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Optimization of cell density and LPS concentration for the evaluation of nitric oxide production on BV-2 cells in a Griess assay

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ABSTRACT

Introduction: Production of nitric oxide (NO) is one of the main responses elicited by a variety of immune cells such as macrophages (e.g. microglia, resident macrophages of brain), during inflammation. Evaluation of NO levels in the inflammatory milieu is considered important to the understanding of the intensity of an immune response; and has been performed using different methods including the Griess assay. To assay NO in culture, an appropriate number of cells are stimulated into an inflammatory phenotype. Common stimuli include lipopolysaccharide (LPS), IFN-γ and TNF-α. However, overt stimulation could cause cell cytotoxicity therefore an ideal concentration of LPS should be used. **Objective:** To set-up a model of BV-2 cell activation that allows the assay of detectable levels of NO. Optimization of BV-2 microglia cell density and LPS concentrations after stimulation by bacterial lipopolysaccharide (LPS) for the Griess assay is demonstrated in this study. **Methods:** BV-2 microglia were cultured at different cell densities, and treated with LPS at three concentrations (1, 5, 10 µg/ml). NO production in culture supernatants were then measured at 18, 24, 48 and 72 hours. Moreover, methyl tetrazolium assay (MTT) was also performed to ensure that NO measurement is performed at no-cytotoxic concentrations of LPS. **Results and Conclusions:** NO production follows a temporal pattern. The density of 25000 cells/well was the ideal seeding density for NO evaluation in BV-2 cells. BV-2 stimulation by LPS is dose dependent, and NO levels are increased proportional to the LPS concentration up to 1.0µg/ml, whereas the higher LPS concentrations are associated with decreased cell viability may be caused by the high toxic levels of LPS or NO. Although Griess assay has been commonly used by the scientists, however, optimization of its parameters on BV-2 cells will be useful for the experiments which will be performed on this particular cell line. The optimized pattern of Griess assay on BV-2 cells was achieved in this study, hence easier and more practical for the future scientists to perform Griess assay on BV-2 cells.

Key Words : Griess assay, Nitric oxide, BV-2 cells, LPS concentration, Cell density

INTRODUCTION

Monocytes which are one of the key players in an inflammation process are crucial in host defense in mammalian innate immune response.[1] Recent evidence shows that they play a role in tissue homeostasis as well as in pathogen defense and repair of tissues.[2] During development, they are released into the bloodstream, and enter tissues to take specialized role. They have been given specific names based on the organ they reside such as Kupffer cells (liver macrophages), mast cells (tissue macrophages), and microglia (brain macrophages).[3] Different from peripheral tissues, existence of the blood brain barrier prevents the entry of other immune cells into the intact central nervous system (CNS) and thus well-being of the CNS is solely monitored by microglia. Microglia elicit responses to trauma or an infection[4,5] through secretion of various inflammatory mediators such as growth factors (GM-CSF and TGF-β), cytokines (IL-4, IL-2, IL-10, TNF-α, IL-3 and IFN-γ) and prostaglandins (PGE2),[3] and soluble factors such as nitric oxide (NO).[6] Microglia are capable of phagocyting pathogens and presenting antigens to CD4+ cells.

Both Gram positive and Gram negative bacteria may cause serious bacterial infection in the CNS like in meningitis.[7,8] Common bacteria which can cause bacterial meningitis resulting in microglial stimulation are *Haemophilus influenzae* type b, *Neisseria meningitidis, Listeria monocytogenes, Streptococcus pneumoniae* and *Streptococcus agalactiae,*[9] and therefore it is of interest to study microglial responses to pathogenic organisms.

