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RESEARCH ARTICLE

PLACENTAL LIPOPOLYSACCHARIDE ENHANCES MACROPHAGE ACTIVATION

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ABSTRACT

The purpose of this study was to assess the effect of human placental lipopolysaccharide (HP-LPS) on activation of macrophages. We isolated and characterized the lipopolysaccharide from human placenta and *E. coli* by Westphal's method. The effects of different concentrations of *E. coli* and HP- LPS were assayed on mice macrophages and nitric oxide production was measured using Griess's method; data were then analyzed. Results showed that using the Westphal method, HP- LPS could be isolated as well as Gram-negative bacterial LPS. The results indicate that the HP- LPS had ability to stimulate mice macrophages to produce nitric oxide. Detoxified LPS Gram-negative bacteria in concentrations of more than 500 ng/ml showed toxicity. Whereas, HP- LPS at concentration of 1000 ng/ml had a significant effect on nitric oxide production. In addition, the isolated HP- LPS had no pyrogenic effects. The findings of this study suggest that the HP- LPS shows activity similar to Gram-negative bacterial endotoxin which stimulates macrophages to produce nitric oxide. These properties indicate that this substance acts as a detoxified Gram-negative bacterial endotoxin. Because of its human origin, it has no toxic effects and may be a safe adjuvant.

INTRODUCTION

Global vaccination depends on the existence of effective and safe adjuvant and several types of adjuvant are available (Steerenberg *et al*., 2004). It is well-established that, the Gram-negative bacterial lipopolysaccharide (LPS), called endotoxin can elicit a wide range of immunological effects and also act as a strong adjuvant, but it´s pyrogenicity has precluded its uses (Switalla *et al*., 2010). However, detoxified endotoxins have acquired approval for use in developed countries as new adjuvants (Casella *et al*., 2008 and Mata-Haro *et al*., 2007). Monophosphoryl lipid-A (MPL- A) is a detoxified form of the endotoxin and is among the first of a new generation of Toll-like receptor agonists, likely to be used as a vaccine adjuvant on a mass scale in human populations (Smit *et al*., 2008). In addition, results of some investigations indicate that supplementation with LPS analog forms are a promising to improve acellular pertussis vaccines (Geurtsen *et al*., 2007). Likewise, LPS is an inflammatory agent causing induction of inducible nitric oxide synthase (iNOS) expression and nitric oxide (NO) production by macrophages (Sareila *et al*., 2008). A recent study has revealed that the synthetic compounds containing lipids linked to a phosphate-containing acyclic backbone are shown to have biological properties similar to LPS which behave as agonists of TLR4, resulting in responses similar to those elicited by LPS (Hawkins *et al*., 2002). Moreover, it has been reported that the LPS from animal and human placentas exhibit strong biological activity with no toxicity (Haiping *et al*., 2008). Thus, there is a possibility that LPS from human placenta could enhance the immune function. If so, the LPS from human placenta might have the potential to stimulate macrophages and enhance their antibacterial activity, especially to increase the nitric oxide (NO) production. The aim of the present study was to isolate lipopolysaccharide from the human placenta and examine its effects on activation of mice macrophages to produce nitric oxide (NO) and to compare it with the standard commercial *E. coli* LPS.

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MATERIALS AND METHODS

Isolation of LPS from human placenta

Human placenta was obtained within 10 min of normal-term delivery. It was stripped into a nylon bag and transferred to the laboratory and washed several times with depyrogenated D.S water. Washing continued until the discharge of bloody plasma stopped. Then the tissue was grinded and the water was removed. After complete removal of surface water, the placental tissue was weighed. The placental tissue was cut into segments with a sterile knife. Using a meat grinder, the segments were crushed and 10 g of tissue was transferred to a sterile container and stored at -20°C. LPS was extracted from 10 g of crushed frozen placenta tissue thawed for three times by the Westphal *et al*. method (Westphal *et al*., 1952 and Ataee *et al*., 2012) with some minor modifications. Then, 10 g of crushed placenta was homogenized in 20 ml PBS buffer (0.02 M, pH 7.2 ± 0.2) 1:2 (w/v) and mixed for 2 min in a Sorvall Omni-Mixer

