The Biological Influence of *Salmonella enterica* Lipopolysaccharide on Excretion of Inflammatory Mediators by Fibroblasts in an *in vitro* Wound Healing Model

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Inflammatory mediators produced by fibroblasts as the main reagents of wound healing have fundamental roles in this process. Due to stimulatory effects of lipopolysaccharide (LPS) on different biological mechanisms of mammalian cells and its influence on pro-inflammatory cytokines, this bacterial endotoxin has been surge of interest in immunological and inflammatory studies. Skin is the first barrier in immune response which is more susceptible to inflammation. In this investigation, the effects of *Salmonella enterica* LPS on skin fibroblast cells viability was evaluated. Nitric oxide (NO), cyclooxygenase-2 (COX-2) and hydrogen peroxide (H_2O_2) levels were assessed after LPS treatment of fibroblasts. Human foreskin fibroblasts were treated by different concentrations of *Salmonella enterica* LPS (100μg~0.01μg). Effects of LPS on cell viability and NO, COX-2 and H_2O_2 levels were examined respectively by XTT assay and related kits as per the manufacturer's protocols. Results of present survey illustrate that there is a dose and time dependent significant difference between control and treated cells in cell proliferation. Results obtained from assays indicate that LPS stimulates NO, COX-2 levels and reduces H_2O_2 levels in fibroblast cells (p-value<0.001). According to LPS effects on cell proliferation in dose and time dependent manner and increasing nitric oxide as a vital factor in the healing process, it will be considered theraputical potential of this bacterial endotoxin.

Key words: *Salmonella enterica* LPS, Inflammation, Nitric oxide, Cyclooxygenase-2, Hydrogen peroxide, Skin fibroblasts, Cell viability.

Lipopolysaccharide (LPS) called endotoxin has the ability of promoting various cellular mechanisms in mammals[1, 2]. According to the different characteristics of interfering with immune system, it is also known as exo-hormone[3]. Production of 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[4-6]. 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

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extracellular matrix and acting as a signaling cell by releasing growth factors[7-12].

Bacterial lipopolysaccharide (LPS) is one of the major inducers of inflammatory mediators. COX-2 is the inducible isoform of COX enzyme which its expression in normal conditions is very low, but is strongly induced in response to bacterial products or cytokines[13, 14]. LPS is one of the stimulus that induce COX-2 expression by mitogen activated protein kinase (MAPK) signal transduction pathway[15-17]. According to previous findings, the intracellular hydrogen peroxide as a signal for white blood cell increased accumulation after tissue damage to begin the repair process[18]. Since ROS at low concentrations provide signaling pathways for immunity against microorganisms, Low levels of these mediators can be beneficial in acute wound healing process. High levels of ROS exert oxidative damage which leads to impaired repairing; this abnormality is explained due to a deficiency in ROS detoxifying enzymes[19]. Nitric oxide is the most effective antioxidant that is produced in the oxygen dependent manner by nitric oxide synthase[17]. Nitric oxide is the detoxifier of high amounts of ROS and also can switches off the important transcriptional activator of inflammatory proteins, nuclear factor-kB (NF-kB)[20, 21].

Nitric oxide (NO) acts as a vasodilator, antimicrobial compound, chemo-attractant, inhibitor of platelet accumulation, stimulator of vascular permeability in inflammation. NO is up regulator and down regulator of inflammatory phase of wound healing. High levels of NO may contribute as an anti inflammation during the late phase of inflammation[22-24]. NO activates factors like VEGF, bFGF, TGF-² which are required for enhancing angiogenesis[25]. These growth factors stimulate migration, adhesion and proliferation of endothelial cells. NO produced by iNOS is essential for proliferation of keratinocytes. Attraction of IL-1 by NO indicates indirect effects of NO in re-*epithelialization*. Most in vitro investigations shows that NO enhances collagen synthesis in both wound model fibroblasts and normal skin fibroblasts. NO converts latent TGF-β_1 to the active form which is necessary for activation of fibroblasts[26-28].