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NO secretion is one of the major mechanisms of bacterial killing by macrophages, microglia cells and other immune cells.[10] Apart from the crucial antimicrobial activity, NO has been shown to possess anti-tumor activity and tissue damaging effect. NO is a radical gas which is water soluble,[11] and is derived from the amino acid L-arginine by inducible nitric oxide synthase (iNOS or NOS2).[10] Beside NO production, other researchers also looked at other inflammatory cytokines which can be induced by LPS (10 ng/ml), such as TNF, IL-1β, expression of IL-10 receptor; IL-6, IL-12, and IL-10.[10]

Measuring NO is rather difficult due to its short half-life and reactivity with free oxygen resulting in the generation of nitrite (NO_2-). However, this end product (NO_2-) is relatively stable and can be measured using various assays[12] such as, Griess assay. Different parameters can affect the Griess assay such as cell type, seeding density, stimulant type, stimulant concentration, and time points. Here in the present article, we narrate the variations in NO level that could arise upon changing the experimental parameters such as time point, seeding density and LPS concentration using Griess assay. On the other hand, in order to authenticate the experiment, it was required to test the cytotoxicity of the various experimental conditions tested here to avoid any misinterpretation of data. Hence, cell viability was measured by a colorimetric assay called methyl tetrazolium assay (MTT assay).

METHODS

Cell Culture

Transformed microglia cells (BV-2 cells) which were immortalized by genetic alternations (Horvath, Nutile-McMenemy, Alkaitis, & DeLeo, 2008), were cultured in Dulbecco's Modified Eagle Medium- high Glucose, with 5% Fetal Bovine Serum at 37°C with 5 % CO_2 in a humidified incubator. The cells were rinsed with 1X phosphate buffered saline (PBS) twice and harvested with 0.25 % Trypsin-EDTA on reaching 80-90 % confluency. Cells with more than 95 % viability (measured by trypan blue staining) were used for all downstream experiments. The passage number of cells used in experiments was restricted between 19 to 24 in order to minimize any difference in NO production across different passage numbers and the parameters of the Griess assay (cell seeding density in 96-well plate, LPS concentration, and time points) were as followed: 25000 cells/well, stimulated with 1 µg/ml of LPS and NO was measured at 18, 24, 48 and 72 hours in order to evaluate the temporal pattern of NO production, 5000 cells/well, 10000 cells/well and 25000 cells/well, stimulated with 1.0 µg/ml LPS and NO was measured at 24, 48 and 72 hours in order to optimize cell seeding density, and 25000 cells/well, stimulated with 1.0, 5.0 and 10 µg/ml of LPS concentrations and NO was measured at 24, 48 and 72 hours in order to optimize LPS concentration, by Griess assay.

Cell Treatment

In order to optimize the seeding density of BV-2 cells to be used for Griess assay, BV-2 cells were seeded at 5000 cells/well, 10000 cells/well and 25000 cells/well in a 96-well plate and allowed to attach overnight. Media were replaced with phenol red free media and the cells were allowed to acclimatize for 4 hours before treatment. The cells were then treated with bacterial lipopolysaccharide (LPS; *E. coli* serotype 026:B6; Sigma Cat. No. L2762) with the concentration of 1.0 µg/ml, and culture supernatant were analyzed for NO levels using Griess assay.

BV-2 cells were seeded at a density of 25000 cells/well of a 96-well plate and stimulated with 1 µg/ml of LPS and analyzed at different time points (18, 24, 48 and 72 hours), to perform the temporal pattern of NO production. Different LPS concentrations (1.0, 5.0 and 10.0 µg/ml) were tested to identify the optimal concentration of the stimulant, through Griess assay and MTT assay as well.

Griess Assay

Griess reagent was prepared by dissolving 1 % sulfanilamide (Sigma, Batch No. 126K0001) and 0.1 % N-1-naphthyl-ethylenediamine hydrochloride (Sigma, Cat. No. 222488) in 3 % phosphoric acid freshly at each time point. Fifty microliter of culture supernatant was transferred to a fresh 96-well plate at respective time points and an equal volume of freshly prepared Griess reagent was added. Plates were incubated in the dark for 10 minutes and absorbance was read at 530 nanometers by Dynex MRX II microplate reader at 18, 24, 48 and 72 hours. Nitrite concentration was calculated with reference to a standard curve of freshly prepared sodium nitrite (0–100 µM).

