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Comparison of lipopolysaccharide *Agrobacterium tumefaciens* and *E. coli* on TNF- α and Nitric Oxide Production via Mice Macrophages

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The purpose of this study was to isolate and purify lipopolysaccharide (LPS) from *A. tumefaciens* and *E. coli* and compare its ability to produce nitric oxide and TNF-a in peritoneal mice macrophages. We isolated and purified LPS from *A. tumefaciens* and *E.coli*. The endotoxin activity of LPS extracted from *A. tumefaciens* and *E.coli* were examined via the Limulus Ambocyte Lysate Test (LALT). The effects of different concentrations of lipopolysaccharides were assayed on mice macrophages as stimuli to produce nitric oxide and TNF- a production. Then, they were measured by Griess and Enzyme Linked Immunosorbent Assay respectively. Data were analyzed by SPSS version 19.0. Using the Westphal method LPS can be isolated from both aforementioned Gram negative bacteria. The results suggest that the quantity of extraction and purification of LPS from *A. tumefaciens* and *E. coli* was dependent on culture volumes; 5 to 10 mg of LPS can be obtained from 1 liter of 24 hour culture respectively. The results indicate that the stimulating effects of 500 ng/ml LPS concentration extracted from *E. coli* has the same effect as 1000 ng/ml concentration of LPS *A. tumefaciens*. *E. coli* LPS was more effective in stimulating production of TNF-α and to produce nitric oxide. The findings of this study suggest that the effect of 1000 ng LPS from *A. tumefaciens* was equal to 500 ng LPS from *E.coli* in stimulating macrophages to produce nitric oxide. This demonstrated that the immunomodulatory effect with less toxicity.

Key words: Macrophages, nitric oxide, TNF-α, LPS, *A. Tumefaciens* and *E.coli.*

Recent studies have shown that using safe biological adjuvants such as MPL, a derivative of endotoxins, have therapeutic effects for bacterial and viral infections as well as in animal cancer[1-4]. However, to reduce the toxicity and complement activation of bacterial endotoxins, retaining immunoajuvant properties and ability to stimulate macrophages is desirable[5,6]. Some methods have

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developed forming LPS mutations creating endotoxins without toxicity[6,7]. Such mutations include novel LPS species that are more suitable for human vaccines[8]. Studies show that the toxic properties of lipid A can be removed without eliminating immunomodulating activity. In addition, certain forms of lipid A can overcome immunologic lesions of immunodeficient and hyporesponsive animals[9]. Some investigators have reported the advantages and therapeutic potential of endotoxin antagonists[10]. They have shown that inactive disaccharide analogs of lipid A the essential structure of LPS, may act as LPS antagonists effective against septic shock induced by Gram-negative bacteria endotoxins[11]. Popa showed that, *Bartonella quintana* LPS is a natural antagonist of toll- like receptor 4 (TLR4). Based on microarray studies they revealed that LPS of *Bartonella quintana* blocked the interaction of *E. coli* LPS with TLR4 in transfected cell lines[12]. Significant differences in endotoxin structure are seen in Gram-negative bacteria explaining different biological activities (including different ability to trigger the adaptative immune response)[13,14].

Most of these studies have been devoted to endotoxin isolated from human pathogenic bacteria disregarding pathogenic bacteria from plants. Considering that the plant pathogenic bacteria are not capable of causing disease in human. it assumed that isolated endotoxins from plant pathogenic bacteria have lower toxicity. Thus, we sought to isolate and purify LPS from *A. tumefaciens* and *E. coli* to assess their impact on nitric oxide and TNF-α production by macrophages in mice.

MATERIALS AND METHOD

Bacterial strains

Agrobacterium tumefaciens (A. tumefaciens) strain LB 4404 and *E. coli* strain ATCC 3521 (Tarbiat Modaress University, Iran) were acquired. *Agrobacterium tumefaciens* was cultured at 28°C in liquid shake culture (150 rpm) in 5 liters of fresh sterilized broth medium (Tryptone 5g/l ,yeast extract 1g/l, nutrient broth 5g/l, sucrose 5g/l, Mg so4 with 7H2O 0.49g/l) for 32 hours.

E. coli was also grown in 5 liters of BHI broth at 37°C for 24 hours. Bacterial cells were then harvested by centrifugation (5000 rpm for 10 min at 4°C). The cell pellets were washed with cold phosphate-buffered saline (PBS), pH 7.4 and centrifuged (5000 rpm for 10 min at 4°C) twice.