In this study we tried to stimulate these inflammatory factors by bacterial LPS. Fibroblasts were treated with different concentrations of LPS for detection of inflammatory mediators. According to the results, LPS can induce the production of NO and COX-2 levels and reduces H_2O_2 level in fibroblasts in the dose dependent manner. Due to central role of these mediators in repairing process and the effect of *Salmonella enterica* LPS on fibroblasts proliferation, this endotoxin may be useful in drug designing for cutaneous wound therapies.

MATERIALS AND METHODS

Materials

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, lysis buffer, Nitric Oxide assay kit (Biovision, Ca, USA), H_2O_2 assay kit (Biovision, Ca, USA), COX-2 assay kit (Assay designs & Stressgen Inc. Michigan, USA), and all other cell culture regents were obtained from Gibco Life Technologies (Pasley UK) and Sigma.

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.

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, we designed two groups. In the first group cells treatment were immediately but in the second group the cells were cultured over night and then treated with different concentrations of LPS (0.01, 0.1, 1, 10, 100 µg/ml). Treated cells were incubated for 24, 48, 72 hours and prepared for cell viability assay and evaluation of NO, COX-2 and H_2O_2 levels.

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 ¼l of (PMS) and 6ml of XTT were required. After addition of reactive solution to all of the wells, microplates 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.

Determination of NO production

Although NO is unstable but the content of its stable products namely nitrate and nitrite is a suitable index of NO production. NO levels in the above mentioned samples were determined using colorimetric assay kit (Nitric Oxide assay kit, Biovision, Ca, USA). The assay principle for measurement of NO in the supernatant was Griess

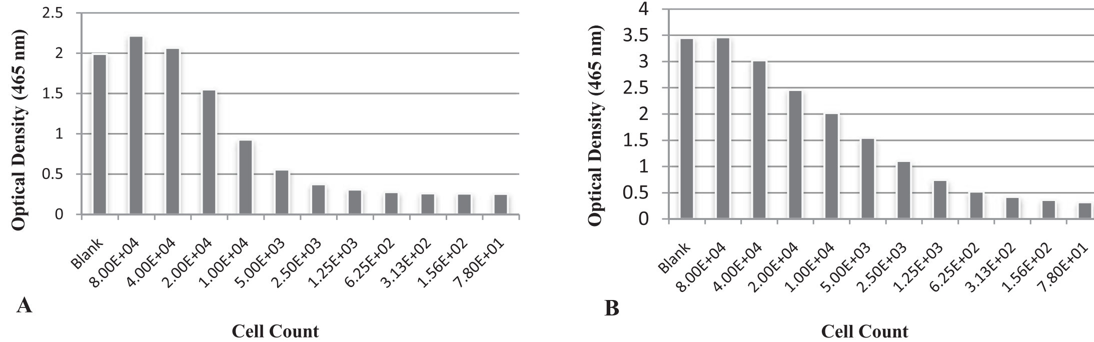

Fig.1. Determination of cell viability without LPS treatment after 24 hours (A). Determination of cell viability without LPS treatment after 48 hours (B)

