DonnishJournals

Donnish Journal of Biomedical Research
Vol 4(1) pp. 001-005 November, 2017.
http://www.donnishjournals.org/djbr
ISSN: 2984-8954

Original Research Article

Lipopolysaccharide-induced Alterations of Immune-related Genes in Goat

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Accepted 15th November, 2017.

Background: ***Blood samples (8-10 ml) collected in heparinized sterile screw-capped tube from the jugular vein of twenty Egyptian goats were used in this study to be treated with Lipopolysaccharide (LPS) which is an endotoxin used as a model of inflammation. LPS offers an attractive model for inducing inflammation due to its ability to provoke secretion of tumor necrotic factor TNF-a and multiple cytokines (e.g. IL-1β, IL-6, and IL-10) so LPS is an important model used as an immune stimulant. The importance of endotoxin is based on a variety of biological responses which this molecule provokes both in vitro and in vivo. The biologically active moiety of endotoxin, the lipopolysaccharide (LPS) has been identified and biochemically characterized for a variety of enteric organisms. Further, the toxic principle of LPS lipid A has been defined and synthesized. Genes in LPS-stimulated monocytes is evaluated by quantitative real-time PCR using beta-actin for normalization.***

Results: ***Four genes (TNF-a, IL1-B, IL6 and IL10) were quantified by RT-qPCR in LPS-stimulated goat monocytes in vitro and viability of monocyte cells treated with LPS were evaluated using MTT assay resulted in decreased cell viability with increasing LPS concentrations in contrast to control group while there was an increase in gene expression of IL1-B, IL-6 and TNF-a but no change for IL10.***

Conclusions: ***LPS incubated with monocyte with different concentration resulted in decreased cell viability and increase gene expression.***

Keywords: Toxin, Inflammation, Goats, Lipopolysaccharide.

INTRODUCTION

Cells of the mononuclear phagocyte system play main roles in the pathophysiological progressions of inflammation and infection (Auffray et al., 2009), (Serbina et al., 2008). LPS act as a component of cell membrane of gram-negative bacteria and play as a strong stimulator of immune responses of the mononuclear phagocyte system (Brandtzaeg et al., 2001). Stimulation of monocytes by LPS is often engaged as a model system to study inflammatory responses (Toltl et al., 2008). One methodology to examine cellular processes is by gene expression studies.

Due to its high sensitivity, specificity, dynamic range and straightforwardness, quantitative reverse-transcription polymerase chain reaction (RTqPCR) has become one of the greatest methods used to detect gene expression. For comparison between gene expressions levels in definite conditions of a cell or organ, gene expression quantities need to be normalized to a standard. Several approaches have been proposed to achieve adequate normalization, but expression levels of internal reference genes, habitually called housekeeping genes, are mainly used (Huggett et al., 2005).

Lipopolysaccharide is the main component of the outer cell membrane compound of gram-negative bacteria. Its relevant role in bacteria encouraged diseases and various uses in altered types of cell stimulation provide a theoretical basis for studies concentrated on the isolation and purification of LPS. The problem with LPS purification protocols is the contamination of the end product by proteins and nucleic acids in different proportions which could possibly interfere with the applications.

In this study we have a simple technique for purification of LPS from Escherichia coli microorganism as a gram-negative bacteria with high purity and very low contamination with DNA, RNA and proteins depends on the hot phenol-water extraction protocol (Westphal and Jann 1965).

MATERIALS AND METHODS

Bacterial strains and growth conditions

E.coli O157:H7 (Animal Research Reproduction Institute, Giza, Egypt) were grown in Modified tryptic soy broth medium at 37°C in shaker incubator overnight. The culture broth media was centrifuged and the pellet of bacteria was gathered for extraction of purified LPS.

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LPS extraction and purification

LPS was extracted by hot phenol-water method (Westphal and Jann 1965). In brief, 3-4 g of washed bacterial pellet was re-suspended in 20 mL water and preheated in the oil-water bath with continuous stirring for 10 min. Protein and nucleic acids contamination eliminated by adding proteinase K, DNase and RNase was performed post or prior to the extraction step. To achieve this process, proteinase K (100 *µg/mL*) was added to the cell mixture and the tubes were kept at 65°*C* for an additional hour. The mixture was consequently mixed with RNase (40 *µg/mL*) and DNase (20 *µg/mL*) in the presence of 1 *µL/mL* 20% MgSO4 and 4 *µL/mL* chloroform and incubation was continued at 37°*C* overnight. The same volume of heated (65-70 °C) 90% phenol was added slowly to the mixture while stirring the cell suspension followed by vigorously shaking for 30 min.

