic morphology, with multi-lobed nuclei and granular cytoplasm (Figure 2). These findings confirm that the isolation procedure yielded a highly pure rPBMCs fraction suitable for downstream immunological and molecular assays.

Comparing the Effects of Different Mitogens on Cell Viability and Proliferation

To determine the more effective mitogen for rPBMCs stimulation, the cells were treated with different concentrations of ConA (1.25, 2.5, 5, and 10 µg/mL) or PHA (1 and 2% (v/v)), for 3 and 5 days, and then collected and evaluated for cell viability. As shown in Figure 3 and Table 1, the assessment of rPBMCs viability following mitogen stimulation revealed a clear differential response to ConA and PHA. The results demonstrated that the highest cell viability was observed on day 3 at ConA concentrations of 1.25 and 2.5 µg/mL. This increase in viability was statistically significant compared to the untreated control group ($p < 0.0001$). However, viability at these concentrations decreased on day 5. Regarding the 5 µg/mL ConA concentration, a significant increase in cell viability was observed at both time points compared to the untreated PBMCs ($p < 0.01$). Furthermore, the viability level at this concentration on day 5 was similar to that measured on day 3. In the case of PHA, an increase in cell viability was noted at the 2% concentration. However, this increase was not statistically significant. A higher viability was observed for this group on day 5 compared to day 3.

ConA Increased Cytokine Secretion from rPBMCs

To assess the cytokine response at a later stage of lymphocyte activation, TNF-α secretion was measured on day 5 of rPBMCs culture following stimulation with ConA or PHA using ELISA. The results demonstrated that ConA markedly enhanced TNF-α production compared with unstimulated rPBMCs. Treatment with ConA at 2.5 µg/mL increased TNF-α level relative to the control, and a similar inductive effect was observed at 5 µg/mL. In addition, stimulation with 2% PHA produced a moderate increase in TNF-α secretion (Table 2).

Discussion

The present study established a modified and efficient protocol for isolation and stimulating rPBMCs using a standard Ficoll (1.077 g/cm³) gradient combined with a short-term monocyte adherence step. Previous investigations have demonstrated that PBMCs exhibit a higher density in rats compared to human. Accordingly, GE Healthcare, a leading manufacturer of separation media, recommends using a higher-density gradient for the effective isolation of PBMCs from rat blood (Healthcare, 2010). In this study, we employed a standard Ficoll gradient, which successfully yielded a high cell recovery rate. The purity of the isolated lymphocytes (over 98%) was determined by CBC analysis and Giemsa staining through microscopic evaluation of cell morphology. The average lymphocyte concentration in the rat blood samples was determined to be approximately 6 million cells per milliliter (Otto et al., 2015).

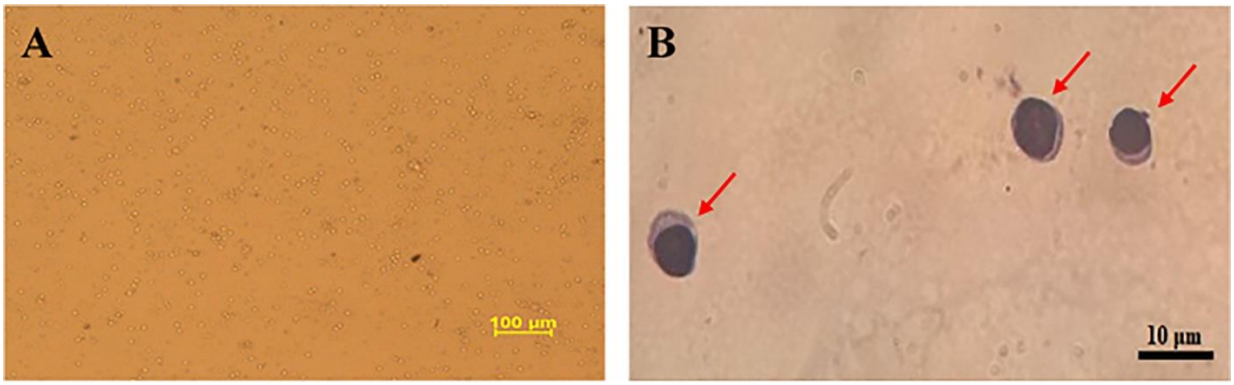

Figure 2. Morphological analysis of rPBMCs. (A) Light microscopy image of rPBMCs at 100× magnification (Nikon, Japan, scale bar represents 100 µm). (B) Giemsa-stained rPBMCs from the same sample, showing lymphocytes at 1000× magnification (indicated by arrows, scale bar represents 10 µm).

