ISSN: 0067-2904

Effect of Lipopolysaccharide Extracted from *Pseudomonas aeruginosa Bacterium* on the Number of Immune Cells and Some Cytokines in White Rats

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Received: 16/12/2023 Accepted: 29/4/2025 Published: 30/4/2026

Abstract

Lipopolysaccharide (LPS) has a role in activating the immune system, including immune cells and interleukins. The current study aimed to estimate the levels of immune factors compared with control. LPS was extracted from *Pseudomonas aeruginosa* by a modified EDTA method. Three concentrations, 625, 1250, and 2500 µg/100gm of weight were prepared. Rats injected with a calculated dose, followed by a booster dose for two weeks. The results showed a significant increase in the number of neutrophils (3.009±0.002, 2.054±0.001, and 2.719±0.010) $\times 10^3$ µL, and the number of lymphocytes (5.954 ± 0.003, 4.902 ± 0.02, and 5.423 ± 0.1) $\times 10^3$ µL at the concentrations of 625, 1250, and 2500µ g/100gm, compared to control (2.775±0.100**)** and (4.207 ± 0.007) $\times 10^3$ µL, all respectively. In addition, the results also revealed a considerable increase in the number of monocytes (0.319 ± 0.001, 0.362 ± 0.002, and 0.329 ± 0.002) ×103 µL for concentrations respectively compared to control (0.080±0.0003) ×103 µL. Moreover, the levels of interleukin-8 and interleukin-12 were measured in the serum samples; the outcomes displayed a remarkable difference in the level of (IL-8) as (354.00 ± 0.04) pg/ml at a concentration of 2500 mg/100gm compared to control (313.330 ± 0.04) pg/ml. Furthermore, the levels of (IL-12) revealed a significant rise (11.766 ± 1.738, 34.600 ± 3.05, 12.400 ± 0.556) pg/ml at all used concentrations, respectively, compared to control (6.600 ± 0.360) pg/ml. The current study concluded that lipopolysaccharides at different concentrations affected the total number of white cells and increased the number of lymphocytes, mononuclear cells, and the levels of immune cytokines compared to the control.

Keywords: Lipopolysaccharide; *pseudomonas aeruginosa*; neutrophils; lymphocytes; monocytes; IL-8; IL-12.

تأثير عديد السكاريد الدهني المستخلص من بكتيريا الزائفة الزنجارية على عدد الخلايا المناعية وبعض السيتوكينات في الفئران البيضاء

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للسكريات الدهنية الدهنية (LPS) دور في تنشيط جهاز المناعة مثل الخلايا المناعية والإنترلوكينات. هدفت الدراسة الحالية إلى تقدير مستويات العوامل المناعية مقارنة بالسيطرة. تم استخراج LPS **من بكتيريا الزائفة الزنجارية**بواسطة طريقة EDTA المعدلة. تم تحضير ثلاثة تراكيز: 625، 1250، 2500 مايكروغرام/100غم من وزن الجسم. تم حقن الفئران بجرعة محسوبة، تليها جرعة معززة لمدة أسبوعين. أظهرت النتائج زيادة كبيرة في عدد العدلات (3.009 ± 0.002، 2.054 ± 0.001، و 2.719 ± 0.010) × 103 مل، وعدد الخلايا الليمفاوية (5.954 ± 0.003، 4.902 ± 0.02، و 5.423 ± 0.1) × 103 ML عند التراكيز625، 1250، 2500 مايكروغرام/100غم مقارنة بالسيطرة (2.775±0.100) و (4.207 ±0.007)×103 مل، جميعها على التوالي. بالإضافة إلى ذلك، كشفت النتائج أيضًا عن زيادة كبيرة في عدد الوحيدات (0.319 ± 0.001، 0.362 ± 0.002، و 0.329 ± 0.002) × 103 مل عند 625، 1250، و 2500مايكروغرام / 100 غم، على التوالي مقارنة بالتحكم (0.080 ±) (0.0003 ×103 مل. علاوة على ذلك، تم قياس مستويات الإنترلوكين-8 والإنترلوكين-12 في عينات المصل، وأظهرت النتائج فرقاً ملحوظاً في مستوى (IL-8) حيث بلغ (354.00 ± 0.04) بيكوغرام/مل عند التركيز 2500 مايكروغرام/100غم مقارنة بالسيطرة (313.330 ± 0.04) بيكوغرام/مل. علاوة على ذلك، أظهرت مستويات (IL-12) ارتفاعاً معنوياً (11.766 ± 1.738، 34.600 ± 3.05، 12.400 ± 0.556) بيكوغرام/مل في جميع التراكيز المستخدمة، على التوالي، مقارنة مع السيطرة (6.600 ± 0.360) بيكوغرام/مل. خلصت الدراسة الحالية إلى أن عديدات السكاريد الدهنية بتراكيز مختلفة أثرت على العدد الإجمالي للخلايا البيضاء، وزيادة في عدد الخلايا الليمفاوية، والخلايا وحيدة النواة، ومستويات السيتوكينات المناعية مقارنة بالسيطرة.

