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ÖZGÜN ARAŞTIRMA

RE-EVALUATION OF LPS CONCENTRATIONS ON THE 293T HUMAN RENAL CELL LINE

293T İNSAN RENAL HÜCRE DİZİSİNDE LPS KONSANTRASYONLARININ YENİDEN DEĞERLENDİRİLMESİ

[1]Aris Çakiris, [1]Atilla Cakar, [1]Neslihan Abacı, [1]Sema S. Ekmekci, [1]Zeliha Emrence, [2*]Duran Üstek

ÖZET

Amaç: *Hücrelerin LPS (Lipopolisakkarit)'ye enflamatuvaryanıtları in-vitro deneylerde sıklıkla kullanılmaktadır. Buna rağmen hücre kültürü çalışmaları için LPS 'nin doğru dozunun belirlenmesinde zorluklarla karşılaşılmaktadır. Maksimum yanıt veren LPS konsantrasyonunun belirlenmesi in vitro enflamatuvar deneyler için kritik bir noktadır. Bu çalışmanın amacı LPS'nin 293T insan renal hücre dizisindeki konsantrasyon bağımlı etkisinin yeniden değerlendirilmesidir.*

Yöntem: *Bu çalışmada LPS ile stimule edilmiş 293T hücre hattı Xcelligence Real-Time Cell Analyzer (RTCA) sisteminde incelendi. LPS 'nin artmış konsantrasyonlarının 293T hücre hattı üzerindeki etkisi RTCA cihazında hücre proliferasyonu ölçümleri yapılarak araştırıldı.*

Bulgular: *Sonuçlarımıza göre 2, 4 ve 8 µg'lık LPS konsantrasyonlarını hücre bölünmesini inhibe ederek hücreleri kararlı duruma yönlendirdiğini gösterdi. 1 µg 'lık LPS konsantrasyonu hücreleri mitotik faza sürüklerken daha düşük LPS konsantrasyonlarının ise hücre proliferasyonu üzerinde bir etki göstermedi.*

Sonuç: *Bu çalışmadaki sonuçlara göre LPS konsantrasyonlarının hücre proliferasyonu üzerinde farklı etkilerinin olduğu ve deneysel çalışmalardan önce LPS konsantrasyonlarının optimize edilmesi gerektiği gösterilmiştir.*

AnahtarKelimeler: *Lipopolisakkarit (LPS), hücreproliferasyonu, enflamasyon*

ABSTRACT

Objective: *The inflammatory responsiveness of the cells to Lipopolysaccharides (LPS) is commonly used for in vitro experiments. However, the correct dose of the LPS for cell line experiments is elusive. The LPS concentration that gives the maximal response is a critical point of in vitro inflammatory experiments. The aim of this study was to reevaluate the concentration dependent effect of LPS on the 293T human renal cell line.*

Methods:*We evaluated the cell-detachment assay of LPS-stimulated 293T cell line monitored by xCELLigence Real-Time Cell Analyzer (RTCA) system. We applied increasing concentration of LPS followed by Roche xCELLigence Instrument based on the Real-Time Cell Analysis System.*

Results:*Our results demonstrated that the 2, 4 and 8µg of LPS inhibit cell division which diverts cells to a steady-state phase, 1 µg acts as mitogen. Lower concentrations are no effect on cells.*

Conclusion:*These work showed that LPS concentrations had various effects on cell proliferation and need to be estimated for each experiment before carry out the experiments.*

Keywords:*Lipopolysaccharides(LPS), cell Proliferation, inflammation*

[1]Genetics Department, Institute for Experimental Medicine, Istanbul University, Istanbul, Turkey

[2]Department of Medical Genetics, School of Medicine, Istanbul Medipol University, Istanbul, Turkey

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SorumluYazar
Duran Üstek,
Department of Medical Genetics, School of Medicine, Istanbul Medipol University, Istanbul, Turkey

Tel:
+90 212 414 20 00 / 33316

e- posta:
acakiris@istanbul.edu.tr

Introduction

Lipopolysaccharides are located in the outer membrane of gram negative bacteria. They contains two parts; lipids which consist of fatty acids and long polysaccharides which have two sections O antigen and a core oligosaccharide[1]. LPS is an exogenous pyrogen, induces a strong response from normal animal immune systems and act as endotoxins[1]. Endotoxins are powerful mitogens to some mammalian cell types and lines. Endotoxins can also inhibit cell division and as with stimulation of mitosis, these effects depend on the dose and origin of the Endotoxin[2-4]. The inflammatory responsiveness of the cells to LPS is commonly used for *in vitro* experiments[5]. There is no consensus at the LPS concentrations for *in vitro* experiments. Concentration depend effect of LPS can miss lead the experiments, ranging from promotion of mitosis to inhibition, and even toxic at higher concentrations.

