



The effect of lipopolysaccharide extracted from *Escherichia coli* on total WBCs, granulocytes and on phagocytic activity in female rats

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Abstract

The present study was conducted to compare the total white blood cells count, granulocytes and phagocytic activity in female rats immunized with lipopolysaccharide (LPS) extracted from two different isolates of *Escherichia coli*, one normal isolate from the gut of healthy adult and the other was pathogenic strain isolated from patients suffering from urinary tract infection. The study was included 35 female albino rats divided randomly into seven groups, three groups injected with different concentrations of LPS of normal flora of *E.coli* 90, 120 and 150 microgram/kilogram body weight respectively and the other three groups were injected with the same concentrations of LPS extracted from pathogenic strain of *E.coli*, the last group (7th) represented the control group which given normal saline only. The results showed that the LPS from normal isolate causes a significant increase in the total number of leukocytes, granulocytes and phagocytic activity in animals immunized with the concentration of 120 $\mu\text{g}/\text{kg}$ compared with the control group, and the concentration of 150 $\mu\text{g}/\text{kg}$ of the same extract resulted in a high significant increase in phagocytic activity and the concentration of 90 $\mu\text{g}/\text{kg}$ did not stimulate any significant differences in all the variables, while immunization with the extract of pathogenic strain increases the total leukocytes at a concentration of 90 $\mu\text{g}/\text{kg}$ only with a significant increases in phagocytic activity at all concentration, and it also did not cause any significant differences in the granulocytes count.

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Introduction

Human and animal gut represented a good niche for a large numbers of bacteria, viruses and fungi, which may be present as a commensals named as gut microbiota (1). *E.coli* belongs to the family of Enterobacteriaceae, it normally inhabits the intestine, many pathogenic strains of it may cause different diseases as infantile diarrhea, urinary tract infection as 90% of this disease was caused by *E.coli*, especially in female and this strain causing (UTI) and called Uropathogenic *E.coli*, in addition to other infections like burn and wound infections and others (2). Gram-negative bacteria are characterized by an external envelope contains two different membranes: an inner membrane (IM) internal components, and an outer membrane (OM) that

encircles the cell from its surrounding environment, the OM was regarded as the first line of defense against external threats and composed of a highly asymmetric bilayer that contains phospholipids in the inner leaflet and lipopolysaccharide (LPS) molecules in the outer leaflet (3). Lipopolysaccharide is a pyrogen and may have an immune stimulatory activity and is a glycolipid composed of three distinct structural domains: lipid A, the core oligosaccharide part, and the O-antigen, Lipid A moiety considered as a hydrophobic part of the molecule and is an acylated β -1'-6-linked glucosamine disaccharide that forms the outer leaflet of the OM and responsible for the toxic activity of the bacteria (endotoxin) and triggers physiological immunostimulatory activity in mammals and at higher doses can also lead to pathological reactions, The

core oligosaccharide part of OM is a non-repeating oligosaccharide that is linked to the glucosamine of lipid A moiety, This part of LPS was responsible for the immunogenic properties, The O antigen is highly variables an extended polysaccharide that is attached to the core oligosaccharide part. It is composed of a repeating oligosaccharide made of 2-8 sugars (4). After liberation of LPS from Gram negative bacteria cell wall it associates with a glycoproteins present in serum called lipid binding protein (LBP), which can transport the LPS to the surface of antigen presenting cells and then a series of intracellular signals begins through the specific receptor for LPS named as toll like receptor 4 through a pathway called (toll like receptor pathway), increased amounts of lipopolysaccharide in serum may cause vessels destruction and may lead to death through septic shock (5). Leukocytes were considered as a part of the immune system that participate in both innate and adaptive immunity, they circulate in the blood stream and heighten inflammatory and cellular immune response against injury and different pathogens. Neutrophils are multinucleated effector cells that form the first line of defense against invading pathogens. They fight infections through a variety of mechanisms, including chemotaxis, phagocytosis, the release of reactive oxygen species (ROS), and the release of a wide range of cytokines (6).

The aim of present was to compare the some immune parameters included Total leukocytes, granulocytes and phagocytic activity in female rats immunized with LPS of two different strain of *E.coli*.

