Evaluation of effects of *Salmonella enterica* endotoxin on human fibroblast proliferation by XTT assay

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Abstract

Background: Bacterial lipopolysaccharide (LPS) is the major component of outer leaflet of cell wall of most gram negative bacteria that has been surge of interest in immunological and inflammatory studies. The proliferation of fibroblasts is one of the inevitable events in wound healing process. Skin is the first barrier in immune response which is more susceptible to inflammation. The aim of this survey was the evaluation of effects of *Salmonella enterica* LPS on skin fibroblasts proliferation.

Material and methods: Fibroblast cells were plated at 3×10^5 cells/well in a 96 well culture plate. Cells treatment was performed in two groups. The first group treatment was immediately and in the second group After an overnight culture period in standard conditions were treated with different concentrations of *Salmonella enterica* LPS (1, 3.16, 10, 31.6, 100µg and 3.16, 10, 31.6, 100, 316ng) for 24, 48 and 72 hours. Effects of LPS on the activation of mitochondrial dehydrogenase were examined by XTT assay which were more save, accurate, easy to use than MTT assay. Cell viability was determined by Trypan blue.

Results: Data obtained from XTT assay indicated that different concentrations of *Salmonella enterica* LPS had no toxic effects on fibroblasts. ANOVA test showed significant difference in

cell viability in a dose and time dependent manner between control and treated cells. In the groups of immediately treatment, at 72 hours, cell proliferation increased in dose dependent manner. In the groups that the treatment was after overnight incubation, the significant effect was observed at 24 hours for the dose of 31.6µg/ml, at 48 hours for the dose of 3.16µg/ml and at 72 hours for the dose of 100ng/ml LPS (p-value<0.001).

Conclusion: This study showed that LPS increased the cell proliferation in time and dose dependent manner. It is hypothesized that this bacterial endotoxin might be one of the future candidates for repairing process.

Keywords: LPS, fibroblast cells, XTT assay, Trypan blue

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1. Introduction

Lipopolysaccharide (LPS) is the main hydrophobic part of outer membrane of gram negative bacteria that composed of three distinct regions: lipid A, core oligosaccharide and O antigen (1, 2). During the cell division and biochemical processes LPS may release to outer environment. In fact due to stimulating ability of several biological systems in mammalian cells and contributing in the production of proinflammatory cytokines, LPS called endotoxin. The production of inflammatory mediators in low concentrations of endotoxin may lead to beneficial biological effects, but at higher concentrations, cytokines releasing have toxic effects and ultimately cause toxic shock syndrome (3-5).

According to previous studies, LPS as a bacterial product could accelerate wound healing process in the epithelial cells of air way. The effect of LPS in this pathway was dose dependent; at low concentrations accelerated the wound repairing, but at high concentrations was toxic for

the air way epithelium and caused the impaired healing. Determination of responses to lower concentrations of LPS indicated that the air way epithelium has the central functional role in delivering signals from pathogen and activation of host response. In contrast, higher levels of LPS overcome immune responses that it's toxic effects leads to epithelium damage and increased microbial invasion. TLRs have been shown to utilized signaling cytokine for recruitment and activation of neutrophils to clear pathogens. In fact the surface defense mechanism against *Pseudomonas aeroginosa* LPS accelerates wound healing process (6, 7, 17).

Fibroblasts are the main dermal cells that illustrate vital role in wound healing process by accumulation in injury site, deposition of collagen and organization of fibronectin-rich extracellular matrix and acting as a signaling cell by releasing growth factors (8-13). The role of inflammation and immunologic reactions of injured organ are inevitable in cellular phase of wound healing. Occurrence of delayed wound healing process is an important clinical problem which imposes a huge cost on society (14-16).

The application of LPS is important in immunological studies. In the studies of effects of any foreign substance on the cells, ensuring from cytotoxicity effects is inevitable factor. We demonstrated that *Salmonella enterica* LPS has the potential of proliferative effect on skin fibroblasts at dose and time dependent manner. In this study for determination of proliferation effects of LPS, XTT assay was selected. XTT reagent compared to MTT assay (that is frequently used for experimental studies) is more sensitive, safe, and accurate and has one step process.

