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Effect of Lipopolysaccharide (LPS) Extracted from *Salmonella enteritidis* on Production of Hydrogen Peroxide and Total Antioxidant Capacity in Fibroblast Cells

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ABSTRACT

Lipopolysaccharide (LPS) located in the cell wall of most gram-negative bacteria known to have a variety of biological and immunochemical activities. Fibroblasts are spindle shaped cells found in the majority of tissues and organs of the body associated with extracellular matrix (ECM) molecules. The aim of this study was to determine the effect of LPS extracted from Salmonella enteritidis on hydrogen peroxide production and total antioxidant capacity (TAC) in fibroblast cells. In present study LPS was extracted from Salmonella enteritidis cell wall by methanol chloroform method and purified. Electrophoresis and pyrogenicity test for purity of extracted sample was done. Electrophoresis was performed on extracted LPS with standard LPS in agarose 12% gel and was stained by AgNO3. Pyrogenicity of LPS was tested in rabbit before injection, 30 minutes after injection and 1 hour after injection. In this assess we used respectively 75, 100, 125 ng of LPS combination extracted from salmonella enteritidis in MTT test for cytotoxic level measurement on fibroblast cells. 100 ng of LPS was used for assay of hydrogen peroxide and TAC in human fibroblast cells. Human fibroblast cells treated with 100 ng LPS along with non-treated cells were incubated. Our study revealed that LPS can stimulate the increasing of hydrogen peroxide and decreasing of TAC in human fibroblast cells. These finding also confirmed that extracted LPS by methanol-chloroform method has the same activity of standard LPS.

Keywords: *Salmonella enteritidis*, Lipopolysaccharide, Rabbit pyrogen test, Fibroblast cells, Total antioxidant capacity, Reactive oxygen species

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INTRODUCTION

Salmonella is a gram-negative bacilli that belongs to the Enterobacteriaceae family. This bacteria has the capacity to grow under either aerobic or anaerobic conditions, and is non-encapsulated and non-spore forming organism. LPS is a kind of an amphibiotic molecule which by hydrophobe combination is linked to outer membrane of gram-negative bacteria. Outer membrane of LPS affects on linkage between bacterium and its environment [1, 2, 3, 4].

Fibroblasts are spindle shaped cells found in majority of tissues and organs of the body associated with extracellular matrix. Characteristic features of Fibroblasts include expression of vimentin in the absence of desmin and α-smooth muscle actin. When activated fibroblasts exhibit an abundant endoplasmic reticulum and prominent Golgi associated with the synthesis and secretion of ECM molecules including collagens, proteoglycans and fibronectin, as well as, proteases that capable degrading of ECM. Cytoskeletal proteins in association with cell surface integrants and the ECM facilitate cell motility and the generation of contractile forces important in tissue homeostasis and wound healing [5]. LPS induces the activation of intracellular signaling cascade via Toll like receptor that exists in macrophage and endothelial cells and following this process stimulation of inflammatory factors such as TNF-α and IL-1β is occurred [6, 7]. Previous studies have been showed that LPS can involve in stimulation of ROS and free radicals [8].

Reactive Oxygen Species (ROS) are derived from the metabolism of molecular oxygen. ROS include super oxide onion radical, singlet oxygen, hydrogen peroxide and highly reactive hydroxyl radical. The deleterious effects of oxygen are said to result from its metabolic reduction to these highly reactive and toxic species [6, 8]. ROS can damage DNA and division of cells which leads to mutation [9, 10, 11, 12].

Certain levels from ROS is necessary for suitable functions, but the other levels lead to oxidative stress and have harmful effects on cells function such as proteins changes to auto antigen or increasing of their destruction [13, 14]. Surveys show that cells have powerful defense mechanism against oxidative damages that a lot of them are known as antioxidants. When we say antioxidant, refer to a material that exists in low density to substrate oxidation and at a same time it prevents from oxidation substrate or can delay it and according to other definition that is named metabolite antioxidant. Antioxidant is a combination that protects biological tissues from free radical destruction effect. Antioxidant protection inside of cells is occurred in different levels such as: prevention from free radical formation, separation of free radical after formation, repair of oxidative damages that are produced by radicals and absorb increasing of molecules which are damaged [9, 15, 16]. The aim of this study was to determine effects of LPS extracellular matrix from *Salmonella enteritidis* on hydrogen peroxide and total antioxidant capacity in fibroblast cells.

MATERIALS AND METHODS

Salmonella enteritidis, methanol, chloroform, salmonella enteritidis standard LPS, DMEM, human fibroblast cell, PBS, H_2O_2, solution HRP, solution choromogen, TAC cayman kit, MTT test.