LPS extraction

Harvested bacterial cell pellets were re-suspended in 50ml of sterilized distilled water. The hot phenol/water method was used to extract LPS. This method was previously described by Westphal et al [15, 16]. First, 90% phenol- water mixture was prepared and kept in water bath at 68°C. A solution of 90% phenol (w/v) was made via adding 10.8 g of crystalline phenol (newly opened bottle) to a 250-ml graduated balloon and adding 1.2 ml of double-distilled water (prewarmed to 68°C) to bring the volume to 12 ml. The graduated cylinder in the 68°C water bath was and stirred (the phenol crystals liquefy at the 68°C temperature). An equal volume of hot 90% phenol was added to the cell pellet (1g/ 10 ml) suspended and stirred at 68°C for 25 min in a shaking water bath. After that, centrifugation was carried out (8000 rpm for 20 min). Three distinct layers appeared which contained a clear upper layer (aqueous phase), a yellowish lower layer (phenolic phase) and a milky color solid phase. The aqueous upper phase was accumulated in two 50 ml falcon tubes and centrifuged again for removal of debris. The supernatant of this step were dialyzed in sacks with 12000 MW cut off (Dialysis Sacks, Cat No 250-7u, Lot 10H-6134. Sigma) against distilled water at room temperature for 5 days until all the liquid phenol was depleted. The dialyzed solutions were sterilized by 0.45 µm filter and were kept refrigerated as crude LPS.

Purification of crude LPS

To reduce contamination of RNA, DNA and proteins, the crude LPS were treated by 40µl DNase I (1u/µl)and 60µl RNaseA at 37°C for 15 min then 50µl proteinase K (20 µg/ml) was added and incubated at 60°C for 60 min (all enzymes from Fermentas). Next 15 ml pretreated LPS solutions were poured in poly-allomer tubes (14×95mm) and ultracentrifugated at 100000 ´g at 4°C for 4 hours (Beekman-Optima LE-80 K). The supernatants were discarded and the gelly sticky precipitates at the bottom of the tubes resolved by 5ml PBS and then were lyophilized. The purity and estimated molecular weight of the LPS was determined using SDS-PAGE (electrophoresis was carried out at 120 *m*v for 60 min). The slob gel was stained using colloidal Coomassie brilliant blue G-250.

During the various stages of extraction and purification of LPS, by Nano- Drop (Thermo Scientific NanoDrop 2000 Spectrophotometer USA LTD) the absorbance was measured at 200 to 800 nm with a limited amount of impurities.

Limulus amebocyte lysate test (LALT)

Detection and quantization of isolated LPS from *A. tumefaciens* and *E. coli* were performed by using LAL reagent (PyroMed: Lot No 0409240) and control standard endotoxin *E. coli* strain 0111: B4 (Lot No 0505162, PyroMed Co Ltd Iran). The gel clot LALT method was conducted. In this procedure the LAL reagent was mixed with the test specimen and promptly incubated; the mixture was left undisturbed for 60 minutes at 37° C.

Determination of endotoxic activity of LPS

The gel-clot LAL (Limulus Amebocyte Lysate) method was used for determination of endotoxic activity of extracted LPS. At first serial ten-fold dilutions of the specimens were prepared by apyrogenic distilled water and the endotoxin activities were analyzed .Then two serial diluted specimens which showed positive and negative appearances (end point) were selected and serial two-fold dilutions of positive specimens were prepared and end point in gel- clot formation was determined. The *E.coli* 0111 B4 standard endotoxin as a positive control and negative control apyrogenic distilled water was used in every test. Experiments were performed with adding 0.2ml of each test specimens to Ambocyte Lysate tubes and mixed gently until the contents dissolved; then the reaction tubes were placed in a 37°C water bath for 60 min. A positive -result was deduced by formation of a gel clot capable of maintaining its integrity when the test tube is inverted. The sensitivity of used LAL-kits was 0.06 EU/ml of endotoxin units.