(Sorvall Inc., USA). Then, it was maintained in container at 60 °C hot water bath shaker. After equilibration of the temperature, hot phenol–water (1:1, v/v) solution was added in to the homogenated tissue and maintained at 60 °C for 30 min. and centrifuged at 6000×g for 30 min (Keler *et al*., 1986). The supernatant was retained and dialyzed (Dialysis Sacks, Cat No 250-7u, Lot 10H-6134. Sigma) at room temperature against distilled water for at least 36 hours or until the smell of phenol disappeared. Then, 2 units/ml enzyme DNase I (Cinnagen, Iran) was added to the supernatant and maintained at 45°C, for 60 min (pH 5 was adjusted); after that, 2 units/ml RNase (Cinnagen, Iran) at 37 °C and pH 8.0, for 4 h. was dialyzed. The solution was dialyzed against distilled water four times overnight at 4 °C. To remove protein contaminant. Proteinase K was then added to a final concentration of 10µg/ml. The suspension was heated in a water bath at 60°C for 1 h, and then overnight at 37°C, and then dialyzed once again against double-distilled water for five exchanges. Finally, it was lyophilized and until next application was kept refrigerated. All the water used in this study was free of pyrogens.

Lipopolysaccharide measurement

During the proccesses, isolated LPS from placenta was determined by absorption in 220 to 280 nm wavelengths and also according to the methylene blue dying method described previously (Siamon *et al*., 2002). The dying solution was prepared by dissolving 1.6 mg 1, 9- dimethylamine methylene blue (Sigma, USA) in 5 ml ethanol (80%, v/v), adding 3.33 g NaCl and diluted to 100 ml with glycine – NaOH buffer (pH 10.1, 0.057M). It was then stored in a colored glass bottle. Serial standard lipopolysaccharide (1, 10, 50, 100, 200, 300, 400 and 500 ng) from *Escherichia coli* 055:B5 (Sigma, USA) were dissolved in 0.1 ml injectable distilled water and then mixed with 3ml dying solutions. All the mixed solutions were determined spectrophotometrically at 544 nm wavelength. Blank control was made by 0.1 ml injectable-distilled water mixed with 3 ml dying solution. Based on the data, the standard curve of absorbance versus LPS was determined. The sample content was determined using the same method stated above and the lipopolysaccharide content was read from the standard curve. In addition, in order to assess quality of the isolation and purification of LPS, SDS-PAGE was also performed.

Pyrogen test

The pyrogen test was carried out by interaperitoneal injection of different doses in normal New Zealand rabbits. The isolated LPS in our study could not produce fever in rabbits and were considered safe at the doses of this experiment.

Limulus Amebocyte Lysate (LAL) test

Detection and quantization of isolated LPS from human placenta was performed by using LAL reagent Lot No 0409240 and control standard endotoxin *E. coli* strain o111: B4 Lot No 0505162 purchased from PyroMed Co Ltd, Iran. The gel clot LAL test method was conducted based on kit instructions. In this procedure the LAL reagent was mixed with test specimen and the mixture was promptly incubated undisturbed for 60 minutes at 37 °C. A positive response on the gel clot test indicated that there was an amount of endotoxin in the sample which equals the reagent's labeled sensitivity.