2. Material and method

2.1. Material

Human foreskin fibroblast cells were obtained from Tarbiyat Modarres University cell bank, Lipopolysaccharide (LPS; *Salmonella enterica* L6511-100mg. 109k4087) were purchased from Sigma, FBS, PBS, XTT, tripan blue and all other cell culture regents were obtained from Gibco Life Technologies (Pasley UK) and Sigma.

2.2. Cell Culture

Fibroblast cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) high glucose glutamate supplemented with 5% penicillin-streptomycin solution, 10% fetal bovine serum (FBS) in standard culture conditions at 37°C, 95% humidity and 5% CO_2. When the confluence of cells reached to 80%, cells were trypsinized and transferred into another cultivation flask.

2.3. Treatment of the cells

LPS from *Salmonella enterica* was employed for this study. For preparation of LPS stock solution (0.2 mg/ml), sterile distilled water was the solvent. For the evaluation of LPS effects on skin fibroblasts, two groups were designed for this research. In first group the cells were treated immediately with different concentrations of LPS (1, 3.16, 10, 31.6, 100µg and 3.16, 10, 31.6, 100, 316ng) and in the second group the cells were cultured over night and then treated with different concentrations of LPS. Treated cells were incubated for 24, 48, 72 hours and prepared for cell viability assay.

2.4. Cell viability assay

24 hours before performing this procedure, cells were cultured over night. All tests have a blank containing just cell culture media. For preparation of reactive solution for every plate, 120 µl of (PMS) and 6ml of XTT were required. After addition of reactive solution to all of the wells, micro plates were incubates for 2 and 4 hours. The optical density of samples was read by ELISA-reader in 465nm. The number of viable cells was determined by trypan blue staining.

Statistical analysis

Statistical analysis was performed by using SPSS (version 20) software. One-way analysis of variance (ANOVA) followed by Student's *t* test were done between control and treated groups. Values of $p<0.05$ were considered significant differences between groups. Data were expressed as means ± standard errors (SEM).

3. Result

Cell viability assay

There was no treatment for the control groups that were determined after 24 and 48 hours incubation. This group was designed for recognition of accurate cell count for treatment groups (Fig.1 and Fig.2). The maximum cell count in control group was 8×10^4 and minimum cell count was 1.56×10^2 cells. By evaluation of these results, the optimum cell number that was chosen for treated groups was 3×10^5 cells/well. As shown in Fig.3, when the effect of LPS on fibroblasts viability was evaluated immediately after treatment, it was unaffected by 31.6µg/ml LPS challenge up to 24 and 48 hours, but at 72 hours, cell proliferation increased (p-value=0.050). In the groups that the treatment of LPS were after overnight incubation of fibroblasts (Fig.4), the significant effect was observed at 24 hours for the dose of 31.6µg/ml (p-value=0.001), at 48 hours for the dose of 3.16µg/ml (p-value<0.001) and at 72 hours for the dose of 100ng/ml LPS (p-value<0.001).

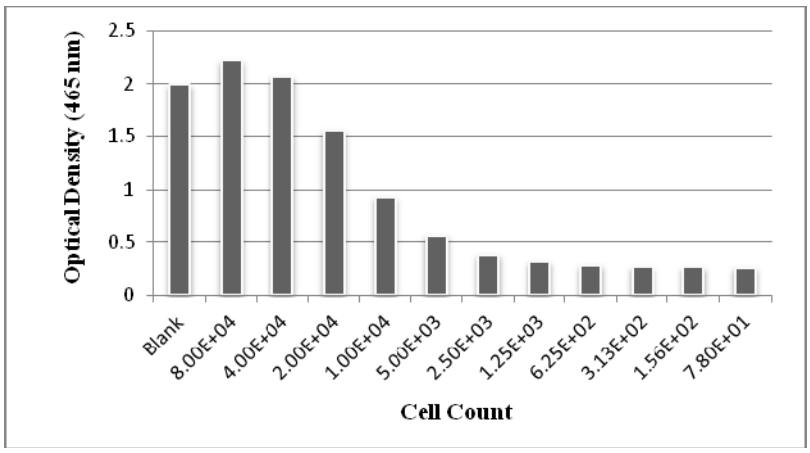

Fig. 1. Determination of cell viability without LPS treatment after 24 hours.