Macrophage preparation

To prepare peritoneal macrophages[17-19], 25 male BALB/C mice with mean weight 20 to 30 g were divided into five groups of five. Each group of mice was anesthetized with diethyl ether and then killed through the spinal cord. Then the mice were placed in 70 % ethanol for one minute. Then the abdominal area of animal was dried by sterile gauze. The abdominal skin was brought up by forceps and a slot was created with a scissor. After that, 10 ml cold phosphate buffer was injected into the peritoneum cavity. The abdominal skin on the peritoneum areas was massaged three to four times. The injected liquid was drawn using a Pasteur pipette and collected in a sterile container. From 10 ml of buffer injected into the peritoneum of mice, 5 ml of liquid containing macrophages was recovered. This cell suspension was centrifuged (1000 rpm, 3 minutes at room temperature). Supernatant was decanted and 20 ml culture medium (RPMI containing 10 percent calf serum and antibiotics) was added to the cell pack. This operation was repeated twice. By using trypan blue dye, the vital cell counting was carried out. The 100 µl of macrophage suspension with 100 µl trypan blue solution were mixed. Then a drop of it placed on the slide Neobar which was covered with glass and the live cells were counted. Finally, 10^4 cells/ml were obtained. Then 1ml of macrophage suspension was added into to each well of 48-well micro titer plate and incubated it for three hours. In this manner adherence of the macrophages to the bottom of the wells were monitored. The supernatant was decanted and the wells were washed three times using warm PBS. Then, 1 ml of cell culture medium (containing 10 % calf serum and 1% pensterp) was added and incubated it for 4 hours at 37° C and 5 percent CO_2. Finally, the supernatant was decanted slowly. The macrophages sticking to the floor were retained and the others were removed. The plates were used for subsequent testing.

Macrophages treated with LPS

Different concentrations of each LPS (62.5ng/ml, 125ng/ml, 250ng/ml, 500ng/ml and 1¼g/ml) were added to each of the three rows of 24 well micro titre plates containing at least 10^4 macrophage cells/well. In addition, three rows of wells without LPS served as negative controls and three rows of wells with reference LPS served as positive controls. Three rows of wells containing only RPMI with %10 FBS and %1 antibiotics served as blank controls. Then, the plates were incubated for 24 hours under sterile conditions at 37° C and 5 % CO_2 incubator. After that, the supernatant of each well was decanted; centrifuged and the nitrite (Nitric Oxide) measurements were performed.

Nitric Oxide measurement

Nitric Oxide was measured according to Griess methed [20- 22] with a minor modification. According to this protocol the following reagents

were prepared:

1 Sulfanilamide solution 2% (w/v); dissolution of 1 g sulfanilamide in 5 ml phosphoric acid
2 N - 1 - n a p t h y l e t h y l e n e d i a m i n e dihydrochloride (NED); dissolution of 0.01 g of the component in 10 ml D.w or RPMI 1640 culture media
3 Vanadium chloride solution H: dissolution of 8 mg of this compound in 1*M* hydrochloric acid (Vanadium chloride solution should be kept in dark color bottle at 4 ° C maximum for two weeks).

Sodium nitrite standard solutions with concentrations of 0, 20, 40, 60, 80 and 100 micro molar were prepared by using sequential dilution.

The nitrite (NO) measurement was performed on ELISA plates in a 100 µl sample or nitrite standard solution. Then 100 µl vanadium chloride solution was added. In addition, 50 µl NED and 50 µl Sulfanilamide solution was added respectively. In a row plate as blank wells, 100 µl vanadium chloride solutions, 50 µl hydrochloric acid 5% and 50 µl D.W were added. After adding the above materials, the plates were incubated at 37 ° C for 30 to 45 minutes. Then the optical density of dye formed was measured at 540 nm wave length. The blank well absorption values were subtracted from the standard or sample value and by using standard curve obtained samples and the concentrations of nitrite were calculated.

TNF-α measurement

The amount of TNF-α production in culture supernatant of macrophages treated with various concentrations of lipopolysaccharides was performed by using the ELISA Kit specific for the mice TNF-α (BM607/2) measurement. The results of nitric oxide and TNF-α measurement were analyzed with SPSS version 19.0.

RESULTS

The results of cultured *A. tumefaciens* and *E.coli* showed the total wet weight of 5 liter centrifugal sediments were 16.86 gram (3.37 g/l) and 21.23 gram (4.24 g/l) respectively. The average yield of available extracted LPS was 5 and 10 mg/l of purified material monitored at 220 *nm* wavelength absorbance. As shown in Fig1, proteins and nucleic acid contamination (see the peak two; in part A and C Fig1) were treated by enzymes (DNase, RNase and Protienkinase K). The LPS purification results indicated that the proteins and nucleic acid diminished almost completely (see part B and D Fig1).

However, the results suggest that the quantity of extraction and purification of LPS from *A. tumefaciens* and *E. coli* was dependant the culture volumes. Base on this study from 1 liter of 24 hours of culture 5 mg to 10 mg of LPS were obtained respectively.