1. Introduction

Pseudomonas aeruginosa is a Gram-negative pathogenic bacterium. It can be found in various environments, including soil and water; it is very risky and resistant to many antibiotics [1-4]. Lipopolysaccharide (LPS) is one of the most important components of the cell wall of *P. aeruginosa*, found in the outer membrane of this bacterium. This structure has a very specific role in the pathogenicity of *P. aeruginosa* and the relationship between this bacterium and its host[5]. LPS consists of three parts that are arranged from outside to inside, starting with O-antigen (O-Ag), core oligosaccharide, and Lipid. A.[6, 7]. O-Ag is a chain containing hundreds of sugars. There are two kinds of LPS, smooth and rough, depending on whether the sugar chain (O-Ag) is present or not. LPScaped with O-Ag is called smooth, whereas LPS uncapped with O-Ag is called rough[8]. Lipid A is the toxic partition of LPS, which mediates inflammatory response-induced endotoxicity[9]. Lipid A contains 12-14 carbon lipids as an acyl chain. Many modifications may happen in the structure of lipid A in response to environmental stimuli, which increase antibiotic resistance [10]. The core oligosaccharide structure is commonly divided into two parts - inner and outer core. The inner core consists of two residues of kdo (3-deoxy-d-manno-oct-ulosonic acid) and two residues of L-glycero-D-manno-heptose. Both heptoses act as phosphorylation sites[11-13]. Innate immunity attacks bacterial infections through soluble immune factors such as TNF- α Interleukins (IL-6-IL-8-IL-10) [14, 15] and insoluble immune factors such as white blood cells[16, 17] The ability of the innate immune system to recognize invading microorganisms is aided by pathogen recognition receptors (PRRs) [18-20] by linking with pathogen-associated molecular patterns (PAMPs) such as lipopolysaccharide (LPS) that are recognized by Toll-like receptor 4 (TLR4) [21-25].

2. Materials and methods

2.1 Extraction of LPS

Using a modified EDTA approach, LPS was isolated from *P. aeruginosa*, which had

previously been identified by the research unit of the College of Science /University of Mosul- Iraq. The *P. aeruginosa* was cultured in four liters of brain heart infusion broth medium (BHI), then incubated at 37ºC for 48–72 hours in a shaker incubator. Bacterial cultures were centrifuged at 5000 rpm for 30 minutes, and the precipitate was washed three times by adding 20ml of 95% ethyl alcohol, then centrifuged again at 3000 rpm for 10 minutes. Following drying, the precipitation cells were taken, and 1 ml of 10% EDTA solution was added to the samples. The cells were left overnight in the refrigerator, and isolated bacterial cells were broken by ultrasonication (ultrasound omni international UK) at a frequency of 20000 vibrations per minute for 30 seconds under refrigerated conditions. Then, the cells were centrifuged in a cooling centrifuge. The supernatant was withdrawn using clean and sterile micropipettes and placed in clean airtight test tubes[26].

2.2 Lyophilization

Acetone was used at a ratio of (1:5 v/v) and kept at 4 ºC for 72 hours. White granules showed the presence of the LPS (modified step) of Subhi *et al.,* and Oursel *et al.,* [27, 28].These granules were dried by lyophilized device (Alpha 1-2 LD Plus/ Germany) and kept at 8 ºC. The purity of LPS was analyzed by using Gas Chromatography-MASS Spectrometry (GC-MS) .

2.3 Animal injection

Fifty animals of white rats were divided in to four groups and placed in cages. Each cage containing three animals ranged from 2 to 3 months. They were placed in suitable environmental temperatures, good ventilation, and healthy food. Rats were injected three times every 48 hours with three concentrations of lyophilized LPS (625, 1250, and 2500 µg/100g **)** of body weight, followed by a last injected dose one week after the last injection [29].