New technologies are also made real time continuously monitoring the cell growth, cell mass and cell index. This technology uses impedance technology as an indicator of cell adhesion. It produces an electronic readout of impedance to quantify cell adhesion, proliferation and viability in real time. Increased cell density leads an increasing in electrode impedance which is the indicator of cell index[6]. The aim of this study was to reevaluate the concentration dependent effect of LPS on the 293T human renal cell line using a Real-Time cell analysis system.

Methods

Tissue Culture and monitoring of cells by RTCA:

The Human embryonic kidney epithelial cell line, 293T (invitrogen), was cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal calf serum (Hyclone, Logan, UT) containing 200 mM L-glutamine, 10 mM MEM non-essential amino acids, 100 mM sodium pyruvate, 100 U/ml penicillin, 100 µg/ml streptomycin at 37 °C, 95% air and 5% CO2 in a humidified incubator. The 25.000 cells/well was seeded into 100 µL of media in 96x well microtiter plates (E-Plate, Roche, medium containing various amounts of LPS (0.25 µg/ml, 0.5 µg/ml, 1 µg/ml, 2 µg/ml, 4 µg/ml and 8 µg/ml) added over cells without distributing the cells. We performed 12 replicas per concentration. The wells starting from well A1 to A12 were set for only cells without LPS.The wells starting from well B1 to B12 were set for 8 µg/ml of LPS.

The wells starting from well C1 to C12 were set for 4 µg/ml of LPS. The wells starting from well D1 to D12 were set for 2 µg/ml of LPS. The wells starting from well E1 to E12 were set for 1 µg/ml of LPS. The wells starting from well F1 to F12 were set for 0.5 µg/ml of LPS. The wells starting from well G1 to G12 were set for 0.25 µg/ml of LPS. The wells starting from well H1 to H12 was set for only medium without cells and LPS.

Statistical Methods

All measurements are presented as the mean +/- the standard error of the mean and significance was determined using the unpaired Student's t-Test and $p<0.05$ was considered significant.

Results

For the statistical consideration, we performed 12 replicas of each well per concentration. We monitor the cell for 19 hours to see no variations between cells. 19 hours later after the cell were seeded into the 96xwell RTCA plate system paused for the LPS addition. System stopped 43 hours later for the analysis and statistics. There was no variation at the time point 19th hours between all wells except the wells without cells (H1-H12). We created 8 models for each concentration of LPS (Model A-B-C-D-E-F-G-H) as shown in Figure 1.

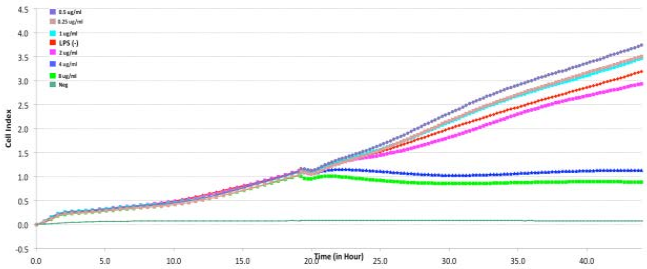

Figure 1. The Cell Index values at the end of the 43rd hour.

To create a negative control, LPS was not added to Model A. It only included 293T cells so there was no deviation. That was biologically expected condition. F Significant Value, that was looked for general significance of Model A, was lower than $\alpha = 0.05$. So, we can say that our Model A is generally significant.

For Model B, 8 µg/ml LPS was added to B wells. When looked for significance values of parameters, only time parameter's significance value was over $\alpha = 0.05$. That means, time parameter is insignificant for Model B. Other parameters' significance values were lower than $\alpha = 0.05$. So, all the parameters except time have significant ratios. There is a cytostatic condition in Model B. In other words, 293T cells don't divide, don't die and reduce their metabolic activity to basal level. They proliferate in a period and then they stop dividing. That cytostatic condition causes insignificance of time parameter. General significance value of the Model B is lower than $\alpha = 0.05$. So, Model B is generally significant.

For Model C, 4 µg/ml LPS was added to C wells. When looked for significance values of parameters, only time parameter's significance value was over $\alpha = 0.05$. That means, time parameter is insignificant for Model C. Other parameters' significance values were lower than $0.05 = \alpha$. So, all the parameters except time have significant ratios. There is a cytostatic condition in Model C. In other words, 293T cells don't divide, don't die and reduce their metabolic activity to basal level. They proliferate in a period and then they stop dividing. That cytostatic condition causes insignificance of time parameter. General significance value of the Model C is lower than $\alpha = 0.05$. So, Model C is generally significant.