Materials and methods

Biofilm formation assay

To confirm the pathogenicity of *E.coli*, the isolates were tested for their activity to grow in a biofilm (7), briefly in this method, brain heart infusion broth (BHIB) containing tubes were inoculated with the isolated bacteria, incubated at 37°C for 24h, then the content of the tubes was poured and drops of crystal violet was added to the tubes with gentle rotation, left for two minutes, then the tubes placed upside down on a filter paper after rinsing with distilled water to remove excess stain.

Hemolytic assay

Hemolytic activity of *E.coli* were tested by cultivation of bacteria on blood agar plates then incubated at 37°C for 24h, a clear zone around the colonies, indicating a positive result (8).

Selection of isolates for LPS extraction

Stool isolate lacking the ability for biofilm formation and hemolytic activity on blood agar were selected for LPS isolation as a Normal Flora, while urine isolate with the highest biofilm formation activity and hemolytic activity were selected for LPS extraction as a pathogenic isolate.

Extraction protocol of lipopolysaccharide

In brief, 250 ml of the 24h bacterial growth in brain heart infusion broth (BHIB) were centrifuged at 6000 rpm for 20 minutes, the sediment washed with phosphate buffered saline (PBS) twice then subjected to 15 ml of lysis buffer containing Tris HCl, MgCl₂, SDS and β-mercaptoethanol, then placed in water bath at 65°C for 1 hour to solubilize the sediment, then, 1 ml of (proteinase K) was added to the preparation to remove contaminants proteins, preparation was incubated at 37°C for 24h, then LPS was precipitated at -20° C using 3M sodium acetate and cold absolute ethanol, after final centrifugation, the pellet was re-suspended in 9 ml of 10 mM Tris-HCl followed by extraction using hot phenol, where 9 ml of phenol at 65°C was added and mixed vigorously and then was immediately placed in an ice bath, preparation was centrifuged at 6000 rpm for 15 min and the top aqueous layer was taken, and the extraction was repeated again, then the aqueous layers of both extract were subjected to dialysis for 48h against distilled water, and the LPS was lyophilized and stored at -20°C until use (9).

Experimental animals

Thirty-five Swiss adult female rats of 8-9 weeks age and weights between 145-170 grams were obtained from the Animals House, College of Veterinary Medicine, University of Mosul were kept under standard laboratory condition (10,11), they were given standard locally prepared diets (12) and were placed in cages and were acclimatized for a week (13,14).

Experimental design

Thirty five female albino rats were used, they were divided into 7 groups (5 rats each), six groups were the experimental groups and the last was the control group, the first 3 groups were injected intraperitoneally with three different concentrations of LPS extracted from the pathogenic strain, while the second three groups were injected intraperitoneally with the same concentrations of LPS extracted from the Normal floral isolate, the last 7th group was left as a control and was given Normal saline, all concentrations were injected with 3 repeated doses 48h between them, after 7 days of immunization with LPS, the blood was collected using retro orbital blood collection technique and placed in EDTA tubes.

Tests

Leukocyte and granulocytes count were done using automated blood analyzer (celltac α japan) and phagocytic activity (15), in brief, 0.1 ml of candida spores suspension was mixed with 0.1 ml of whole blood followed by gentle mixing, incubated at 37°C for 30 min, then blood smears were prepared and stained with giemsa stain. The phagocytic activity of neutrophil was examined microscopically and calculated by (the number of

neutrophils with the engulfed candida/ total number of neutrophils)*100.

Statistical analysis

The data was summarized, analyzed, and presented using the Statistical analysis for Social Science version 23 software program and the difference in mean of quantitative variables between groups was investigated using a one-way ANOVA, which was accompanied by a post hoc Duncan multiple range test.

Results

Table 1 showed the results of the immunogenic effect of intraperitoneal injection of three concentrations 90, 120 and 150 µg/kg body weight of LPS extracted from normal flora and uropathogenic *E.coli* on total leukocyte counts and granulocytes $10^3/\mu\text{l}$, the results showed that there were a significant differences between groups immunized with LPS extracted from Normal flora *E.coli* and groups immunized with LPS extracted from uropathogenic isolate. At concentration 90µg/kg body weight, the animals group immunized with LPS of uropathogenic isolate showed highest significant increase in total leukocytes $24.93 \times 10^3/\text{mm}^3 \pm 0.72$ compared with the control group $16.07 \times 10^3/\text{mm}^3 \pm 1.28$ and animals group immunized with the same concentration of LPS extracted from Normal isolate flora $17.17 \times 10^3/\text{mm}^3 \pm 1.57$. in contrast, LPS