MTT assay

In order to choose suitable LPS dose, MTT assay was done on LPS treated and resting BV-2 cells parallel to Griess assay, at 48 and 72 hours as well. In order to perform viability test, cells were seeded in 96-well plates, and incubated overnight to be attached. Cells were treated with LPS with concentrations of 0.5, 1.0, 5.0 and 10 µg/ml. MTT reagent was prepared by dissolving 5 mg/ml of MTT powder in distilled water (Amresco, LOT No. 1247B021). MTT reagent was added to the attaching cells at 48 and 72 hours and the cells were incubated for 3 hours at 37°C with 5 % CO_2 in a humidified incubator. Afterwards, the culture supernatant was removed and DMSO was added to the wells and plates were incubated in the dark for 10 minutes and absorbance was read at 490 nanometers by Dynex MRX II microplate reader. Viability percentage was calculated with reference to a comparison of treated cells to untreated cells as control.

RESULTS

Temporal Pattern of NO Production

Figure 1 illustrates that unstimulated BV-2 cells produced NO with minimal levels and the levels remaining lower than 20µM at all time points tested. LPS stimulation induced NO production at 18 hours 48 and 72 hours. NO levels were increased significantly to 95.76 ± 23.44µM by 48 hours and126.93 ±9.69 µM by 72 hours. Therefore, further experiments were conducted at 48 and 72 hours. Viability of the cell at these time points were 85.04 ± 0.42 and 71.83 ± 0.25 respectively (Table 1).

Table 1. Determination of ideal LPS concentration by measuring cell viability through MTT assay on BV-2 cells. LPS induction had the lowest toxic effect on the cells at the concentration of 1 µg/ml in BV-2 cells.

	Time	
	48 hours	**72 hours**
	Viability (% ± SD)	
BV-2 (25000 cells/well)	100 ± 0.21	100 ± 0.77
BV-2 + 1.0 µg/ml LPS	85.04 ± 0.42	71.83 ± 0.25
BV-2 + 5.0 µg/ml LPS	89.76 ± 0.38	64.86 ± 0.12
BV-2 + 10.0 µg/ml LPS	75.35 ± 0.34	67.54 ± 0.41

BV-2 cells at the cell density of 25000 cells/well were treated with different LPS doses (1.0, 5.0 and 10 µg/ml) and evaluated for viability at 48 and 72 hours via MTT assay. Cell viability showed the highest percentage at the concentration of 1 µg/ml, while the viability started to decrease to below 70 % at the LPS concentrations of 5.0 and 10 µg/ml. Values are shown in mean ± SD from triplicate readings.

