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Evaluating the Proliferation of Human Peripheral Blood Mononuclear Cells Using MTT Assay

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Abstract

Introduction: 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay is a safe, convenient, and low-cost technique with high applications for the measurement of cell proliferation rate in researches and clinical laboratories. Our aim was to evaluate the proliferation rate of human peripheral blood mononuclear cells (PBMCs) and production rate of Tumor necrosis factor alpha (TNF-α) by these cells after various mitogens stimulation in different situations.

Methods: The MTT test was performed with various concentrations of mitogens including concanavalin A (ConA), lipopolysaccharide (LPS) and phytohemagglutinin (PHA) on the PBMCs. The cells were incubated for 24, 48, 72, and 96 hours in the culture medium and TNF-α cytokine assay was performed on the supernatant of the cultured splenocytes using the enzyme-linked immunosorbent assay (ELISA) method.

Results: The optimal time and incubation of the PBMCs with the mixture of PHA-ConA were 5 µg/mL and 72 hours, respectively. The TNF-α level increased significantly after PHA-ConA and PHA stimulation.

Conclusion: The results showed that the mixture of PHA-ConA (at the concentration of 5 µg/mL) can give rise to the optimal results on stimulation of the PBMcs using the MTT assay after 72 hours incubation.

Keywords: Cytokine, Mitogens, Peripheral blood mononuclear cells, Tumor necrosis factor alpha

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Introduction

Human lymphocytes are the peripheral blood mononuclear cells (PBMCs) functionally evaluated by proliferation, survival, and cytotoxicity analysis.[1,2] In this regard, there are 4 types of methods for analyzing cell proliferation based on the detection of DNA synthesis, metabolic activity, antigens stimulation, and ATP concentration. Techniques such as, lymphocyte transformation (LTT) and 5-bromo-2'-deoxyuridine (BrdU) tests measure cell proliferation by analyzing the DNA synthesis with high accuracy, but these methods are expensive. Furthermore, hazardous materials are produced through the 3H-thymidine incorporation assay.

The 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay is a safe and convenient colorimetric method, with high applications in research and clinical laboratories. The MTT is a kind of tetrazolium salt that is used to evaluate cell proliferation as a function of redox system. In this way, the yellow water-soluble tetrazolium salt is changed to an insoluble purple formazan crystal that is dissolved by detergents like dimethyl sulfoxide (DMSO) or acid isopropanol.[3-6]

To evaluate the cell proliferation rate, lymphocytes should be triggered by using several stimulators, including specific antigens, cytokines, and different mitogens. The mitogens influence various lymphocyte subtypes. Concanavalin A (ConA), lipopolysaccharide (LPS) and phytohemagglutinin (PHA) comprise the most common used mitogens.[7] The LPS toxins, which are extracted from the cell wall of gram-negative bacteria, lead to B cell activation both in vivo and in vitro directly. The LPS induces mitosis in memory B cell

with its differentiation to plasma cells.[8] Another mitogen is ConA, which is a lectin (carbohydrate-binding protein) that leads to T-cells stimulation.[6,9-11]

Assessment of immune responses against various microorganisms can be conducted by the evaluation of cytokine production in response to mitogens. One of the most important cytokines that triggers the acute phase of immune response reactions is tumor necrosis factor alpha (TNF-α). This cytokine is derived from the activated macrophages, monocytes, and T cells.[12-14]

Among all types of proliferation assays, MTT provides a safe, convenient, and low-cost technique with many applications in investigations and clinic.[15] Thus, this study aimed to perform the MTT assay to find the best incubation time and concentration of different mitogens for the human PBMCs proliferation. The second aim was to analyze the immune responses of PBMCs to different kinds of mitogenic factors by TNF-α measurement.

Methods

Sample Preparation and Cell Culture

The PBMCs were isolated from 5 mL of whole blood consisting of anti-coagulant EDTA (Sigma, MO, USA) from a healthy adult donor on a Ficoll-Hypaque (Hornby, Ontario, Canada) density gradient by centrifugation at 400 × g at the room temperature for 30 minutes according to the manufacturers' protocol. The cells were cultured within T25 culture flask (SPL Life Sciences, Pocheon, Korea) for overnight in the supplemented RPMI-1640 (Sigma, MO, USA) with fetal bovine serum (FBS), 100 U/mL penicillin, 100 µg/mL streptomycin (Sigma, MO, USA) and 2 mM L-glutamine (Gibco, NY, USA) at 37°C for 24 hours before any treatments. Before performing the experiment, the medium was discarded; the separated cells were washed and counted. The cell viability was measured by trypan blue staining to greater than 95% viability.