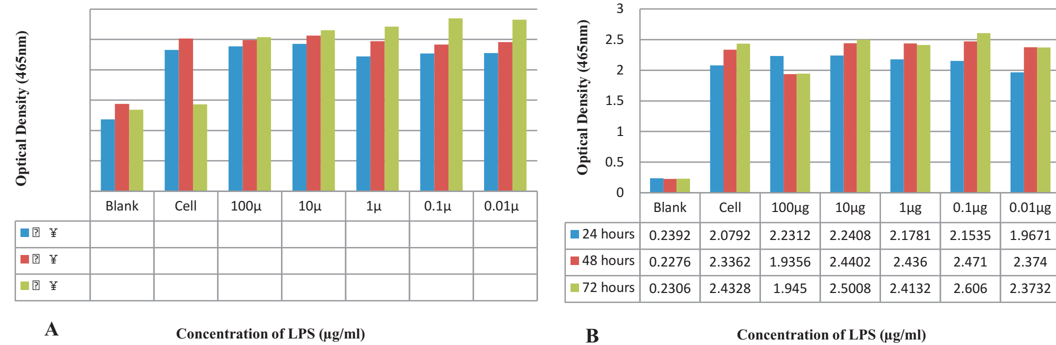

Fig.2. Determination of cell viability when the treatment was immediately (24, 48, 72 hours after LPS treatment) (A). Determination of cell viability when the treatment was after overnight incubation (24, 48, 72 hours after LPS treatment) (B).

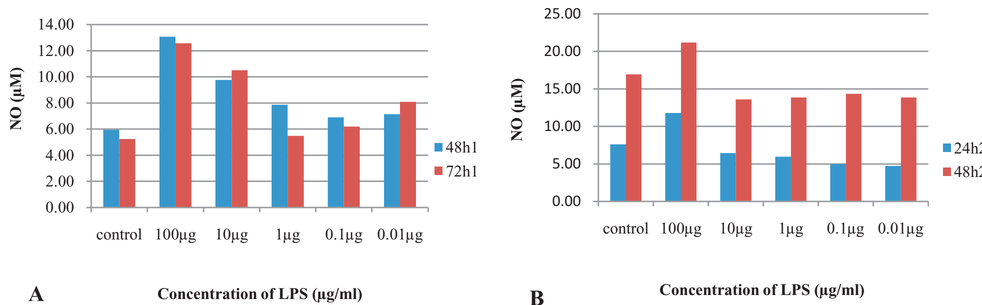

Fig. 3. Determination of NO production in the groups of immediately LPS treatment of cells (A).Determination of NO production in the groups of after overnight treatment of cell with LPS (B)

reaction. An equal volume of cell supernatants were mixed with Griess reagent and the absorbance was read at 540nm. The assay sensitivity was 0.2nM and the intera assay coefficient of variation was 3.4%.

Determination of COX-2 level

The activity of COX-2 was measured by Enzyme Linked Immuno Sorbent assay kit (Assay design & Stressgen Inc. Michigan, USA) according to the recommendation of the manufacturer insert. The measured optical density in 450nm was proportional to the concentration of COX-2 in either standards or samples.

Hydrogen Peroxide Measurement

Hydrogen peroxide levels in the supernatants of cell culture samples were determined using colorimetric assay kit (Hydrogen peroxide assay kit, Biovision, Ca, USA). The assay was performed based on the kit insert. The assay principle was based on peroxidase reaction and destroying of hydrogen peroxide. The assay sensitivity was 0.1nM and the intera assay coefficient of variation was 2.8%.

Statistical analysis

Statistical analysis was performed by using SPSS (version 20) software. Levels of NO, COX-2 and H_2O_2 between control and test samples were analysed using ANOVA, followed by Student's *t* test. Values of $p \leq 0.05$ were considered significant differences between groups. Data were expressed as means ± standard errors (SEM).

RESULTS

Cell viability assay

When the effect of LPS on fibroblasts viability was evaluated immediately after treatment, it was unaffected by 0.1 ¼g/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, the significant effect was observed at 24 hours for the dose of 10µg/ml (p-value=0.001), at 48 hours for the dose of 0.1µg/ml (p-value<0.001) and at 72 hours for the dose of 0.1µg/ml LPS (p-value<0.001).