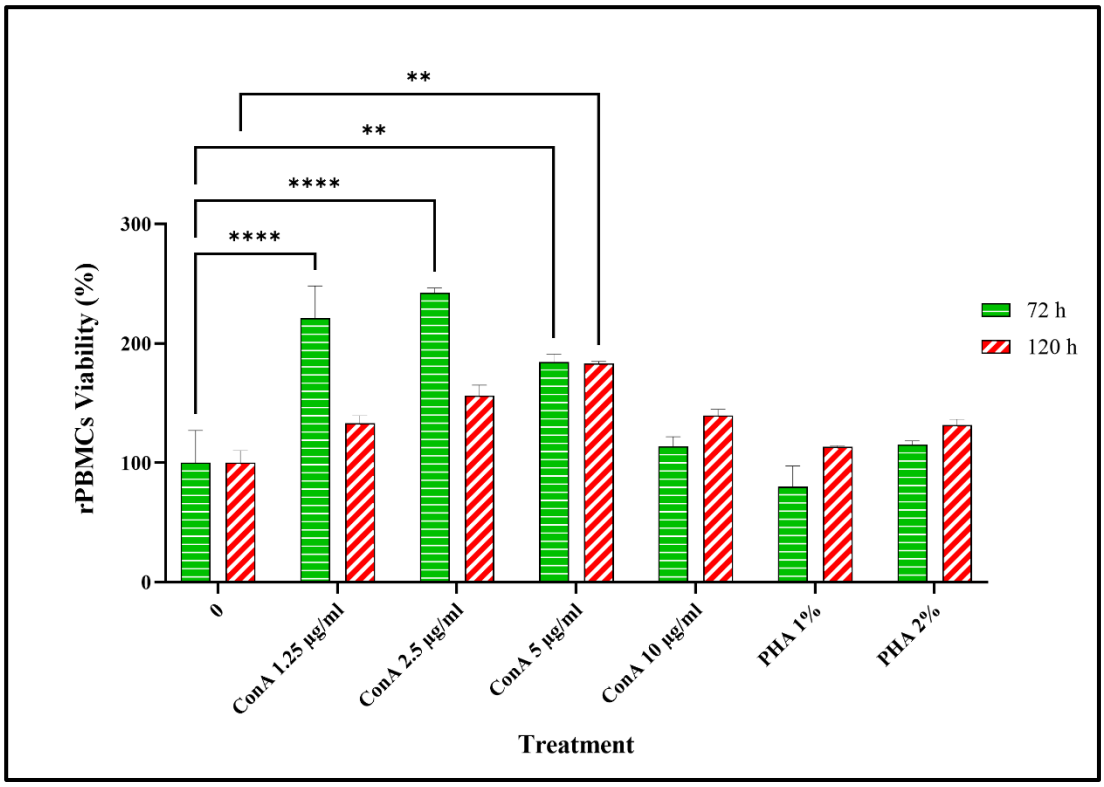

Figure 3. rPBMCs viability in response to different concentrations of ConA and PHA on Day 3 and Day 5. rPBMCs were exposed to different concentrations of ConA and PHA, and cell viability was assessed on Day 3 and 5 using the MTT assay. Triplicate measurements were obtained for each cell group. Data are represented as mean of viabilities ± SEM. $p < 0.01$ (**) and $p < 0.0001$ (****) vs. control.

Table 1. Percentage of rPBMCs viability after treatment with different concentrations of ConA and PHA on Days 3 and 5. Data are reported as mean ± SEM.

Treatment	rPBMCs	ConA 1.25 µg/ml	ConA 2.5 µg/ml	ConA 5 µg/ml	ConA 10 µg/ml	PHA 1%	PHA 2%
Day 3	100±27.7	221.33±26.7	242.53±3.9	184.53±6.4	113.73±8.0	80.13±17.3	115.10±3.3
Day 5	100±10.2	133.08±6.36	156.07±0	183.45±1.4	139.70±5.0	113.23±1.2	131.67±4.6

Table 2. TNF-α concentration in rPBMCs culture supernatants following stimulation with ConA and PHA. TNF-α levels were measured by ELISA on Day 5 of culture.