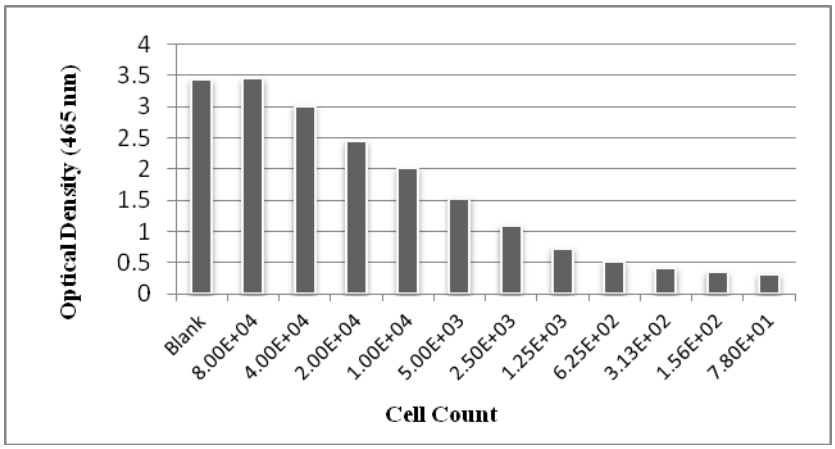

Fig. 2. Determination of cell viability without LPS treatment after 48 hours.

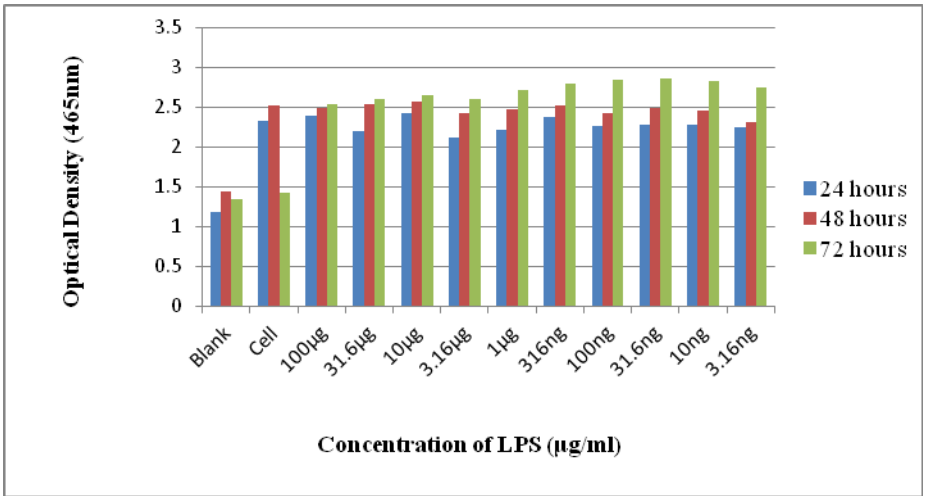

Fig. 3. Determination of cell viability when the treatment was immediately (24, 48, 72 hours after LPS treatment).

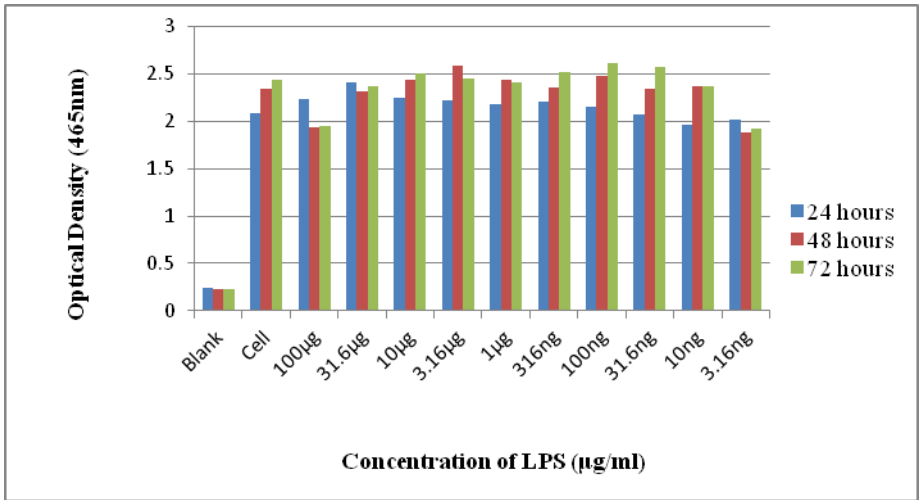

Fig. 4. Determination of cell viability when the treatment was 1 day after overnight incubation (24, 48, 72 hours after LPS treatment).