2.4 Blood collection

Blood samples were drawn from the injected animals, and experiments were performed on them.

250 Estimation of the total and differential number of white blood cells

A portion of the drawn blood was placed in the EDTA tubes, and the total number of white blood cells (WBCs) was calculated using a hemacytometer chamber slide. The following equation was used to determine the total number of W BCs in the blood:

$$Number\ of\ W.B.Cs\ in\ 1\ mm3 = \ sum\ the\ number\ of\ W.B.Cs\ in\ 4\ squares \quad (1)$$

[30]

Blood smears were prepared and stained with Giemsa stain to obtain the differential number of WBCs in the examined samples[31, 32].

2.6 Determination of cytokines levels

ELISA kits manufactured by (Elabscience Biotechnology Inc. Rat IL-8 and IL-12) were used to determine the levels of IL-8 and IL-12 in blood sera drawn from injected animals with three different concentrations of LPS [33].

2.7 Statistical analysis

All the significant differences between the obtained data were statistically analyzed at the percent using the ANOVA program.

3. Results

LPS was analyzed using the GC-Mass technique, and the results implied there are many compounds of LPS in the cell wall of *P. aeruginosa*, such as (11,14 Hicosadienoic acid Methyl ester, cis-13-Octadeconoic acid, n-Hexadecenoic acid, 9.12-Octadecadienoyl chloride, Octadecanoic acid, 9.12-Octadecanoic Methyl ester), as shown in Table 1 and Figure 1. They have an effect on the total and differential count of WBCs, where the percentages were 37.03, 24.98,10.05, 24.98, 10.05, 3.77, 6.61, and 3.42), respectively. However, there was no remarkable increase in the total numbers of WBCs (9.074 ± 1.627, 8.363 ± 1.215, and 9.200 ± 1.176) $\times 10^3$ ML at all used concentrations: 625, 1250, and 2500 µg/100 gm of body weight, respectively, comparing with the control (10.0960) $\times 10^3$ ML at a significant level of 0.05. The results are in agreement with Markazi *et al.,* and Saadat *et al.,* [34, 35].

Table 1: The structure of LPS compounds of *P. aeruginosa,* which analyzed by GC-MS.

Peak#	R. Time	Area	Area%	Name
1	18.371	64567	0.83	Hexadecanoic acid, methyl ester
2	18.898	778.346	10.05	n-Hexadecanoic acid
3	20.142	264645	3.42	9,12-Octadecadienoic acid, methyl ester
4	20.216	129992	1.68	cis-13-Octadecenoic acid, methyl ester
5	20.288	29777	0.38	6-Octadecenoic acid, methyl ester, (Z)-
6	20.710	2869249	37.03	11,14-Eicosadienoic acid, methyl ester
7	20.765	1935402	24.98	Cis-13-Octadecenoic acid
8	20.968	511954	6.61	Octadecanoic acid
9	21.117	80514	1.04	2,4,6,8,9,10-Hexathiatricyclo[3.3.1.1(3,7)]decane,1,1'-dithiobis[3,5,7-trimethyl-]
10	21.180	92343	1.19	2-Methoxy-4,6-bis[2,2,2-trifluoro-1,1-bis(trifluoromethyl)ethyl]-1,3,5-traazine
11	21.233	43270	0.56	2-{[62-Benyl-2-[(dicyclohexylcarbamoyl)-methoy-methyl]-3-phnyl-proxy}[36]-N ,N-dic
12	21.571	119573	1.54	9,12-Octadecadienoic acid (Z,Z)-
13	21.650	35541	0.46	2-Acetyl-1,3-benzlidene-4,5-di(O-p-toluenesulfonyl)-d-arabitol
14	21.722	54218	070	
15	22.155	80886	1.04	Docosanoic anhydride
16	23.299	107593	1.39	9,12-Octadecadienoic acid (Z,Z)-, 2-hydroxy-1-(hydroxymethyl)ethyl ester
17	23.358	41098	0.53	Oxazol-2-thione[4,5-o]ergost-7,22-dien-3-ol, acetate(ester)
18	23.720	292021	3.77	9,12-Octadecadienoyl chloride, (Z,Z)-
19	23.767	165616	2.14	trans-13-Octadecenoic acid
20	23.850	52084	0.67	
		7748359	100.00	