extracted from Normal flora *E.coli* induced highest significant increase in total leukocytes only at concentration 120µg/kg body weight $20 \times 10^3/\text{mm}^3 \pm 0.53$ compared with control group and animal group immunized with the same concentration of LPS extracted from uropathogenic strain $16.33 \pm 0.77 \times 10^3/\text{mm}^3$, the results also showed a significant increase in granulocytes in animal groups immunized with both 120 and 150 µg/kg body weight of LPS of Normal isolate 15.50 ± 2.79 , $13.1 \pm 2.42 \times 10^3/\text{mm}^3$ compared with the control group $7.47 \pm 1.81 \times 10^3/\text{mm}^3$ and the animals groups immunized with the same concentrations of LPS extracted from uropathogenic strain 7.73 ± 1.76 , $9.63 \pm 1.57 \times 10^3/\text{mm}^3$.

Table 2 showed the comparative effect of LPS of both strain on phagocytic activity of female rats, the results showed that LPS extracted from normal floral isolate induced a highest significant increase in phagocytic activity after immunization with concentration of 120 and 150µg/kg body weight $92.67\% \pm 0.88$ and $90.67\% \pm 0.67$ respectively compared with control group $70\% \pm 1.15$ and groups immunized with the same concentration of LPS of uropathogenic isolate $86\% \pm 1.15$ and $80.67\% \pm 0.67$ for both concentrations respectively.

In contrast, LPS of uropathogenic isolate induced highest significant increase in phagocytic activity after immunization with the concentration 90µg/kg body weight $92\% \pm 1.86$ compared with control group and animals group immunize with the same concentration of LPS of normal isolate $72.33\% \pm 0.88$.

Table 1: Total Number of leukocytes and granulocytes counts in animal's groups injected with LPS of Normal flora and uropathogenic *E.coli* compared with control

	Total WBC ($10^3/\text{mm}^3$)±SEM		Granulocytes ($10^3/\text{mm}^3$)±SEM	
	Normal flora	Uropathogenic	Normal flora	Uropathogenic
90µg/kg	17.17±1.57 ^{ab}	24.93±0.72 ^c	5.43±2.71 ^a	8.07±0.52 ^{ab}
120µg/kg	20±0.53 ^b	16.33±0.77 ^a	15.50±2.79 ^c	7.73±1.76 ^{ab}
150µg/kg	15.43±0.72 ^a	15.83±0.81 ^a	13.1±2.42 ^{bc}	9.63±1.57 ^{abc}
Control	16.07±1.28 ^a		7.47±1.81 ^{ab}	

Letters are used to express comparison; dissimilar letters indicate a significant difference.

Table 2: Percentage of phagocytic activity in animal's groups injected with LPS of normal flora and uropathogenic *E.coli*

	Phagocytic activity%±SEM	
	Normal flora	Uropathogenic
90µg/kg	72.33%±0.88 ^a	92%±1.86 ^d
120µg/kg	92.67%±0.88 ^d	86%±1.15 ^c
150µg/kg	90.67%±0.67 ^d	80.67%±0.67 ^b
Control	70%±1.15 ^a	

Letters are used to express comparison; dissimilar letters indicate a significant difference.

Discussion

Our results, showed that LPS of normal isolate at most concentrations leads to increase in number of granulocytes and this results was in agreement with a study Faas *et al.* (16) who showed that LPS of wild type *E.coli* induced an increase in neutrophil and total leukocytes. The LPS of pathogenic strain of *E.coli* induced a decrease in the numbers of neutrophils after 24h of immunization with no significant differences in total leukocytes and lymphocytes (17), while the single high dose of LPS of pathogenic strain induced leukopenia (18).

The differences in total WBCs, granulocytes and phagocytic activity in our study may be due to the structural differences in lipid A structure of LPS in normal flora and

pathogenic *E.coli*. Lipid A, a portion of pathogenic and commensal outer membrane structure, is recognized by Toll-like receptor 4 (TLR4), when an animals or subject exposed to LPS, TLR4 recruits sequentially two signaling adaptors, MyD88 and TRIF which in turn induce a complex network of proteins that mediate TLR signaling and ultimately activate transcription of several genes to induce broad range of pro-inflammatory mediators, the relationship between immune response and LPS structure was correlated with the numbers of fatty acyl chains in endotoxin LPS. The structure of lipid A (hexa-acylated 1, 4'-bisphosphate) in LPS varies slightly between bacteria species (19).