Macrophage preparation

To prepare peritoneal macrophages, 25 male white mice with a mean weight of 30g were purchased from the Pasteur Institute of Iran, and divided into five groups of five mice each. Each group of mice was anesthetized with ether and then they were sacrificed by spinal cord section. Mice were placed in 70 % ethanol for one minute. Then the abdominal area of animal was dried using sterile gauze. Under aseptic conditions, the abdominal skin was lifted by forceps and with scissor a slot was created. After that, using a plastic pasture pipette, 5 ml cold RPMI 1640 containing sodium bicarbonate was injected into the peritoneal cavity. The abdominal skin on the peritoneum areas was given massage several times. Then the injected liquid was drawn using a Pasteur pipette and collected in a sterile container. In this case, a little change of color occurred in the liquid. From 15 ml of medium injected into the peritoneum of mice, almost 15 ml of liquid containing macrophages was recovered. This cell suspension was centrifuged (2000 rpm, 3 minutes at lab temperature). Supernatant was decanted and then 20 ml culture medium (cell culture containing 10 percent calf serum and antibiotics) was added to the cell pack. This procedure was repeated twice. Using trypan blue dye, the vital cell counting was carried out. The 100 µl of macrophage suspension with 100 µl trypan blue solution was mixed. Then a drop of it was placed on the slide, covered with glass cover slip and live cells were counted using 40 X magnification. Finally 10^4 cells per ml were obtained. Then 100 µl of macrophage suspension was added to each well of a 48-well microtiter plate. In addition, 100 µl of cell culture medium (containing 10 % calf serum) was added and incubated for 4 hours at 37 °C and 5 % CO_2. The supernatant was then decanted slowly. In this case the macrophages which were sticking out at the floor well remained and others were removed. Then, the 200 µl of sterile cell culture medium was added to each well. The plates were incubated (24 hours in 37 °C and 5 % CO_2). After that, the supernatant was removed and the plates were maintained in -80 °C for subsequent testing.

Nitric oxide measurement

Nitric oxide was measured by the method of Griess with some modifications (Grisham *et al*., 1996; Misko *et al*., 1993 and Green *et al*., 1982). According to this protocol, the following reagents were prepared:

- Sulfanilamide solution 2% (w / v); by the dissolution of 1 gram Sulfanilamide in 5 ml phosphoric acid
- N-1-napthylethylenediamine dihydrochloride (NED); by the dissolution of 0.01 gram of the component in 10 ml d.w. or RPMI 1640 culture media
- Vanadium chloride solution ш: by the dissolution of 8 mg of this compound in 1M hydrochloric acid.

Vanadium chloride solution should was kept in a dark colored bottle at 4 °C maximum for a maximum two weeks. Sodium nitrite standard solutions with concentrations of 0, 20, 40, 60, 80 and 100 micromolar by using sequential dilution were prepared. The measurement of nitrite (NO) was performed in 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 a blank well, 100 µl vanadium chloride solutions, 50 µl hydrochloric acid 5% and 50 µl d.w. was added. After adding the above, 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 substracted from the standard or sample value and by using standard curve obtained from the samples the concentrations of nitrite was calculated.

Macrophages treated with LPS

In order to evaluate the effect of human placental lipopolysaccharide to induce nitric oxide production by macrophages, different concentrations of LPS (10 ng/ml, 50 ng/ml, 100 ng/ml, 500 ng/ml and 1 µg/ml) were added to each of the three rows of 96 well microtitre plate which contained at least 10^3 macrophage cells / well. In addition, three rows of wells without LPS as negative controls and three rows of wells with reference LPS were used as positive controls. Three rows of wells containing RPMI with 20 percent FBS were used as blanks. Then, the plates were incubated 24 hours under sterile conditions at 37 °C and 5 percent CO_2. After that, the supernatant of

each well was decanted and the nitrite (nitric oxide) measurements were performed.

RESULTS

Placental LPS extraction

The results of LPS extraction and purification showed that the hot water- phenol could be a suitable procedure for this purpose. Centrifuge suspension of mixture prepared from human placenta with the same volume of hot water- phenol revealed four distinct phases (Fig. 1), the assay suggested that the LPS as entered an aqueous phase. The volume of the upper phase was almost 12 ml.