However, the results exhibited an important increase in the number of neutrophils and lymphocytes (3.009 ± 0.002, 5.954 ± 0.003) $\times 10^3$ µL, respectively, at the concentration of 625 µg/100 of body weight, while no noticeable rise shown in the number of neutrophils (Figure 2 A) and lymphocytes (Figure 2 B) (2.719 ± 0.01 and 5.423 ± 0.100) ×103 µL, respectively, at a concentration of 2500 µg/100 gm of body weight, comparing with the control (2.775 ± 0.1 and 4.202 ± 0.002) ×103 µL, respectively (Table 2). Moreover, the number of monocytes (Figure 2 C) showed an important increase at all concentrations (0.319 ± 0.001, 0.362 ± 0.002, 0.329 ± 0.002) $\times 10^3$ µL compared with the control (0.080 ± 0.0003)

$\times 10^3$ µL (Table 2). On the other hand, the current results showed remarkable differences in the cytokine (IL-8, IL-12) levels in sera of the injected animals, as shown in Table 2. The results revealed a considerable rise in the IL-8 level, and it reached(354.00 ± 0.040) pg/ml at a concentration of (2500) mg/100 of body weight, while no significant increase was noticed in the level of IL-8 at (1250, 625) mg/100gm of body weight as the levels were (312.7 ± 2, 316.0 ± 1.00) pg/ml, respectively, comparing to control (313.3 ± 1.00) pg/ml (Table 3). Finally, the levels of cytokine (IL-12) were increased at all used concentrations (11.76 ± 1.73, 34.60 ± 3.051, 12.40 ± 0.556) pg/ml, respectively, compared to the control (6.600 ± 0.360). The highest level of IL-12 was at the concentration of 1250 mg/100 gm of body weight, as illustrated in Table 3.

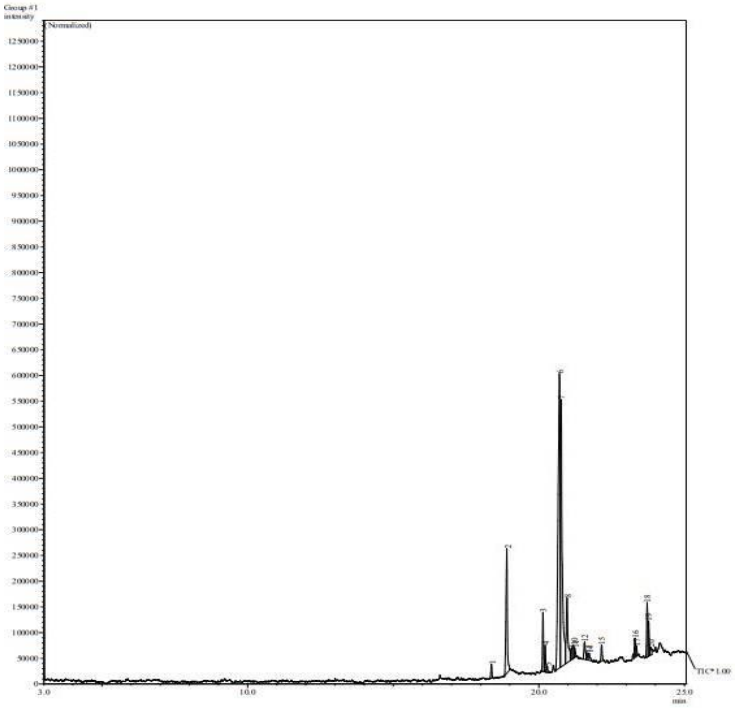

Figure 1: GC-Mass analysis of LPS compounds.