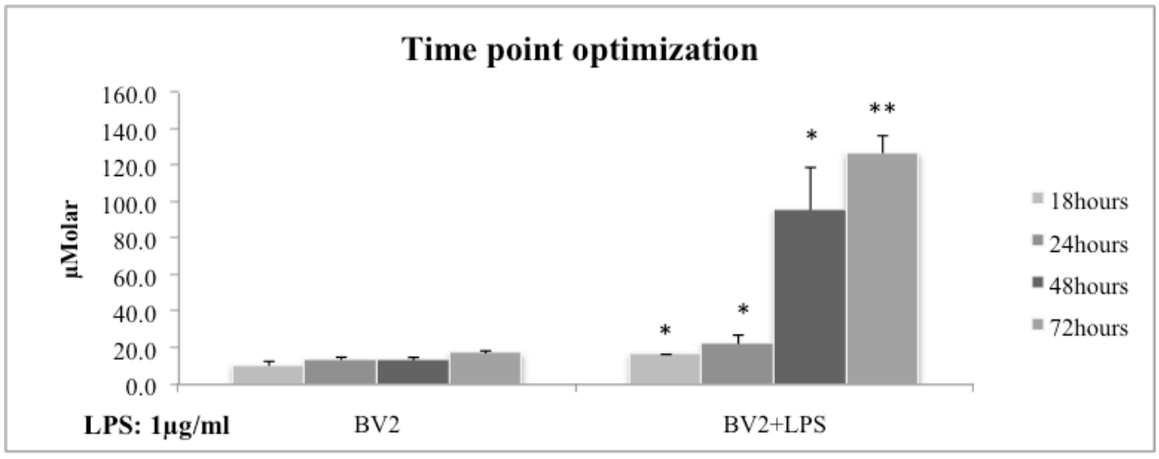


Figure 1.Time point analysis for production of nitric oxide (NO) by BV-2 cells: NO levels increased significantly at 48 and 72 hours, following LPS stimulation. BV-2 cells were seeded in 96-well plate at the density of 25000 cells/well and allowed to adhere overnight. Cells were stimulated with 1 µg/ml of LPS and NO levels were measured at time points mentioned in the graph by Griess assay. *p<0.05 compared to untreated BV-2 cells at the corresponding time points. **p<0.01 compared to untreated BV-2 cells at the corresponding time points. Values are shown in mean ± SD from triplicate readings.

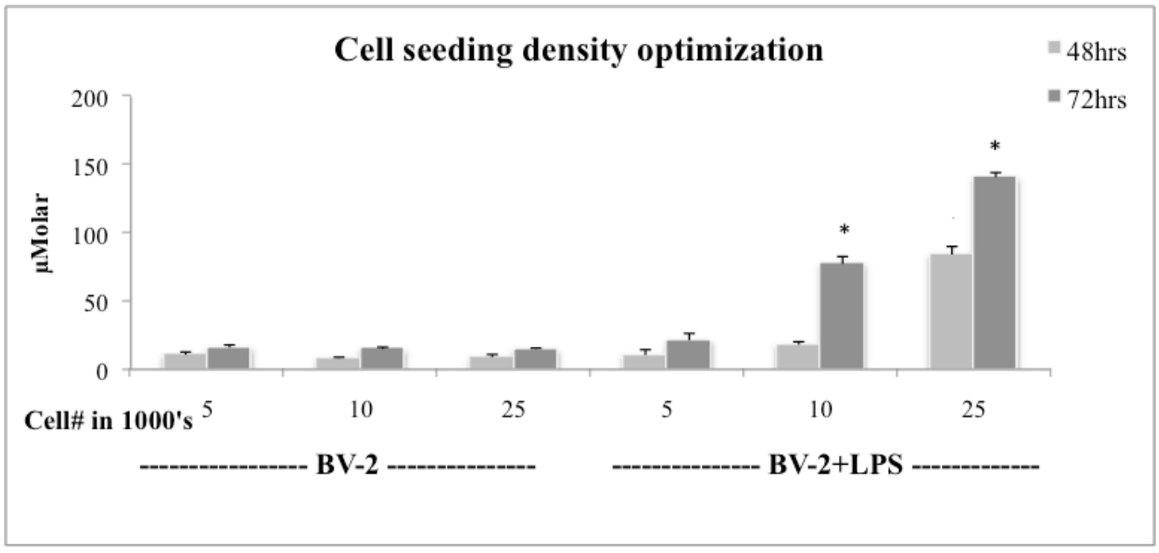
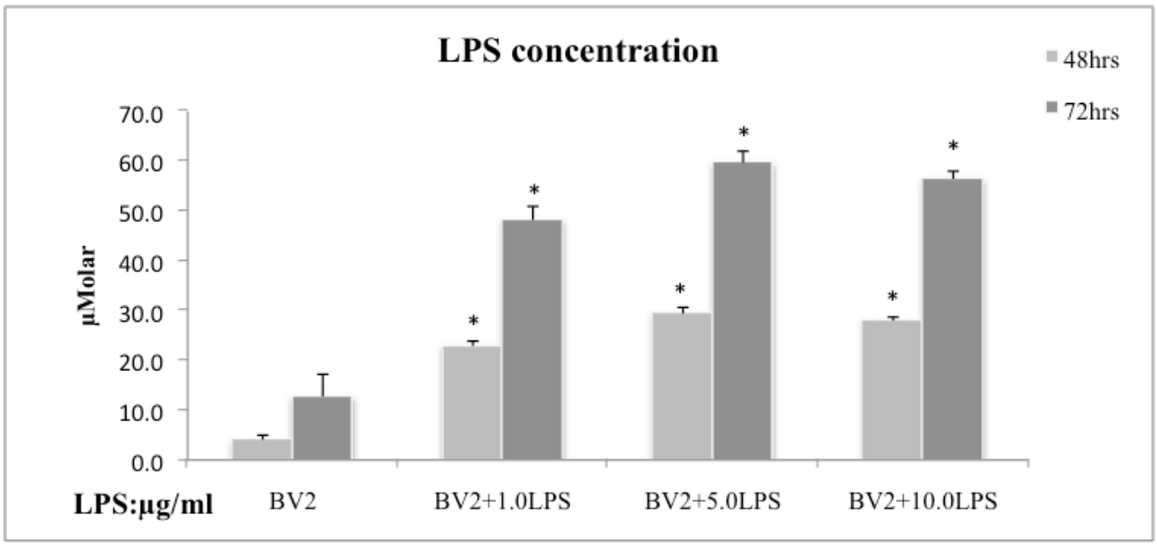