The dose response test results showed a dilution of less than 1 microgram per milliliter of LPS provoked the macrophage response. Accordingly the final working dilution of 1000, 500, 250, 125.62, *ng*/ml of each LPS was prepared and used.

The result of preparation of mice peritoneal macrophages suggests that the injection of a cold phosphate buffer solution into the peritoneum cavity can isolate macrophages from the peritoneum. Injection of 10 ml of cold buffer recovered 5 ml macrophages suspension. However, the results of enumeration of macrophages suspension showed at least 10^5 cell/ml was obtained. The macrophage cell suspensions were washed using a centrifuge with RPMI culture medium to remove particles of fat. Then, further dilution of this suspension with 10^3 or more per ml was prepared.

The endotoxin activity of the LPS extracted from *A. tumefaciens* and *E.coli* was examined by Limulus Ambocyte Lysate test (LALT). The results indicated that 200 µl of ten-fold serial dilutions of each sample added to the lysates reference tubes formed a firm gel capable of maintaining its integrity when the test tube was inverted. This condition constituted a positive reaction. The end point of endotoxin activity was seen in $2.5x10^{-6}$ and $2x10^{-5}$ dilutions of LPS *E .coli* and *A. tumefaciens* respectively. Based on LALT the results showed that endotoxin activity of *A. tumefaciens* LPS was about 12.5 fold less than that for *E. coli* LPS.

The ANOVA analysis results demonstrated that measuring the amount of nitric oxide induced by LPS in macrophages was not significant (Table 1 part A: $P < 0.697$). However, the comparative results suggest that the amount of nitric oxide from macrophages treated with various concentrations of LPS with control samples have shown increases in nitric oxide concentration.

The overall analysis of data from the measurement of TNF-α in particular was significant (Table 1 part B: P < 0.002). Table 1 shows the effect of LPS on TNF-α production was significant (P <0.008). Similarly, the results suggest that the effect of LPS was significant (P <0.013). In addition, various concentrations of LPS on TNF-α production was significant over time (P <0.014).

As, shown in Table 2 the stimulating effects of 500 ng LPS concentrations extracted from *E. coli* had influence (Part A) while, the concentration of 1000 ng of the toxin had the most effective influence in stimulating the production of TNF-α. However, the effects of various concentrations of LPS extracted from *A. tumefaciens* had lesser effects.

Table 1. The results of Univariate Analysis of Variance of the effects of different concentrations of extracted LPS on Nitric Oxide (part A) and TNF- α production from mouse macrophages

Tests of Between-Subjects Effects
Part A: dependent Variable : Nitric Oxide

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	.692a	87	.008	2.060	.000
Intercept	3.064	1	3.064	793.671	.000
LPS	.003	2	.001	.362	.697
concen	.029	5	.006	1.513	.190
time	.163	2	.082	21.164	.000
LPS * concen	.036	10	.004	.931	.508
LPS * time	.022	4	.005	1.405	.236
concen * time	.039	10	.004	.998	.449
LPS * concen * time	.078	20	.004	1.016	.449
Error	.494	128	.004	-	-
Total	4.250	216	-	-	-
Corrected Total	1.186	215	-	-	-

a. R Squared = .583 (Adjusted R Squared = .300) - —

Tests of Between-Subjects Effects
Part B: Dependent Variable: TNF- α

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	3.336[a]	67	.050	10.998	.000
Intercept	10.558	1	10.558	2331.787	.000
LPS	.067	2	.034	7.445	.008
concen	.054	2	.027	5.968	.016
time	1.145	3	.382	84.257	.000
LPS * concen	.034	4	.009	1.880	.179
LPS * time	.092	6	.015	3.393	.034
concen * time	.119	6	.020	4.386	.014
LPS * concen * time	.081	12	.007	1.500	.247
Error	.054	12	.005	-	-
Total	16.125	80	-	-	-
Corrected Total	3.391	79	-	-	-

a. R Squared = .984 (Adjusted R Squared = .895)

Table 2. The results of the effects of different concentration of extracted LPS *E. coli* and *A. tumefaciens* on Nitric Oxide (part A) and TNF- α production from mouse macrophages?