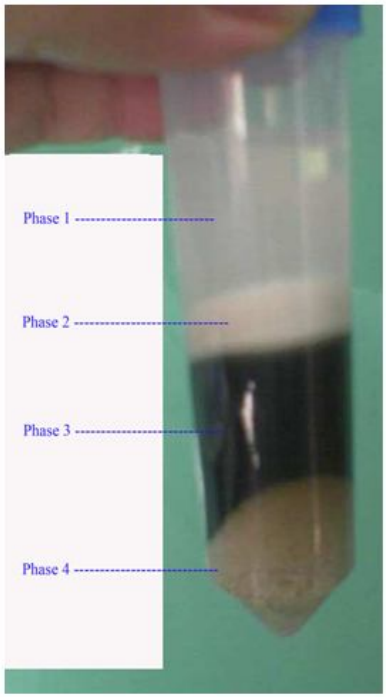
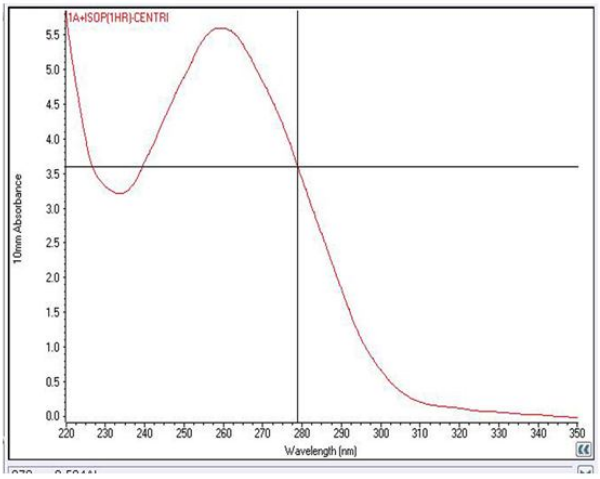
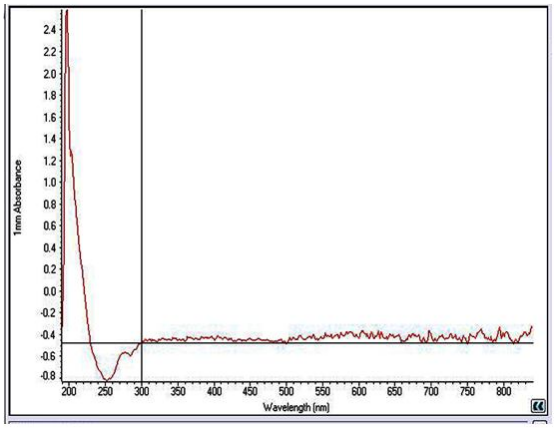


Fig 1. The test tube contain 20 ml D.W which was added to 20 g of human placental tissue. Then, it was placed in boling water bath for 30 min, the temperature was brought to 68 °C, after that, the same volume of phenol 90% was added and maintained in a water bath 68 °C for 30 minutes. Then centrifugation was carried out. As shown, the centrifuge has four phase. The upper phase is LPS water soluble. The second phase are insoluble lipids in water. The third phase are packed color components of placenta cells and the fourth phase contained cellular debris.



A)



B)

Fig 2. part A) a large peak of absorption in 220 t0 280 nm representing protein and nucleic acid impurities. As showed in part B, the results of LPS solution treatment with DNase and Proteinase K enzyme after one hour at 60 °C and overnight incubation at 37 °C indicated the removed protein and nucleic acid contamination.

Dialysis of the upper phase solutions against distilled water was done during 48 hours and the dialysate replaced six times until the smell of phenol disappeared. In other words, the phenol solution was removed completely. The results of UV scan of dialysated of upper phase solutions at wavelengths from 200 to 1000 nm, showed considerable protein and nucleic acid impurities. After protease and nuclease treatment, they were removed (Fig 2A & B). LAL test results showed that LPS extracted from human placenta as well as *E. coli* endotoxin is able to react with amebocyte lysate reagent and create a gel clot which expressed a positive response. The test result was achieved at neutral pH. Also, it was shown that formation of gel clots were dependent on LPS concentration and time consumption so that whenever LPS concentration was increased, the gel clots occurred in less time.

Nitrite Measurement

Results of measurements of nitrite (as nitric oxide) of macrophages supernatant culture are shown in Table 1.