Treatment	rPBMCs	ConA 2.5 µg/ml	ConA 5 µg/ml	PHA 2%
Concentration (pg/mL)	31	42.63	42.4	34.24

The recovery of > 5 million rPBMCs per mL of blood demonstrates the high efficiency and reproducibility of the density gradient centrifugation method employed. It is important to note that lymphocyte counts are highly dependent on the pathological and physiological conditions of the rat. However, the high and consistent yield achieved in this study indicates that our isolation protocol was

efficient and resulted in minimal cell loss, ensuring that subsequent analyses were performed on a representative and robust cellular population.

Lectins such as concanavalin A (ConA) and phytohemagglutinin (PHA) are plant-derived carbohydrate-binding proteins that play a crucial role in modulating immune cell activation through their ability to recognize and cross-link glycan structures on the surface of lymphocytes. Our data showed that ConA induced a clear, concentration-dependent increase in rPBMCs proliferation, reaching its peak at 3 days. Consistent with previous findings (Fasanmade and Jusko, 1995), ConA stimulated a stronger proliferative response in rat lymphocytes than PHA in whole blood. Fasanmade and Jusko also observed that rat lymphocytes display a rapid proliferative phase peaking at 72 hours under ConA stimulation, which aligns with our data showing maximum viability and proliferation on day 3. The optimum mitogen incubation time reported in the literature varies. According to Molaee et al., a 72-hour incubation period was determined to be optimal for the mitogens PHA and ConA in human PBMCs (Molaee et al., 2017). In contrast, other research has advocated for a longer 96-hour incubation for several mitogens, including ConA and PHA (Norian et al., 2015).

The sustained proliferation observed at 5 µg/mL ConA between day 3 and 5 further suggests that this concentration supports both early activation and maintenance of cellular metabolic activity, potentially through continuous TNF-α secretion as detected in our ELISA assays. Gholamnezhad et al. also demonstrated that stimulation of rat splenocytes with PHA and ConA significantly enhanced cell viability, proliferation, and cytokine secretion. They reported that the mitogenic effect of ConA was more potent than that of PHA, which they attributed to its specific and strong affinity for T-cell receptor complexes on spleen lymphocytes. Furthermore, their data indicated a preferential stimulation of Th2 lymphocytes by ConA, evidenced by a significant reduction in the IFN-γ/IL-4 ratio compared to unstimulated cells (Gholamnezhad et al., 2015). Therefore, the higher potency of ConA in the rat PBMCs model in our study may be related to its stronger ability to cross-link T-cell receptors and glycoproteins on lymphocyte surfaces, thereby triggering more efficient downstream signaling and cytokine release. In contrast, stimulation with PHA produced a weaker and delayed proliferative response, reaching its maximum on day 5. This delayed effect may reflect the slower kinetics of PHA-induced activation, as reported in human PBMCs studies such as Jiao et al., where PHA required higher concentrations (up to 50 µg/mL) to achieve optimal proliferation comparable to CD3/CD28 stimulation (Jiao et al., 2019).

Previous studies have demonstrated that mitogenic compounds stimulate PBMCs to produce cytokines (Molaee et al., 2017; Norian et al., 2015). To assess the rPBMCs response to mitogenic stimulation, TNF-α secretion was quantified after 5 days of incubation with the mitogens. TNF-α, a key inflammatory cytokine, is primarily produced by activated macrophages and T lymphocytes. Cytokine analysis confirmed that ConA at 2.5–5 µg/mL increased TNF-α secretion compared with both control and PHA-stimulated groups. This indicates that ConA not only enhances lymphocyte proliferation but also drives a more potent proinflammatory cytokine release. Molaee et al. demonstrated that TNF-α levels increased in human PBMCs in response to ConA and PHA, with PHA inducing a significantly stronger TNF-α response than ConA (Molaee et al., 2017).

Overall, our data highlight the importance of considering both dose and time-dependent effects when evaluating the impact of mitogens like ConA and PHA on rPBMC cultures. These findings could have important implications for the design and interpretation of *in vitro* studies using these stimulants.

Conclusion

In conclusion, our results demonstrate that ConA is a potent stimulator of both proliferation and TNF-α production in rat PBMCs, highlighting its strong immunostimulatory potential. In contrast, rPBMCs, treated with PHA, exhibited moderate increase in cell viability and TNF-α production, indicating a relatively limited immunostimulatory effect compared to ConA. These differential responses underscore the importance of lectin type and concentration in designing *in vitro* immunomodulation studies using rodent models. The consistent performance of 5 µg/mL ConA concentration in maintaining cell viability across both Day 3 and Day 5 is noteworthy. This indicates that this ConA dose was able to sustain a favorable cellular response over the duration of the experiment.

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Conflict of interest

None.

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