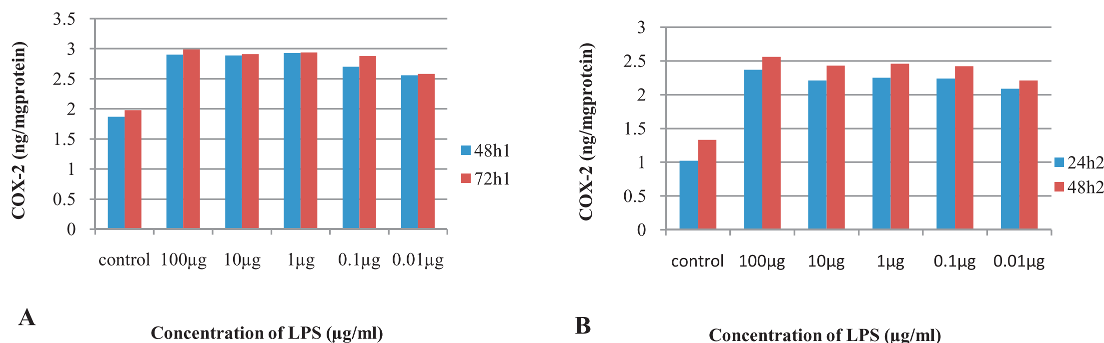

Fig. 4. Determination of COX-2 level in the groups of immediately LPS treatment of cells (A). Determination of COX-2 level in the groups of after overnight treatment of cell with LPS (B)

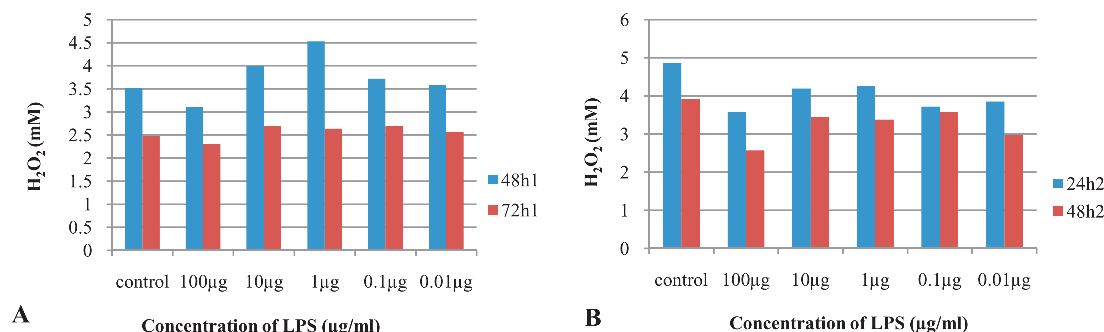

Fig. 5. Determination of H_2O_2 level in the groups of immediately LPS treatment of cells (A). Determination of H_2O_2 level in the groups of after overnight treatment of cell with LPS (B)

Determination of NO production

The results of determination of NO production in the groups that the LPS treatment was immediately indicated that the significant increase in NO level was at the dose of 100¼g/ml for 48 and 72 hours (p-value<0.001). For the groups of after overnight treatment, the maximum increase in NO level was observed at the dose of 100¼g/ml for 24 and 48 hours (p-value<0.001).

Determination of COX-2 level

In the groups that the treatment of LPS was immediately, after 48 and 72 hours incubation, the doses of 1µg/ml and 100µg/ml were respectively showed the maximum increase in COX-2 level (p-value<0.001). In the groups that the LPS treatment was after overnight incubation, the significant increase in COX-2 level was observed at 24 and 48 hours for the dose of 100µg/ml (p-value≤0.001).

Hydrogen Peroxide Measurement

In the case of immediately LPS treatment of cells, the treated group with the dose of 1µg/ml compared to the control group showed the significant increase in hydrogen peroxide level at 48 hours (p-value ≤ 0.001). But in the groups of after overnight treatment, hydrogen peroxide level in the group of treated cells was significantly decreased compared to control group (p-valueÂ0.001).