The suspension was cooled directly on ice bag as possible. Then centrifuged at 3500 rpm at 10°C for 45 minutes which result in three separate and clear distinct layers. The first upper layer was transmitted to another 50 mL polypropylene Falcon tube. This layer is the phenol saturated water layer, smooth and semi-rough LPS extracts are in this layer. Water was preheated at 68 °C to extract the residual phenol phase (bottom layer) and interphase (insoluble cellular components) with additions of 20 mL of the preheated water at 68 °C for 30min. The cellular extract was cooled and centrifuged as mentioned before. The upper layer was pooled from both extraction and dialyzed against 4L of water using 12,000 MWCO dialysis tubing. The final product, purified LPS was lyophilized by dry freeze lyophilize and stored at 4°*C*.

Commassie blue and ethidium bromide stainings

The purified LPS was dissolved in sample buffer to the wanted concentration (1 *mg/mL*), and boiled for 5 *min*. 16 *µL/well* was added on 15% SDS gel with a 4% stacking gel under reducing condition at 100 *mA* for 2 *hr* using mini-PROTEAN electrophoresis instrument (Bio-Rad Laboratories, California, USA). Coomassie blue staining of the gels (Figure 1) was followed according to the standard protocol (Anderson et al., 1991). Staining of agarose gel with ethidium bromide was done as well in order to show if any contamination with nucleic acids persists(Figure 2). To do this, 10 *µL/well* of reconstituted LPS from E-coli and also 10 *µL/well* of Molecular weight marker, were loaded on agarose gel then stained with ethidium bromide (Boffey et al., 1984).

Isolation of monocytes and cell culture

Isolation of PBMC from heparinized blood samples obtained from Egyptian goats by centrifugation through Ficoll-Hypaque (Lymphocyte Separation Medium). Cells were washed two times and resuspended in complete RPMI 1640 medium (RPMI 1640 with 10% heat-inactivated FBS). For purification of monocytes, the PBMC were resuspended at 2×10^6 and 10 ml of the cell suspension was added per 75-cm^2 flask and incubated in 5% CO_2 incubator for 48 hr with replacement of RPMI-1640 media + 10% FBS every 12 hour to maintain the viability of cells and increase its numbers, The medium was aspirated which contains non-adherent cells, the adherent cell layer was washed twice each time with 10 ml RPMI-1640 (to remove any residual non-adherent cells) and replaced with 10 ml fresh RPMI-1640 with 2 mM L-glutamine and 50 µg/ml gentamicin.

Trypsin was added to dissociate adherent monocyte cell layer and incubated for 2 minutes then 10 ml RPMI-1640 + 10% fetal bovine serum were added. Cells were transferred to a 15-ml conical tube and centrifuged 10 min at 300 × g, room temperature, to remove the media. Then, the pellet was suspended in 1ml RPMI-1640 and assessed for cell number and viability by staining with trypan blue. Cell purity was assessed by flow cytometry (Scand et al., 1968).

MTT Assays

For measurement cell viability, the colorimetric MTT metabolic assay was used. Suspended cells were diluted to 1.0 ml with RPMI 1640 media. Cells were counted per ml: $5x10^3$ cells/mL prepared in RPMI 1640 medium. In 96-well plates, cells were added in triplicate in each well and incubated in 5% CO_2 at 37 ºC overnight. Cells treated with medium only served as a negative control group and cells treated with LPS different concentrations (LPS25, LPS50 and LPS100 ug/ml). After eliminating the supernatant of each well, 20 µl of MTT solution (5 mg/ml-PBS) and 100 µl of medium were then added. incubation for another 4 h, the resultant formazan crystals were dissolved in MTT solvent (150 µl) and the absorbance intensity measured by a microplate reader (Bio-RAD 680, USA) at 490 nm with a reference wavelength of 620 nm. The relative cell viability (%) was expressed as a percentage relative to the untreated control cells.

RNA extraction and real-time quantitative PCR (RT-qPCR)

RT-qPCR was implemented for detection the relative copy numbers of different mRNA. Cell suspensions were incubated with different concentration of LPS (LPS 25, LPS 50 and LPS 100 ug/ml) and control group for 3 hours and total RNA was extracted with TRIZOL reagent accordance to the manufacturer's protocol.The concentration and purity of isolated tRNA were measured using a spectrophotometer. For cDNA synthesis, 1 µg of total RNA was prepared by reverse transcriptional reaction with oligo (dT) for all mRNAs. After reverse transcription and cDNA purification, RT-qPCR was run with gene-specific primer pairs for amplifying the target segment of cDNA (table 1).

RESULT

Cell Viability

Result of cell viability is reported normalized to control group as presented in figure 3. Exposure of monocyte cells to LPS revealed a significant difference ($p<0.01$) between the treated groups with different dose of LPS (G2, G3, G4) and control group (G1) which treated with BPS for 24 hour, cells viability in G2, G3 and G4 showed a significant decrease as compared with G1. On the other hand, the statistical analysis comparison between the three LPS treated groups showed that the cell viability recorded significant change ($p<0.01$) throughout the experiment.