MTT Assay

At first, we cultured 1×10^5 cells/well and treated them using various concentrations (2.5, 5, 10, and 20 µg/mL) of PHA, ConA, and LPS. Moreover, we cultured cells in binary mixtures of mitogens (PHA-ConA, PHA-LPS, and ConA-LPS). Then, the cells were incubated for 24, 48, 72, and 96 hours at 37˚C under 5% CO2 and 95% humidity. The PBMCs in complete RPMI-1640 were considered as a control for each group. After incubation with the mitogens, the cells were left with MTT reagent (5 µg/mL final concentration) at 37°C for 4 hours. Formed formazan as an indicator of cell viability was solubilized by adding 100 µL DMSO into each well. The extent of formazan production was determined by an ELISA (enzyme-linked immunosorbent assay) reader (Model ELx800; Bio-Tek Instruments, Winooki, USA) at 550 nm, while 630 nm served as the reference wavelength. The results are presented as stimulation index (SI) calculated using following equation:

$$SI=\frac{\text{Mean OD560 of stimulated PBMCs-Mean OD560 of blank}}{\text{Mean OD560 of unstimulated PBMCs}}$$

Cytokine Assay

The cytokine measurement was performed on the supernatant of the cultured splenocytes by the TNF-α ELISA kit (eBioscience, Vienna, Austria) according to the manufacturer's instruction. The absorbance values of the samples were measured at 450 nm. The concentrations of the samples were calculated by converting the absorbance values, using the standard curve prepared with the serial dilutions of the recombinant TNF-α standards.

Statistical Analysis

The statistical analysis included the independent sample *t* test (exploring meaningful differences between variables in each group). The comparisons between the studied groups were scrutinized applying the Mann–Whitney U test. SPSS software version 16.0 (SPSS, Inc., Chicago, Illinois, USA) was utilized for statistical analysis. Descriptive measures were represented as mean ± standard deviation (SD). The P value < 0.05 was considered as statistically significant.

Results

Different Concentrations of Mitogens

In this study, the prepared cells were stimulated with various concentrations of different mitogens, including 2.5, 5, 10 and 20 µg/mL (Figure 1). The MTT results showed that the maximum SI values were obtained (3.3 ± 0.12) at 5 µg/mL of PHA-ConA (P value < 0.01); however, SI values of PHA (2.7 ± 0.09) and PHA-LPS (2.5 ± 0.12) were significantly higher than the other groups (P value < 0.05).

MTT Results in Different Incubation Times

The MTT assay was done at different incubation periods (24, 48, 72, and 96 hours) at the optimal concentration (5 µg/mL) of each mitogen (PHA, ConA, and LPS) and binary combinations of the mitogens (PHA-ConA, PHA-LPS, and ConA-LPS) (Figure 2). The results showed that 72 hours incubation time was optimum for each mitogen (P value < 0.05).

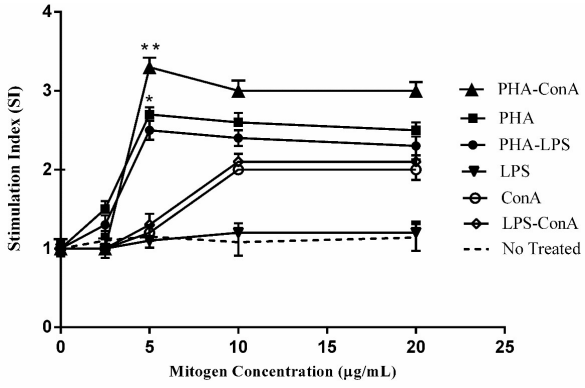

Figure 1. The Effect of Different Concentrations (2.5, 5, 10, and 20 µg/mL) of Each mitogen (PHA, ConA, and LPS) and Their Binary Combinations (PHA-ConA, PHA-LPS, and ConA-LPS) on the MTT Assay. * P value < 0.05, ** P value < 0.01.

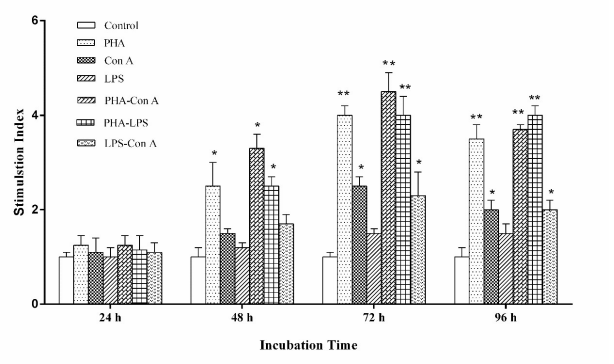

Figure 2. MTT Results at Different Incubation Periods and Optimal Concentrations of Various Mitogens. * *P* value < 0.05, ** *P* value < 0.01.