The differences in acylation of lipid A in different strains of *E.coli* induce different effects on the outer membrane permeability and innate immune response system recognition by human and murine TLR4/MD2, lipopolysaccharide, the higher acylation is more stimulatory than LPS with less amount of acyl chain, the lipopolysaccharide with few acyl chains was less stimulators in human compared with murine TLR4/MD2 (20), and the phagocytosis of strains lacking the core region or the myristoyl chain (i.e. pentaacylated lipid A instead of the wild form hexaacylated type) is reduced (21).

In the present study, the animals groups immunized with relatively high concentrations of LPS extracted from Normal strain of *E.coli* induced more phagocytic activity compared to those immunized with LPS of pathogenic isolate, this result was highly related to the increase in the number of granulocytes in this group. Another study showed that LPS of *E.coli* induced increase in phagocytic activity of macrophage (22). LPS of Normal flora induce an increased production of interleukin-8 from the monocyte that induced by LPS of the pathogenic type, which may result from the minor differences in LPS structure which affect the signaling process (23). Interleukin-8 was regarded as a chemokine activating neutrophils after secretion by LPS-stimulated monocyte, this cytokine plays a crucial function in sepsis pathophysiology through their activation of neutrophil and migration to the site of infection and it was considered also as a good chemokine for neutrophils and triggers phagocytosis (24). While the modulation endotoxin structure as O-antigen and the decrease in the numbers of fatty acyl chains in lipid A moiety may be regarded as a cause to avoid the immune response including phagocytosis (25).

Conclusion

It was concluded from this study that the LPS extracted from a pathogenic isolate and another from a natural isolate stimulated different immune responses and LPS of natural isolate was a strong inducer of the phagocytic activity of macrophages

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Conflict of interest

There are no competing interests regarding publishing or funding this articles.

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تأثير متعدد السكريات الدهني المستخلص من بكتيريا الإشريكية القولونية على التعداد الكلي لكريات الدم البيضاء، الخلايا الحبيبية والفعالية البلعمية في إناث الجرذان

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الخلاصة

أجريت هذه الدراسة لمقارنة التعداد الكلي لكريات الدم البيضاء، الخلايا الحبيبية والفعالية البلعمية في إناث الجرذان الممنعة بمتعدد السكريات الدهني المستخلص من عزلتين مختلفتين من بكتيريا الإشريكية القولونية، أحدها طبيعية التواجد معزولة من أمعاء الأشخاص البالغين الأصحاء والأخرى سلالة مرضية معزولة من المرضى الذين يعانون من التهاب المسالك البولية. اشتملت الدراسة على استخدام ٣٥ من إناث الجرذان البيضاء، قُسمت عشوائياً إلى سبعة مجاميع، تم حقن ثلاث منها بتركيز مختلف من متعدد السكريات الدهني للعزلة الطبيعية لبكتيريا الإشريكية القولونية ٩٠، ١٢٠، ١٥٠ ميكروغرام/كيلوغرام من وزن الجسم على التوالي، وحقنت المجموعات الثلاث الأخرى بنفس التراكيز من مستخلص متعدد السكريات الدهني المستخلص من السلالة المرضية لبكتيريا الإشريكية القولونية، المجموعة الأخيرة (السابعة) مُثلت مجموعة السيطرة وحقنت بالمحلول الملحي الفسلجي فقط. أظهرت النتائج أن مستخلص متعدد السكريات الدهني للعزلة الطبيعية التواجد تسبب بزيادة معنوية في التعداد الكلي لكريات الدم البيضاء والخلايا الحبيبية والفعالية البلعمية في الحيوانات الممنعة بتركيز ١٢٠ ميكروغرام/كيلوغرام مقارنةً بمجموعة السيطرة، كما أن التركيز ١٥٠ ميكروغرام/ كيلوغرام من المستخلص ذاته أدى إلى زيادة معنوية عالية في الفعالية البلعمية، بينما لم يحفز التركيز ٩٠ ميكروغرام/كيلوغرام أي فروق ذات دلالة معنوية في جميع المتغيرات، بينما أدى التمنيع بمستخلص السلالة المرضية إلى زيادة في التعداد الكلي لكريات الدم البيضاء عند التركيز ٩٠ ميكروغرام / كيلوغرام فقط مع زيادة معنوية في الفعالية البلعمية عند جميع التراكيز، كما أنه لم يسبب أي اختلافات معنوية في عدد الخلايا الحبيبية.