Gene Expression

Result of cytokines gene expression levels quantification is presented in figure 4:

- Result of IL-1 gene expression levels were increased significantly in G2, G3 and G4 as compared with G1, on the other aspect in G3 and G4 IL-1 gene expression levels were increased significantly as compared to G2.
- Result of IL-6 gene expression levels showed no significant difference between G2 and G1 but there was a significant increase in IL-6 gene expression levels in G3 and G4 as compared to G1.
- Results of IL-10 gene expression levels showed no change in G2, G3 and G4 as compared with G1.

Result of TNF-a gene expression levels were increased significantly in G2, G3 and G4 as compared with G1, on the other hand, TNF-a gene expression levels showed increase upon LPS concentration increase.

DISCUSSION

In this study, we have chosen lipopolysaccharide (LPS) as a form of infection-induced change which have main impact of immune stress on reproductive system activity (Reitschel et al., 1994). LPS is the constituent of the outer membrane in the cell wall of gram-negative bacteria, and is known to have a broad spectrum of biochemical and immunochemical activities (Jirillo et al., 1984)**.** LPS is a potent activator of immune system capable of triggering cytokine release from cells of different origin so it offers an attractive model for inducing inflammation which leads to secretion of multiple cytokines (e.g. IL-1β, IL-6, and IL-10) (Rand et al., 1997).

Firstly, we have extracted LPS from gram-negative bacteria (E.coli O175 strain) by hot phenol-water method introduced by Westphal. Purified LPS were characterized by SDS-PAGE electrophoresis followed by commassie blue staining. Secondly, we have investigated the effect of LPS on gene expression and cells viability on monocyte cells isolated from peripheral blood of goat.

Table 1: The list of primers for amplification of RT-qPCR of goat

Gene	Forward primer	Reverse primer
TNF-α	CAACAGGCCTCTGGTTCAGAC	GGACCTGCGAGTAGATGAGG
IL-10	GTGATGCCACAGGCTGAGAAC	GAAGATGTCAAACTCACTCATGG
IL-1 B	CCGTGATGATGACCTGAGGAG	CAAGACAGGTATAGATTCTTGTC
IL-6	CGAAGCTCTCATTAAGCACATC	CCAGGTATATCTGATACTCCAG
B-actin	GGCATTGTCATGGACTC	CCGTGGTGGTGAAGCT

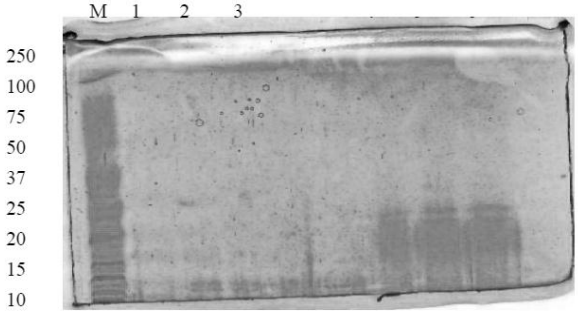

Figure 1: Coomassie blue stainings of LPS.

There's no band in commassie blue staining as shown in Lane 1, 2 &3. This indicates no contamination of purified LPS with proteins,
Lane M: Molecular weight marker.

Figure 2: Ethidium bromide stainings of purified LPS.

The absence of a band in LPS extracted from E.coli in ethidium bromide staining. Lane 1 and 3 shows no contamination with nucleic acids in purified LPS products.
Lane 2: DNA ladder.

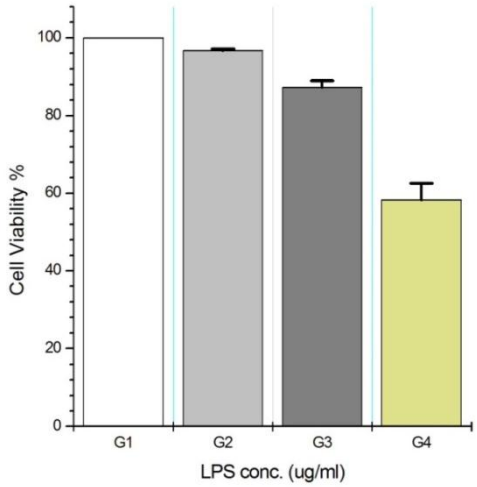

Figure 3: Effect of LPS on monocyte cells viability in vitro.

Group 1 (Control) Monocyte cells were treated with PBS as a control group for 24 hour.
Group 2 (LPS**25**) Monocyte cells were treated with lipopolysaccharide at a dose of 25 µg/ml for 24 hour.
Group 3 (LPS**50**) Monocyte cells were treated with lipopolysaccharide at a dose of 50 µg/ml for 24 hour.
Group 4 (LPS**100**) Monocyte cells were treated with lipopolysaccharide at a dose of 100 µg/ml for 24 hour.
Different significant ($p < 0.01$) between groups.