Table 1. Average concentrations of nitrite in supernatant macrophages culture three times and two repeat experiments as controls

	Nitrite concentration		
Plate A	Well 1	Well 2	Well 3
	9.7	5.9	7.3
Plate B	10.5	10.3	8.7
mean	10.1	9.9	8

Table 2. Results of measuring nitrite concentration (µmol/ml) in macrophages culture supernatant which was treated with various concentrations of LPS of different origins

Concentration / Kined of LPS	1µg/ml	500ng/ml	100ng/ml	50ng/ml	10ng/ml	1 ng/ml	0 ng/ml
HP- LPS	48.4	48.2	39.5	32.1	30	26.5	9.8
E.coli LPS	46.4	62.5	66.3	45.7	38	27.2	9.4

As shown in Table 2, the measurement results of nitrite concentration in macrophages culture supernatant revealed that macrophages treated with various concentration of LPS extracted from human placenta could stimulate the production of nitrite oxide as well as commercial LPS from *E. coli* strain 0111 B4. Univariate analysis of variance and one sample Kolmogorov Smirnov test showed significant data (P- value ≤ 0.001).

The effects of different concentrations of LPS isolated from placenta and LPS *E.coli* on the macrophages to induce nitric oxide production are shown in Table 2.

DISCUSSION

Our study demonstrated that human placental lipopolysaccharide has the ability to stimulate peritoneal mice macrophages to produce nitric oxide almost equivalent to reference LPS and results showed no significant difference between them. Perhaps the human placental lipopolysaccharide could act as a natural adjuvant. Although, further investigations are required. In some Asian countries the animal placentas are dried and prescribed as a drug for treatment patients with physical weakness. In recent years, some researchers have shown that LPS isolated from cow placenta has antitumor and immunomodulatory activity [9]. Because we could not find any published data, it was not possible for us to compare our data with other researches. In other words, no published report exists in this field. One interesting finding of this study was that the macrophages treated with concentrations more than 500 ng/ml of *E.coli* LPS reduce nitric oxide production (Table 2). While increasing the concentration to 1000 ng/ml of placental LPS had no effect on n nitric oxide production. It is well established that the hallmark of classical activation of macrophages in mice and probably in humans is the generation of nitric oxide by an inducible nitric oxide synthase in LPS- challenged cells (Marletta *et al*., 1988).

Thus, there are many studies showing that LPS stimulates the macrophages and increases their activity (Pioli *et al*., 2006; Sun *et al*., 2010 and Su *et al.*, 2006). In this investigation, by the use of the standard extraction LPS method, a lipid was extracted from placenta which acts similar to bacterial LPS which is interesting as well as controversial. Because it might be thought that this lipid-derived from placenta causes bacterial infection. To reject this hypothesis use of standard bacteriological tests failed to show bacterial contamination. A demographic study revealed that a woman in the last trimester did not have bacterial infection and had vaginal delivery. These results demonstrated that separated lipids from the source was not because of contamination and most likely of placental origin. The results of some studies indicated that nitric oxide is a gaseous free radical with a short half-life in vivo of a few seconds (Tsikas *et al*., 2005). Therefore, the levels of the more stable NO metabolites, nitrite (NO_2) and nitrate (NO_3), have been used as the indirect measurement of NO in biological fluids (Wennmalm *et al*., 1993 and Manukhina *et al*., 2000). In this study, we prepared consecutive concentration of sodium nitrite and then performed standard curve. The nitrite concentrations in the supernatant of cultured macrophages as a representative of nitric oxide were measured. Some researchers have shown that the levels of nitric oxide are associated with reproduction and other physiological changes (Rosselli *et al*., 1994). In fact, several researchers have shown that the level of nitric oxide and other immune mediators including TNF-α are increased during the natural childbirth. But the exact mechanisms and initial inducing agent remained unknown. The results of this study revealed that lipopolysaccharide isolated from the placenta could be one of the inducing stimuli as a potential source of nitric oxide.

Conclusion

In this study, human placental LPS has been isolated and examined for its immunomediatory activity on mice macrophages. The results showed that human placental LPS was an effective agent to induce nitric oxide production. Furthermore, it was found to be devoid of pyrogenic response in the rabbits. These results indicate that isolated LPS in this study exhibited significant activity on mice macrophages compared with the reference *E. coli* LPS. However, much remains to be learned about this compound's mechanism of action, but recent developments have made clear that it is unlikely to be simply a weak version of lipopolysaccharide.

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Conflict of Interests

Authors have no conflict of interests.

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