Part A: Dependant variable on Nitric Oxide production					
LPS	Concen	Mean	Std. Error	95% Confidence Interval	
				Lower Bound	Upper Bound
E. coli	Control	.076	.025	.026	.126
	1000n	.127	.025	.077	.178
	500n	.266	.025	.216	.317
	250n	.116	.025	.065	.166
	125n	.104	.025	.054	.154
	62.5n	.102	.025	.051	.152
A.tumefacience	Control	.077	.025	.027	.128
	1000n	.225	.025	.055	.315
	500n	.172	.025	.152	.252
	250n	.111	.025	.060	.161
	125n	.088	.025	.038	.138
	62.5n	.091	.025	.040	.141
Part B: Dependent Variable on TNF- α					
LPS	Concen	Mean	Std. Error	95% Confidence Interval	
				Lower Bound	Upper Bound
E. coli	Control	.292	.024	.240	.344
	1000n	.509	.024	.458	.561
	500n	.456	.024	.404	.508
	250n	.370	.024	.318	.422
	125n	ND	ND-	ND	ND
	62.5n	ND	ND	ND	ND
A.tumefacience	Control	.290	.024	.240	.344
	1000n	.393	.024	.341	.445
	500n	.372	.024	.320	.424
	250n	.348	.024	.296	.400
	125n	ND	ND	ND	ND
	62.5n	ND	ND	ND	ND

DISCUSSION

In this study, production and purification of LPS showed that the method of Westphall can be appropriate for isolation of LPS from *A. tumefaciens* and *E.coli*. In comparison one of the interesting findings was that the obtained product was much less than other studies[23]. But in terms of biological activity was much more effective than other studies. Results of this study showed that the effect of 1000 ng LPS *A. tumefaciens* stimulating macrophages to produce nitric oxide was equivalent of 500 ng LPS *E.coli*. Nitric oxide produced by macrophages treated with 1000 ng LPS *E.coli* was similar to the control groups. This suggests that macrophage cells exposed to 1000 ng LPS-mediated nitric oxide production capacity is low. This may be due to toxic effects of LPS *E.coli* on macrophage activity. Hence, the effects of LPS *E. coli* on inducing macrophages to produce TNF-α was more than that of the effects of LPS of *A. tumefacien* (1000 ng LPS of *E.coli* was 0.509 TNF-α production, while the effects of 1000 *ng* of LPS *A. tumefaciens* on TNF-α production was 0.392). This probably indicates that toxicity of LPS *A. tumefaciens* is less than that of LPS *E.coli*.

However, this study was designed to answer the question, whether or not the effect of LPS derived from *A. tumefaciens* as a plant pathogenic bacterium can stimulate macrophages