Figure 2. Determination of suitable BV-2 cell seeding density for assay of nitric oxide (NO): BV-2 cells produce the highest NO at the cell density of 25000 cells/well. BV-2 cells were seeded in 96-well plate at the different cell densities and allowed to adhere overnight. Cells were stimulated with 1 µg/ml of LPS and NO levels were evaluated at time points mentioned in the graph by Griess assay. *p<0.01 compared to untreated BV-2 cells at the corresponding time points. Values are shown in mean ± SD from triplicate readings.

Figure 3.Determination of ideal LPS concentration for assay of nitric oxide (NO) on BV-2 cells: LPS at the concentration of 1 µg/ml had the highest stimulation of NO production in BV-2 cells. BV-2 cells were seeded in 96-well plate at the density of 25000 cells/well and allowed to adhere overnight. Cells were stimulated with different LPS concentrations (1.0, 5.0 and 10 µg/ml), and NO levels were evaluated at time points mentioned in the graph by Griess assay. *p<0.01 compared to untreated BV-2 cells at the corresponding time points. Values are shown in mean ± SD from triplicate readings.



Optimization of BV-2 Seeding Density

We then sought to determine whether seeding density of BV-2 cells can influence this model of NO induction. For this, BV-2 cells were seeded at three different seeding densities of 5000 cells/well, 10000 cells/well and 25000 cells/well. NO levels were increased by 7 fold to 140.9 ± 3.47 µM in 25000 cells/well, and by 3fold to 77.56 ± 5.24 µM in 10000 cells/well seeding densities by 72 hours. The cell density of 25000 cells/well was chosen for downstream experiments as NO was induced as early as 48 hours and increased to the highest level at 72 hours.

Optimization of LPS Concentration

Measuring Nitric Oxide (NO) Production by LPS Treated cells in order to select the suitable LPS concentration

As 25000 cells at 1µg/ml LPS induces sufficient amounts of NO at 48 and 72 hours, we next wanted to determine whether increasing LPS concentrations induces further NO induction. Therefore, effect of LPS doses 1, 5 and 10 µg/ml on 25000 BV-2 cells/well were examined at 48 and 72 hours. At 72 hours, NO levels increased to 48.22 ± 2.69 µM with 1.0 µg/ml of LPS concentration, 59.67 ± 2.33 µM with 5.0 µg/ml of LPS concentration and 56.4 ± 1.54 µM with 10.0 µg/ml of LPS concentrations, which showed that NO production by BV-2 cells was LPS dose dependent. However, increasing doses of LPS affected cell viability, particularly at 72 hours. Cell viability dropped to 64.86 ± 0.12 for 5 µg/ml LPS and 67.54 ± 0.41 for 10 µg/ml LPS.