DISCUSSION

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[7-12]. The role of inflammation and immunologic reactions of injured organ are inevitable in cellular phase of wound healing[29,30]. In this survey fibroblasts were treated with different concentrations of LPS for detection of inflammatory mediators. According to the results, LPS can induce the production of NO and COX-2 and reduces H_2O_2 level in the dose and time dependent manner. The highest level of NO after LPS treatment was at the dose of 100µg/ml (Fig. 3). COX-2 level was increased after LPS treatment in both of the groups that the highest level was at 100µg/ml (Fig.4). Also in the first group, fibroblasts proliferation was increased after 72 hours treatment (Fig.2A) and increased by 24, 48 and 72 hours in the second group (Fig.2B). H_2O_2 level was increased in the first group at specific doses of LPS that the highest level was at the dose of 1µg/ml (Fig.5A) but in the second group it decreased compared to control group (Fig.5B). It might be considered that LPS had increasing effect on H_2O_2 level at early stage of cell cycle but at late stage cause the decreasing effect on this mediator.

In 2012, Anta Ngkelo and *et al*. reported that LPS induced pro inflammatory cytokines such as CXCL8 and IL-6 in human peripheral blood mononuclear cells. In their study, after LPS treatment, cells were incubated for 16 hours and the culture supernatants were removed for determination of IL-6 and CXCL8 levels[31]. In 2009, Wan Lia and *et al*. suggested that LPS can effect on fibroblast proliferation, TGF-β and INF-γ excretion in human dermal fibroblasts of hypertrophic. According to their result, lower concentrations of LPS (0.005-0.1µg/ml) can increase fibroblasts proliferation. However at higher concentrations of LPS, the results showed opposite effects. So this effect was in dose dependent manner. Collagen synthesis, proliferation and TGF-β, IFN-γ had no significant difference with control group in the dose of 0.1µg/ml[32]. In 2012, Zhengyu and *et al*. indicated that LPS can promote the proliferation of lung fibroblast. LPS effect on TLR4 signaling pathway leads to PI3K-Akt pathway activation and PTEN expression down-regulation. The proliferation of fibroblast cells were detected by BrdU assay. The dose of 1µg/ml LPS after 24 hours had no effect on proliferation but after 72 hours the proliferation was increased significantly[33]. In 2002, Roger S. Smith and *et al*. evaluated the induction of COX-2 and production of prostaglandin E_2 in human lung fibroblasts via *Pseudomonas* Autoinducer N-(3-oxododecanonyl) homoserine lactone. They demonstrated that 3O-C_{12}-HSL not only was essential for the regulation of bacterial virulence factor but also regulate inducible immunity by stimulating the activities of eukaryotic cells important for immune response against *P. aeruginosa*. PGE_2 produced by COX pathway acts as an immunomodulatory lipid mediator that induces mucus secretion and vasodilation[14]. In 2010, Xiao-jun Cai and *et al*. reported that LPS treatment (10µg/ml) of adventitial

fibroblast for 24 hours induce the proliferation. They demonstrated that adiponectin prevented adventitial fibroblasts proliferation by reduction of LPS-induced NO production[34]. 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. The production of NO in the periodontal ligament fibroblasts culture was higher than NO levels in gingival fibroblasts culture at high concentration of LPS (1000ng/well) (35). In 2011, Hossein rastegar and *et al*, demonstrated that the reduction of COX-2 in HepG2 cells by *Salmonella enteritidis* LPS was correlated with cell density and time of incubation. They confirmed that the decreasing effect of LPS on COX-2 can be used as the helper of COX-2 inhibitors such as celecoxib and NS-398 in variety of diseases and cancers[36].

In conclusion this investigation indicates the invitro mechanism of inflammatory mediators' induction by *Salmonella enterica* LPS that has stimulatory effects on NO and COX-2 and reduces H_2O_2 levels in dose and time dependent manner. Considering central role of these mediators in wound healing process and proliferative effect of this bacterial LPS on fibroblasts, it can be worthwhile in development of new methods for wound therapies.

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