Experimental Procedures:

Preparation and extraction of *Salmonella enteritidis* lipopolysaccharide

Suspension of *Salmonella enteritidis* grown in Tryptic soy broth moved to test tubes and centrifuged for 30 minutes in 2500-3000 rpm. Then supernatant was added to a test tube which included precipitation of bacteria. 2 ml of alcohol 95% was added to the test tube possessing sediment and vortexed till the bacteria sediment and alcohol were mixed properly and sediment was separated from the bottom of the test tube. After that tubes were centrifuged in 2000 rpm for 10 minutes and precipitation washed with 2 ml alcohol again (for 3th). At the end, we combine the tubes which include precipitation and for the last time it was centrifuged in 2000 rpm for 10 minutes and after that we remove alcohol and then put the tubes under the hood and then the rest of alcohol were evaporated. Dried bacteria with 1ml EDTA 10% mixed and put in the eppendorf tubes for sonication. Then methanol and chloroform in the ratio of 1:2 inserted to separate tube for saturation. Following 1ml of this solution added to bacteria and EDTA for 2 hours put on the shaker and after this time the tube was centrifuged in 2000 rpm for 10 min and 3 layers were created respectively include: methanol as a first layer, bacteria as a second layer and chloroform as third layer. Tube which include chloroform and methanol removed under the hood for evaporation and rest of it was extracted LPS [17, 18].

Chemical and analysis

SDS-PAGE:

Extracted LPS was electrophoreses with standard LPS which provided by Sigma Company (L6011-100MG - 088K4014) using 12% SDS-Page method and then stained with AgNO3 [9, 14, 19]. In the electrophoresis method we use 20 ml acryl 30%, 5 ml TBE, 600 landa AMS, 30 landa TEMED.

Fig 1. Electrophoresis of extracted LPS and standard LPS from salmonella enteritidis after staining with $AgNO_3$

Pyrogenicity test in rabbits:

The procedure of test is the measurement of body temperature increasing after injection of LPS. In the survey Pyrogenicity was tested in 6 rabbits. Rabbits were given an intravenous injection of 30µg/ml of purified LPS. Rectal temperatures were measured and recorded before injection, 30 minutes after injection and 1 hour after injection [18, 19].

MTT test:

We can assess the respond of different cells to external agents such as growth factors, cytotoxic drugs and the other chemicals agnates by MTT test which is simple and exact. MTT is a yellow tetrazolium and solution in water that by alive cells it can be revived to an insoluble Formazon production in water solution. This assessment is upon capacity of mitochondrial dehydrogenase enzyme in changing of yellow tetrazolium to dark blue Formazan productions. Formazan level which is produced has a direct ratio to the number of alive cells [20, 21]. In this assess the effect of LPS on viability and proliferation of fibroblast cells were examined by the MTT assay. We used respectively 75, 100, 125 ng of LPS combination extracted from *salmonella enteritidis* for cytotoxic level measurement on fibroblast cells.

Fibroblast cells culture:

human fibroblast cells were cultured in Dulbecco modified Eagles medium (DMEM) supplemented with FBS and 100 U/ml penicillin and streptomycin in mixture sterile condition and at 37°C and in a humidified 5% CO_2 atmosphere [8, 22].

H_2O_2 measurement:

In this method H_2O_2 combination indirectly and instead of ROS were measured. 10 µl from H_2O_2 solution is combined with 1ml water until standard solution 100 ml is produced and then from this standard solution dilute is done and finally 10 ml from this dilution in 96 well and 10 µl treatment cells and 10 µl control sample (untreatment cells) are added to next wells. Then 10 µl HRP, 50 µl chromozhen solution were added to all the wells and finally it reads by E reader [23, 24].

TAC measurement:
For capacity measurement of TAC we used TAC kit from American Cayman Company with 709001 catalog number. In this method the antioxidant capacity was evaluated according to the monitoring of ABTS (2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid)) solution decoloration [25, 26, 27].

RESULTS

As it has been shown in fig1, to examine the purification of extracted LPS, in electrophoresis method in well 1 and 2 (on the left) 0/003 gram of extracted LPS sample and in wells 3 and 4 the same amount of standard LPS sample from *Salmonella enteritidis* in 12% agarose electrophoresis and then have been colored with $AgNO_3$ .The bands which relate to extracted sample from salmonella are similar to standard LPS sample and this indicates high efficiency output of LPS extraction with chloroform-methanol method.

Pyrogenicitiy test of extracted LPS was performed in rabbit by injection of 3 µg LPS. If body temperature of rabbit increased up to 0.6°C or the sum of increase of temperature was >1.4°C for the relevant rabbits, the result of pyrogenicity considered positive and our sample was pyrogen. These results showed that LPS has pyrogenicity, because one of the biological effects of this combination is pyrogenicity that by doing this test extraction of LPS from *Salmonella enteritidis* is approved and results are showed in table 1. The extracted LPS induced hydrogen peroxide (H_2O_2) generation in human fibroblast cells. When the human fibroblast cells were incubated with 100 ng/ml LPS for 4h, the H_2O_2 decreased. In the presence of ferrous ion (fe^{2+}), OH is generated via the iron catalyzed fenton reaction from hydrogen peroxide. In this survey the results of control samples and treated samples with 100 ng/ml LPS were read by the ELISA reader in 450 nm.