DISCUSSION

Lipopolysaccharides have been used as a stimuli in *in vitro* experiments to stimulate microglia into an inflammatory phenotype (Tran, McCoy, Sporn, & Tansey, 2008). LPS is a microbial-derived stimulant as it is a component of gram negative bacteria cell wall. Utilising LPS rather than inflammatory stimuli such as TNF-α or IFN-γ simulates bacteria-induced inflammation. As a response to inflammation, microglia secrete various mediators including nitric oxide (NO). NO is assayable with the Griess assay and can reveal the extent of an inflammatory response. However parameters such as cell number, time points and stimulant doses require optimization. This is because insufficient cells, stimulant, or time to assay may result in minimal NO produced. Conversely, stimulated doses that are too high may decrease cell viability. Therefore, this study describes the ideal parameters to allow NO in BV-2 microglia cultures to be assayed.

It was shown by this study that LPS has dose-dependent effect on NO production by BV-2 cells, which followed the previous research (Amiraslani, Sabouni, Abbasi, Nazem, & Sabet, 2012; Keller, Gehri, & Keist, 1992; Lee *et al.,* 2001; Roy, Fung, Liu, & Pahan, 2006). LPS concentration optimized by Roy *et al.* which showed that the concentration of 0.75 µg/mL induced the highest NO production on BV-2 cells at 24 hours (Roy, *et al.,* 2006), therefore, in this study, higher LPS concentrations were examined in order to find higher concentration which induce NO production.

It was shown in previous research that NO production was initiated after 12 hours of stimulation (Lee, *et al.,* 2001). Furthermore, it was shown by a study that NO production initiated after 18 hours (Ring, Braun, Nizet, Stremmel, & Shenep, 2000). Results of time point analysis in the present study showed that NO values produced by BV-2 cells increased with time duration, particularly by 48 hours and 72 hours. Hence 48 and 72 hours were chosen as the suitable time points due to NO levels which were negligible at earlier time points even after LPS stimulation.

Results of cell density optimization demonstrated that NO production by BV-2 cells was cell density dependent. Considerable increase in NO production occurred at the cell density of 25000 cells/well in LPS treated cells, unlike resting BV-2 cells which did not show substantial NO production in none of cell seeding densities. However, resting BV-2 cells showed an insignificant NO production values between time points in resting BV-2 cells. Therefore, the results indicated that the best cell density for NO production by BV-2 cells is 25000 cells/well.

It has been shown by studies that cell density has a positive time-dependent effect on the color which is developed through the assay, as the viable cells have the mitochondrial enzyme of formazon production(Gerlier & Thomasset, 1986). Hence, we have performed MTT assay to authenticate the cells which are being used are alive.

Results of optimization in LPS concentration indicated that NO production by BV-2 cells is LPS dose dependent. However, there was a decrease in viability in the LPS concentration of 5 µg/ml and 10µg/ml, at72 hours, after performing MTT assays. LPS treated cells did not produce a remarkable concentration of NO at 24 hours across all of the LPS concentrations, and the values remained approximately equal. Therefore, the results demonstrated that NO production by BV-2 cells is LPS dose dependent up to 1µg/ml, while the cells remained more than 70% viable.

CONCLUSION

Production of NO by both CNS and peripheral macrophages are widely studied. The findings here demonstrate the importance of monitoring the experimental variables very closely. Within this study, BV-2 microglial production of NO varies temporally based on the seeding density and concentration of the stimulant (LPS). NO levels showed a linear relationship with time, seeding density and LPS concentration. However, viability of BV-2 cells decreased as the LPS concentration was increased. Thus, the study narrates the importance of optimization of variable parameters for individual cell types in similar assays.

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