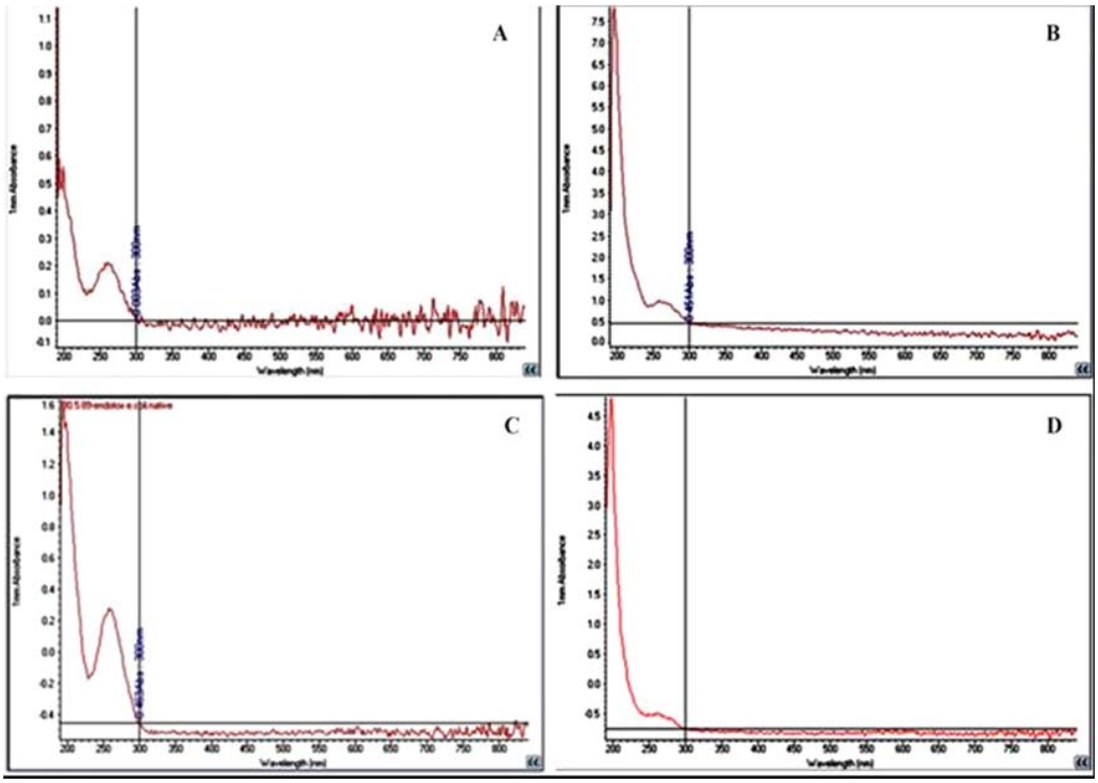

Fig. 1. The results of absorbance scanning extracted LPS solution are shown. Sections A and C, demonstrated the solution of crude LPS extracted from *A. tumefaciens* and *E. coli* before enzymatic treatment respectively, are shown. The area under curves revealed the concentration of the product. As scanning the wavelength range 200 to 800 nm with Nanodrop the second peak in the range of 260 to 280 nm which were protein and nucleic acid contamination indicated that after enzymatic treatment and dialysis solution of purified LPS, the second peaks are completely removed (see section B and D)

in the same manner of LPS extracted from *E. coli* as a human pathogenic bacteria. Although, a lot of research had been carried out on the effect on LPS-stimulated macrophages, the effects of plant pathogenic bacteria LPS have not been compared with LPS of human pathogenic bacteria to assess the stimulatory effect on macrophages. Hence, this study was unique in this regard.

In this study, two important mediators of immunity namely nitric oxide and TNF- α were addressed. Because previous researchers have shown that, the two principal immunological mediator secreted by activated macrophages are TNF- α and nitric oxide[24-27]. TNF- α is a multifunctional cytokine involved in many different pathways, in homeostasis and pathophysiological of mammals[28]. The effects of some biological substances on macrophage stimulatory cytokine production have been investigated. It has been shown that an adjuvant stimulated macrophage IL-10, IL-6, and IL-1beta production, whereas it decreased IL-12, TNF- α and nitric oxide production[29].

In our study, the LPS of *A. Tumefaciens* produced low levels of immune mediators. This indicates that, *A. Tumefaciens* LPS could be considered an adjuvant candidate. This issue however, warrants more research.

Furthermore, the actions of TNF- α on the endothelium and leukocytes are critical for local inflammatory responses to microbes[30]. If inadequate quantities of TNF- α are present a consequence may be failure to contain infections. However, the ability of this cytokine to cause necrosis of tumors, which is the basis of its nomenclature, is mainly a result of thrombosis of

tumor blood vessels[31]. High circulating levels of TNF- α cause's severe metabolic disturbances, such as a fall in blood glucose concentrations to levels incompatible with life. For the aforementioned reasons, the endotoxin-induced large amounts of TNF-α cannot be considered for an adjuvant. This is also true for endotoxin extracted from *E. coli*.

Nitric oxide has potent antimicrobial activity; it also can combine with the superoxide anion to yield even more potent antimicrobial substances. Recent evidence suggests that much of the antimicrobial activity of macrophages against bacteria, fungi, parasitic worms, and protozoa is due to nitric oxide and substances derived from it.

It is well established that, macrophages activated by exposure to lipopolysaccharides (endotoxins) produce nitric oxide, a free radical that is a mediator of the host response to infection[32]. Incubation of mouse macrophages with LPS extracted from *E. coli* and *A. tumefaciens* at concentrations 10 to 1000 ng/ml resulted in different amounts of nitric oxide secreted. In one study, 1-10 µg/ml LPS was used for enhanced inducible nitric oxide[33]. This amount was 10 times more than the amount used in this study.

Other researchers used 100 to 1000 ng/ml LPS to stimulate macrophages to produce nitric oxide which, is consistent with the amount used in this study[34, 35].

CONCLUSION

The extracted endotoxins in this study induced peritoneal mice macrophages to produce nitric oxide and TNF-α. The findings of this study suggest that the effect of 1000 ng LPS from *A. tumefaciens* was equal to 500 ng LPS from *E.coli* in stimulating macrophages to produce TNF-α and nitric oxide. In other words, the endotoxin of *A. tumefaciens* was weaker in stimulating and producing these mediators than the *E.coli* endotoxin; 1000 *ng*/ml endotoxin *A. tumefaciens* produced an amount of less than 500 ng/ml of *E.coli* endotoxin. Thus, demonstrating immuno-modulatory effectiveness with less toxicity.

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Conflict of Interests

Authors have no conflict of interests

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