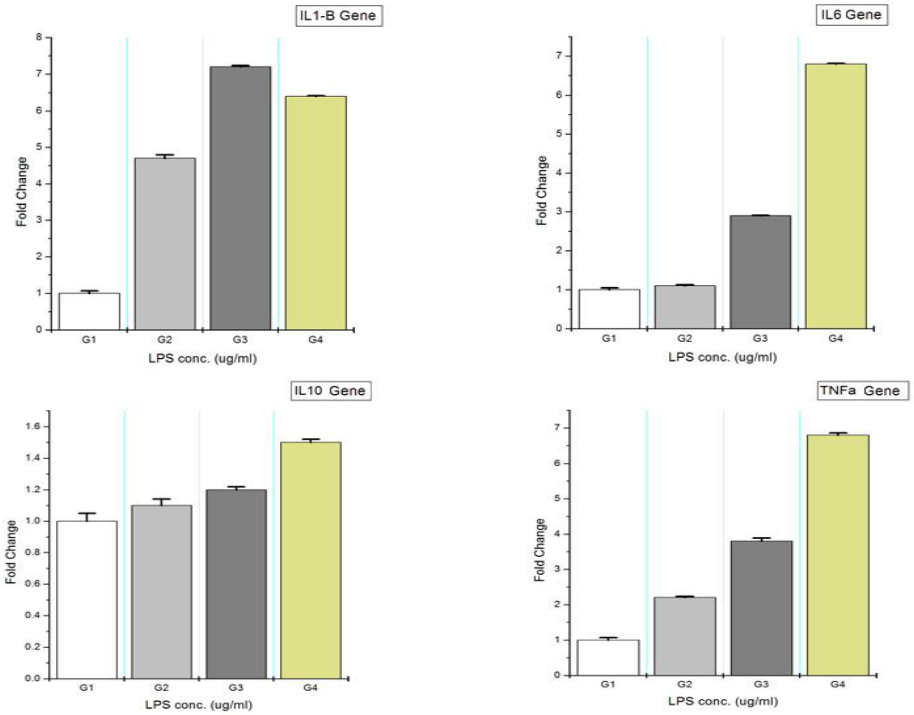

Figure 4: Effect of LPS on gene expression in vitro.

Group 1 (Control) Monocyte cells were treated with PBS as a control group for 3 hours.
Group 2 (LPS**25**) Monocyte cells were treated with lipopolysaccharide at a dose of 25 µg/ml for 3 hours.
Group 3 (LPS**50**) Monocyte cells were treated with lipopolysaccharide at a dose of 50 µg/ml for 3 hours.
Group 4 (LPS**100**) Monocyte cells were treated with lipopolysaccharide at a dose of 100 µg/ml for 3 hours.
Significant difference ($p < 0.01$) between groups except IL-10 showed no significance.

CONCLUSION

Our results indicate that monocytes viability is most significantly affected by LPS. Exposure of monocytes to LPS resulted in significant decrease in cell viability one-day post exposure.

The present findings were in agreement with that of (Lund et al., 2000) who found that the decrease in percentage of viable monocytes was more evident when LPS was added. Also, we have shown that at high concentration (50ug/ml and 100ug/ml) of LPS, the viability of cells was decreased. This result is in accordance with that of (Yildirim et al., 2016), who evaluated LPS effect on esophageal cancer cells viability. The results of the MTT assay showed that LPS, at the higher concentrations of 20, 50 and 100 µg/mL, decreased significantly OE19 cell viability when compared to control after 24 h.

Cytokines IL-1β, IL-6 and TNF-α gene expression levels were significantly increased due to LPS exposure after three hours but IL-10 showed no change, therefore, the present result is in accordance with that of (Waal Malefyt et al., 1991), who found that in response to LPS, monocytes produce large quantities of proinflammatory cytokines, including tumor necrosis factor alpha (TNF-a), interleukin-1 (IL-1), and IL-6, followed by the production of IL-10, which has anti-inflammatory properties. IL-10 was identified in supernatants collected at 7.5 h, but maximal production was observed 20-48 h after activation. In dissimilarity with TNF-a and IL-6 were detected rapidly upon activated and be at maximal levels of production at 3.5 and 7.5 h following activation respectively.

ACKNOWLEDGEMENTS

This work was supported by Cairo University, Animal Reproduction Research Institute and National Organization for Research and Control of Biological product, Giza, Egypt.

CONFLICT OF INTEREST

None of the authors has any conflict of interests that would have influenced the content of the paper or interfered with their objective assessment of the manuscript.

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