Table 1. Measuring of rabbit rectal temperature before injection, 30 min and 1 hour after injection of 30 µg of extracted LPS from *Salmonella enteritidis*

Temperature after 1 hour	Temperature after 30 minutes	Initial temperature
39.1	39	38.5
39.2	39.2	38.6
39.3	39.4	38.7
39.2	39.1	38.5
39.5	39.4	38.8
39.4	39.4	38.7

In this study control samples and treated samples with 100ng/ml LPS, were repeated three times and their light absorption in (OD) 450 nm were examined by ELISA reader. As it is shown in diagram 1, LPS treated samples compared to control samples had higher production of H_2O_2 with $p<0.001$. Also effect of LPS on TAC production in fibroblast cell showed that this combination can reduce the TAC in fibroblast cells. In diagram 2 we observed that LPS treated samples compared to control samples had decreasing amount of TAC which was $p<0.001$.

Diagram 1- Effect of LPS on H_2O_2 in fibroblast cells. LPS treated samples compared to control samples had higher production of H_2O_2

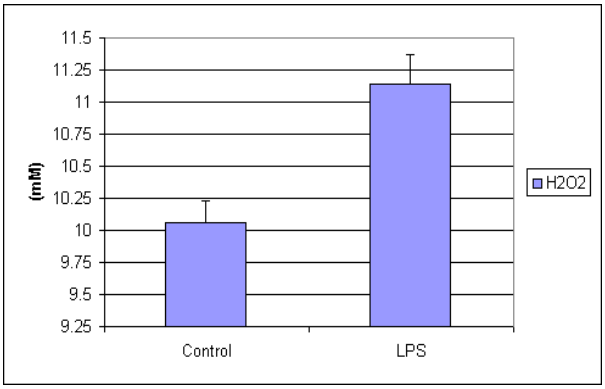
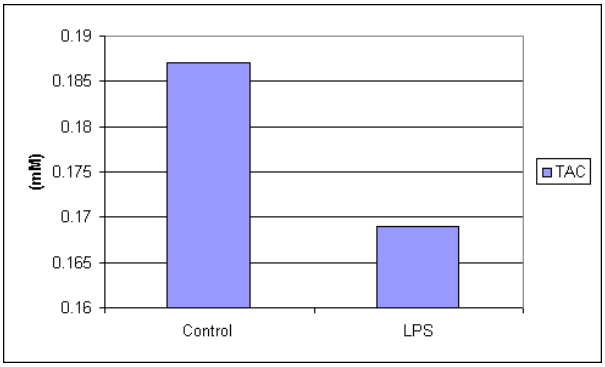


Diagram 2- Effect of LPS on TAC in fibroblast cells. LPS treated samples compared to control samples had low amount of TAC



DISCUSSION

In this survey LPS was extracted from *Salmonella enteritidis* by methanol chloroform method. Electrophoresis and pyrogenicity test for purity of extracted sample was done. According to surveys which were done by Morisson and Leive, it distinguished that phenol extraction method results in cells heavy destroying [28]. But in this method LPS is extracted well. Electrophoresis of *Salmonella enteritidis* extracted LPS with its standard sample showed that extracted LPS bands are very similar to LPS bands of standard sample. Also pyrogenicity test in rabbit shows the accuracy of LPS method extraction. In this survey, we demonstrated that LPS can induce H_2O_2 generation and concomitant decline in TAC in human fibroblast cells. A previous report showed LPS induced ROS generation in a murine DC line Xs52, using flow cytometry with a H_2O_2 sensitive dye, 2, 7 dichlorofluorescein diacetate (DC-FH-DA). However, the fluorescence intensity increased very slightly in LPS stimulated cells, LPS induces the activation of an intracellular signaling cascade via Toll-like receptor 4. The signaling cascade consists of two distinct pathways, The MyD88 dependent and independent pathways. Studies have been shown that LPS can in addition to stimulation of ROS such as H_2O_2, stimulates macrophages, IL-1β, IL-6 and IL-12.

ROS which is produced can create different disease and DNA damaging [9, 12, 29, 30]. Alive organisms have complex antioxidant systems against these harmful combinations. Antioxidant systems include enzymes such as super oxide dismutase, catalase, glutathione peroxidase and macromolecules such as albumin, ceruloplasmin and ferritin and small molecules such as acid ascorbic and glutathione [31]. Stimulation of fibroblast cells with bacteria agents such as LPS, H_2O_2 production is stimulated and cells for confronting to these combination use enzymes and antioxidant combinations that exist in cell, until neutralize these harmful effects and after this process the TAC anti capacity amount in the cells is decreased. In this study it is distinguished that in untreatment human fibroblast cells, in comparison to treatment samples with LPS from *Salmonella enteritidis* can produce less H_2O_2 and this difference is ($p<0/001$). Also this difference in TAC capacity amount in treatment fibroblast cells with LPS in comparison to untreatment cells also is observed ($p<0/001$).

CONCLUSION

In this study we used methanol-chloroform method for extraction of LPS from *Salmonella enetritidis*. Electrophoresis showed similarity among extracted and standard LPS. Pyrogenicity test of LPS was performed in rabbit. We realized that LPS structure and function was maintained. We also observed that LPS from Salmonella enteritidis can induce H_2O_2 production and decrease TAC capacity in fibroblast cells.

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