For Model D, 2 µg/ml LPS was added to D wells. When looked for significance values of parameters, only time parameter's significance value was over $\alpha = 0.05$. That means, time parameter is insignificant for Model D. Other parameters' significance values were lower than $\alpha = 0.05$. So, all the parameters except time have significant ratios. There is a cytostatic condition in Model D. In other words, 293T cells don't divide, don't die and reduce their metabolic activity to basal level. They proliferate in a period and then they stop dividing. That cytostatic condition causes insignificance of time parameter. General significance value of the Model D is lower than $\alpha = 0.05$. So, Model D is generally significant.

For Model E, 1 µg/ml LPS was added to E wells. When looked for significance values of parameters, all the results were lower than $\alpha = 0.05$. In other words, either parameters or the model is significant. In Model E, 293T cells showed mitotic activity by dividing habitually. F significant value that was looked for general significance of the model is lower than $\alpha = 0.05$. Thus we can say that Model E is generally significant.

For Model F; 0,5 µg/ml LPS was added to F wells. When looked for significant values of parameters, only constant parameter's significance value was over $\alpha = 0.05$. That means, constant parameter is insignificant for Model F. Other parameters' significant values were lower than $0.05 = \alpha$. So, all the parameters except constant parameter have significant ratios. It was observed a mitotic activity in Model F that 293T cells divided habitually. . F significant value that was looked for general significance of the model is lower than $\alpha = 0.05$. Thus we can say that Model F is generally significant.

For Model G; 0.25 µg/ml LPS was added to G wells. When looked for significance values of parameters, only constant parameter's significant value was over $\alpha = 0.05$. That means, constant parameter is insignificant for Model G. Other parameters' significant values were lower than $0.05 = \alpha$. So, all the parameters except constant parameter have significant ratios. It was observed a mitotic activity in Model G that 293T cells divided habitually. F significant value that was looked for general significance of the model is lower than $\alpha = 0.05$. So, we can say that Model G is generally significant.Model H included only DMEM. All the parameters have significance values that were lower than $\alpha = 0.05$. In other words, all the parameters are significant. Also, general significance value is lower than $\alpha = 0.05$ and that means Model H is generally significant. LPS doses versus Cell Index graph and Average of

cell index graph at time point 43:58 are given Figure 2, according to averages.

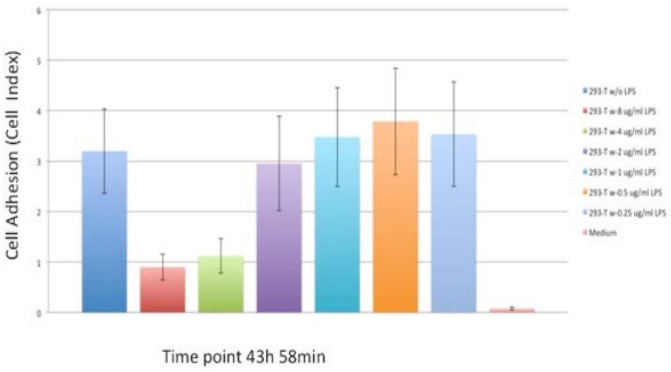

Figure 2. Average of cell index at time point 43:58

Discussion

The inflammatory responsiveness of the cells to Lipopolysaccharides (LPS) is commonly used for in vitro experiments. However, the correct dose of the LPS for cell lines experiments is elusive. The LPS concentration that gives the efficient response is critical for inflammatory experiments. The cell index was used as a measure of response to LPS doses since as doses changed there was a responsive change in the index. For this reason, we performed different LPS doses by stimulating the cells with increasing concentration of LPS followed by Roche xCELLigence Instrument based on the Real-Time Cell Analysis System[6]. According to our results, the best dose is the LPS concentration which gives maximum response to situation that researcher is looking for. There is no a fix dose for every experiment. 1 µg/ml, 0,5 µg/ml and 0.25 µg/ml doses of LPS had mitogen effect on the cells. 1 µg/ml was the optimal dose for the 25.000 293T cells. 8 µg/ml of LPS had a cytostatic effect on the cells. However, Kulahava T.A. et al. reported the mitotic effect of 100 µg/ml of LPS dose on C6 glioma cell line, which was approximately 10 fold more than our cytostatic dose[7]. In our study we can conclude that over dose(4 µg/ml, 8 µg/ml) could be toxic for the cells.In another study that analysedthe effects of LPS on mononuclear phagocytesit has been mentioned both the letal (cytotoxic effects) and the stimulatory effects of LPS[8].In our study we also observed a mitotic activitywhen 0.25 µg/ml, 0.5 µg/mland 1 µg/ml LPS was added to the cells. In conclusion, conventional LPS doses reevaluated with the opportunity of a new technology that based on a Real-Time cell analysis system which gave us the mitotic and cytotoxic doses.

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