4. Discussion

In this research non-toxic effects of *Salmonella enterica* LPS on fibroblasts in culture media was indicated. According to obtained results from effects of different concentrations of LPS on certain count of cells, there was significant difference in cell proliferation in a dose and time dependent manner between control and treated cells. For this study XTT assay was selected. The sensitivity of XTT is significantly promoted due to its activator reagent called PMS (N-methyl dibenzopyrazine methyl sulfate) which is an intermediate electron carrier. PMS is the helper for reduction of XTT and formation of formazan derivative. XTT is the assay without using radioactive isotopes, so is safer than MTT assay. Due to proportionality between the counts of cells in each well and dye absorbance in XTT assay, this method is accurate. XTT assay is a 1-step easy to use process that results in 2-5 hours and is not required additional reagents or cell washing procedures (26-29).

There is considerable controversy in the studies of effect of LPS on fibroblast proliferation. In 2002, Yang H. and et al. investigated the effect of *Escherichia coli* LPS on the proliferation of human fibroblasts and keratinocytes. Cells were treated by different doses of LPS. At

concentrations between 0.05µg/ml and 0.5µg/ml the fibroblast proliferation was significantly stimulated after incubation for more than 6 days (30). In 2010, Xiao-jun Cai and et al. reported that LPS treatment (10µg/ml) of adventitial fibroblast for 24 hours induce the proliferation (31). Similar results were performed by Zhengyu He and et al., that they reported increased lung fibroblasts proliferation by LPS (1µg/ml) challenge for 72 hours after treatment. Regardless of the time of treatment, this result was similar to our first group, but their treatment was after overnight incubation like our second group (32). In 2011, Zhang J. and et al. suggested that LPS had inhibitory effect on human lung fibroblasts through a production of IL-6 and IL-8 (33). In our research, LPS challenge of human foreskin fibroblasts in the first group had no effect on proliferation up to 24 and 48 hours; but unlike the propose of Zhang and et al., fibroblasts underwent significant proliferation at 72 hours after LPS treatment. In 2000, Susilowati H. and et al. detected the rat periodontal fibroblast response to *Escherichia coli* LPS. The results indicated that the response of rat periodontal ligament fibroblasts was differently to LPS challenge. At concentration of 100ng/well, the periodontal ligament fibroblasts proliferation was higher than gingival fibroblasts (34). In 2004, Yi-Hui Luo and et al. evaluated the comparison of *Helicobacter pylori* LPS with *Escherichia coli* LPS treatments on mouse 3T3 fibroblasts. The results showed that low doses of E-LPS and H-LPS (1µg/ml) were toxic for NIH 3T3 fibroblasts. The toxic effect of H-LPS was weaker than E-LPS on fibroblasts (35).

Due to extensive application of lipopolysaccharide in immunological and inflammatory studies, its optimal role in enhancing immune responses against pathogens and diseases has been demonstrated (18, 19). Jalil Tavakkol Afshari and et al. reported that LPS as a mitogenic factor for B lymphocytes has the potential of acceleration of these cells proliferation in the manner of non specific (23-25). In 2001, Tanamoto Ken-Ichi and et al. detected the mitogenic activity of extracted LPS from *Flavobacterium meningospecticum* on mouse hepatocyte cells. The comparison of this LPS with *Salmonella enterica* LPS on hepatocyte cells were determined by [^{3}H] Thymidine method. Their results showed that mitogenic effects of extracted LPS from *Flavobacterium meningospecticum* were 10 fold lower than *Salmonella enterica* LPS and this effect is in dose dependent manner (20).

In conclusion, this research was performed for the evaluation of the effects of different concentrations of *Salmonella enterica* LPS on skin fibroblasts proliferation. XTT assay results showed increased proliferation in time and dose dependent manner. Fibroblast proliferation is an

important part of healing process. It is hypothesized that in the coming years this bacterial endotoxin might be one of the candidates in healing process.

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