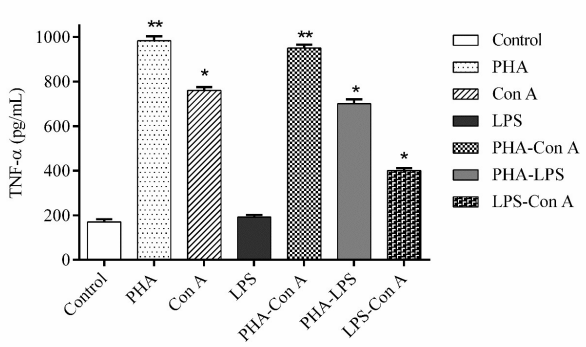

Figure 3. TNF-α Production in Response to Various Mitogens. * *P* value < 0.05, ** *P*-value < 0.01.

Cytokine Concentration

The secreted TNF-α was measured after 72 hours with different mitogens (Figure 3). The results showed that the maximum production of TNF-α was obtained by PHA (983 ± 20) and PHA-ConA (950 ± 15) treatments (*P* value < 0.05).

Discussion

This study aimed to compare the effects of different mitogens on the proliferation rate and TNF-α production of the PBMCs derived from a healthy adult donor. The inducted optimum proliferation induction was seen in 5 µg/mL of PHA-ConA at the concentration of 1×10^5 cells/well. According to the previous studies, selection of suitable mitogen with the best concentration is dependent on the cell type. In this regard, Norian et al reported a higher mitogenic effect for pokeweed mitogen (PWM) respective to PHA and ConA mixture on the same cell count. However, PHA had similar effects with less efficacy in proliferation stimulation of B cells.[15] Wanger et al determined that the best concentrations of PHA and ConA for mitotic response in the canine peripheral blood lymphocytes (PBL) were 4 µg/mL and 1 µg/mL, respectively.[4] On the contrary, Ai et al reported that after stimulation of rat lymphocytes by different concentrations of PHA, ConA, LPS and PWM, higher amount of TNF-α was measured in the presence of LPS.[7] However, selecting the best mitogen for cell proliferation using a lower dose applied in a shorter time, they found PMA/ionomycin as the optimal stimulant that could increase significantly the production of the above-mentioned cytokines within 6 hours.[7]

Our results demonstrated that at the optimum concentration of each mitogen, suitable incubation time was 72 hours. In this regard, Tajima et al demonstrated that 72 hours incubation for PHA was the optimum time.[16] On the contrary, another study showed that the optimal incubation time for PWM, PHA, and ConA was 96 hours.[15] Moreover, Hussain et al showed that 3 days incubation with PHA was essential for the maximal blastogenic responses of the whole blood cells and splenocytes.[17]

According to a previous report, PBMCs produce pro- and anti-inflammatory cytokines in the presence of conventional mitogenic compounds.[15] In this regard, to analyze the response of the PBMCs to the mitogenic agents, TNF-α was measured after 72 hours incubation in presence of different mitogens. The TNF-α is secreted mainly by the activated macrophages and T lymphocytes and is responsible for inflammation. The findings of this study agree with the previous observation which proved that the levels of TNF-α rose in response to all mitogenic factors with the exception of LPS. In addition, the maximum reactions were obtained in presence of PHA and PHA-ConA. Our finding confirmed that coexistence of PHA plus ConA have the best mitogenic effect on cells using the MTT assay.

Our study had some limitations. In this respect, we performed MTT assay with different kind of mitogens on human PBMCs; however, for more evaluations using other mitogens such as PMA/ionomycin and PWK are recommended. On the other hand, evaluation of more cytokines like IL-2, Il-4, and IFN-γ are suggested to interpret the results accurately. Authors suggest more work considering these variables and factors in their studies.

Conclusion

Our study showed that the best incubation time to induce cell proliferation is 72 hours, besides the type of mitogenic agents. In addition, PHA-ConA illustrated maximum stimulatory effect for TNF-α secretion by the PBMCs. The findings of this study can be useful for proliferation, survival, and cytotoxicity studies in researches and practical applications.

Ethical Approval

The study protocol was approved by Ethics Committee of Arak University of Medical Sciences, Arak, Iran.

Competing Interests

Authors declare that they have no competing interests.

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