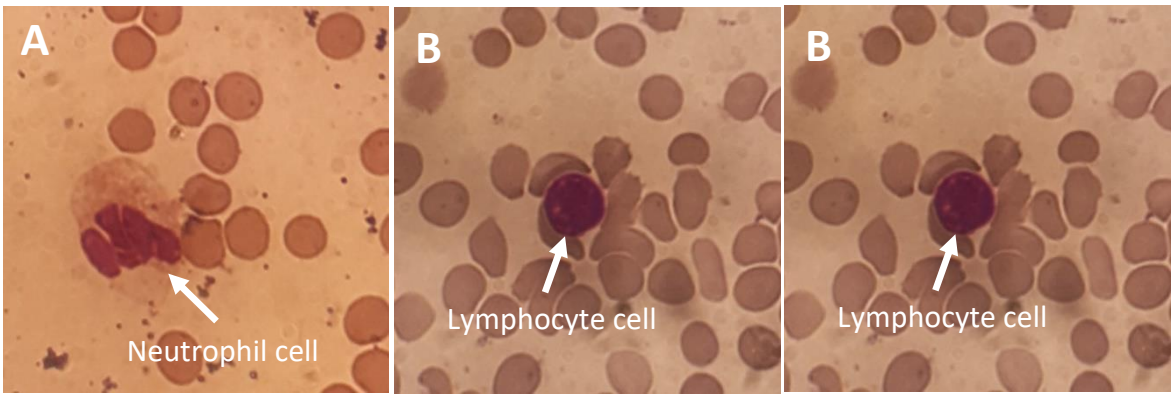

Figure 2: White blood cells. (A) Neutrophil cell. The nucleus is divided into three lobes linked together by chromatin filaments. (B) Lymphocyte cell. Small in size, with a regular nucleus that fills most of the cytoplasm. (C) Monocyte cell. The nucleus is a kidney or crescent shaped, and does not fill all the cytoplasm.

Table 2: Effect of *P. aeruginosa* LPS on the immune cells.

Concentrations μg/100gm for b.w.	Total numbers of WBCs x 10^3 μL	Neutrophils $\times10^3$ μL	Lymphocytes $\times10^3$ μL	Monocytes $\times10^3$ μL
625	9.074 ± 1.627 a	3.009±0.002 a	5.954±0.003 a	0.319±0.001 c
1250	8.363 ± 1.215 a	2.054±0.001 c	4.902±0.002 c	0.362±0.002 a
2500	9.200 ± 1.176 a	2.719±0.010 b	5.423±0.100 b	0.329±0.0002 b
Control	10.0960 a	2.775±0.100 b	4.202±0.002 d	0.080±0.0003 d

Similar letters exhibit no noticeable differences, whereas distinct letters indicate significant differences.

Table 3: Comparative between the levels of IL-8 and IL-12stimulated by *P. aeruginosa* LPS.

Concentration μg/100gm of b.w	IL-8pg/ml	IL-12 pg/ml
625	316.000±1.000 b	11.766±1.738 b
1250	312.730±0.040 b	34.600±3.0512 a
2500	354.000±0.0400 a	12.400±0.556 b
Control	313.330±1.000 b	6.600±0.360 c

4 Discussion

As shown in Table 1, the lipopolysaccharide extracted from the cell wall of *P. aeruginosa* is composed of several compounds. These results were identical to somehow with Wibowo *et al.,* [37]. The current study showed no significant differences in the total number of WBC in all LPS concentrations used. These results differ from the findings of Qin *et al.,* [38], who mentioned that repeated treatment with LPS leads to a significant increase in the total number with a significant reduction of lymphocytes percentage. The findings explained a significant increase in the number of monocytes at all concentrations. Maybe the reason is that repeated exposure to LPS leads to killing the neutrophils as a result of infections, and thus will decrease the total number of white cells in the circulation, and at the same time will stimulate the bone marrow to produce more neutrophils to activate the immune system[31]. The results also illustrated the differences in the effects of LPS produced by *P. aeruginosa* on the levels of IL-8 and IL-12. Based on the current results, we observed that the effect of LPS on IL-12 was evident at all concentrations, where the effect reached double compared to IL-8, see Table 3. The higher level of IL-8 was 34.600±3.0512 pg/ml at a concentration of 250 mg/100gm of body weight compared to control 6.600±0.360 pg/ml. These results agree with Smiechowicz *et al.,* [32]. This effect is related to the infection caused by bacteria or viruses, which leads to resident macrophages in the liver, spleen, and peripheral blood and causes failure of organs, which then leads to activating the immunity system and stimulates and raises the cytokines levels in blood [33, 39].

5 Conclusion

The current study revealed that the LPS has an important role in stimulating the immune system injected white rats. The number of immune cells (neutrophils, lymphocytes, and monocytes) pointed out a remarkable rise, at 0,625 and 1,25 as well as IL_8 raise at 2,5 mg/100gm while IL_12increase at all concentrations.

Conflicts of interest

The author declares that they have no competing interests.

Acknowledgements

We would like to thank the University of Mosul for its kind support, and we would also like to thank Dr. Nazim Ahmed for his scientific technical assistance and looking after the animals.

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