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Determination of IL-6 and TNF- α Levels in Sera of Laboratory Animals Injected by Different Concentrations of Lipopolysaccharide Isolated from *Escherichia coli*

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ABSTRACT

Lipopolysaccharide (LPS) was extracted from *Escherichia coli* by using EDTA method. Three concentrations of LPS have been prepared which are 50, 100, 200 $\mu\text{g}/100\text{g}$ of body weight. Three groups of rats aged 2-3 months were used three animals per each were injected Intraperitoneally (IP) with three doses every 48 hours, followed by two booster doses in two weeks, then blood was withdrawn and serum was separated and concentrations of tumor necrosis factor(TNF- α) and interleukin-6 (IL-6) were estimated by using the enzyme-linked immunosorbent assay (ELISA) method. TNF- α concentrations were 125.566 ± 1.826 , 133.566 ± 1.149 and 150.766 ± 1.954 picogram (pg)/ml for different doses of injected LPS, further the concentrations of IL-6, were 37.733 ± 4.747 , 51.333 ± 4.475 and 63.000 ± 1.982 pg/ml for various injection doses.

Exposure to LPS stimulated production of TNF- α and IL-6, However, we noticed that TNF- α was higher compared to IL-6, the concentration 200 $\mu\text{g}/100\text{g}$ also had a greater catalytic effect than the other concentrations for TNF- α and IL-6.

Keywords: endotoxin, cell wall, immune system, gram negative bacteria, macrophage.

INTRODUCTION

The common constituent of the outer membrane of *Escherichia coli* is endotoxin that called lipopolysaccharide (LPS) (Vishnupriya, 2018). LPS causes septic shock in animals therefore the immune system protects the body by producing many important immune mechanisms developed to detect foreign substances called innate immunity and adaptive immunity (Jinsu *et al.*, 2019). The LPS consists of three main parts, lipid A, a core oligosaccharide, and the O-antigen. Lipid A contains two glucosamine units with hexa- acyl chains and two phosphate groups which are the effective unit that stimulates the immune system in mammals (Han-Gyu *et al.*, 2017).

The induction of cytokine synthesis in response to endotoxin exposure *in vivo* undoubtedly plays a critical role in the host response to septicemia, two cytokine (TNF- α) and (IL-6) were implicated as key mediators in response TNF- α have been linked to leukocyte migration, tissue resorption, acute phase responses, fever, and bacterial killing, its production is regulated at multiple levels (Mortha *et al.*, 2003). LPS binds to the surface receptor CD14, which transmits a signal to Toll-like receptor 4 (Jargalsaikhan *et al.*, 2009).

LPS induces production of IL-6 by macrophages and involved in development of endotoxic shock as a Toll-like receptor (TLR4) ligand. LPS binds TLR4 and activates signal path ways to the expression of early genes (Xiaoyuan and Peter, 2010). TRL4 functions as a dimer, and depends on a small protein MD-2 for the recognition of LPS other proteins such as CD14 and Lipobiprotein (LBP) facilitates the presentation of LPS to MD-2, after activated by LPS TRL4 recruits intracellular adapter molecules such as My D88, Mal, Trif and Tram which in turn activated other molecules including proteins kinases to amplify the signal, and result in the induction or suppression of genes that organized the inflammatory response (Al-Mujamee, 2017). For the previous reasons and the effectiveness of the LPS in stimulating the immune system to defend the body against bacterial infections, the current study was designed to show the effect of different concentrations of LPS on the body and resistance to disease by inducing an inflammatory response with the production of the cytokines under study. This study investigate the effect of the LPS on production of TNF- α and IL-6 *in vivo* and identification of the purity of the LPS and LPA of *E.coli* by using GC/MS technique also determining the best concentration of LPS in stimulating the immune system to produce immune cytokines under study

MATERIALS AND MTHODS

Isolation of Bacteria

E. coli, was isolated from patients attending the Ibn Al-atheer hospital in Mosul with the urinary tract infection. The bacteria were cultured on a selective medium (MacConckey agar) and incubated at 37°C for 24 hours, pink colonies appeared and one isolated colony was taken and inoculated in slant of Nutrient agar to obtain pure isolation then diagnosed with the Vitek 2 device (bio Merieux customer Vitek 2 system version: 08.01) (USA) preserved in the refrigerator for use.

Extraction and Purification of LPS

Preparation of bacterial culture for the isolation of LPS

The bacteria were cultured in four liters of the Brain Heart Infusion broth medium and incubated at 37°C for 48- 72 hours in shaker incubator (A lab Tech. DALHN LAB.LTD. Korea). The cells were centrifuged at 5000 rpm for 30 minutes, the sediment was used for LPS extraction. The precipitate was washed by adding 2 ml of 95% ethanol alcohol and shaken well, then centrifuged at 3000 rpm for 10 minutes. The cells were dried and suspended by adding 1 ml of 10% EDTA solution, bacterial cell walls were destroyed by ultrasonication (Ultrasound 1/96/40/80/3/4 Omni international UK) at frequency of 20000 vibrations per minute for 30 seconds in an ice bath to prevent overheating and damage to the extract. The extract was obtained by centrifuged using the cooled centrifuge. Then the supernatant was taken and placed in clean and sterile glass test tubes

(Shareef and Ismail, 2017). One ml of supernatant and add acetone (1:5) volume for each volume and kept at 4°C for 72 hours. The observation of white granules showed the represented the LPS- (in a modified step) (Delphinis *et al.*, 2007). The extracts were dried by using lyophilized device (ALPHA 1-2 LDplus 19616 Germany) and kept at- 4 ° c . The purity of the LPS has been confirmed by using Gas chromatography- mass spectrometry (GC-MS) (5973 network mass selective detector) (USA) for analyzation of LPS for its original components as it was shown in (Table 2).

Injection of the animals

Swiss Albino Rats 2-3 months old were obtained from the animal's house of College of Veterinary Medicine, University of Mosul. They were bred in cages of four groups each group consisting of three animals, three groups were injected intraperitoneally with the three concentrations of LPS (50, 100, 200) $\mu\text{g}/100$ g of b.w in three doses after 48 hr. for each dose, doses repeated for the three animals for each concentration, the fourth group was used as a control. Blood samples were collected after two weeks of exposure to LPS. (Chrestopher *et al.*, 2017). Blood samples were collected and sera were separated and placed in clean and sterile test tube, kept at- 4°C for use in ELISA (Sandwich- Ab/Ag- ELISA) for estimation concentrations of cytokines TNF- α and IL-6 by using ELISA kits produced by Elabsicence Biotechnology Inc. Rat TNF- α and IL- 6 for three concentrations of LPs. Results were analyzed using A nova spss.

RESULTS AND DISCUSSION

E. coli under the study was diagnosed using the Vitek 2 and the results showed that isolated bacteria was diagnosed is *E. coli* according to the results in (Table 1) and the probability were 98%

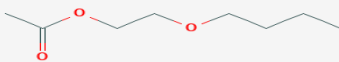
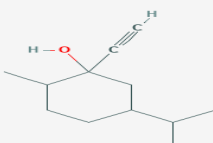


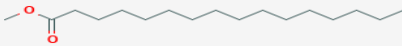
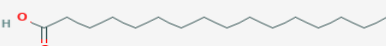

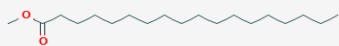
Table 1: The Biochemical tests results for *E. coli*

Number of test	Biochemical test	Result		Biochemical test	Result
2	APPA	-	33	SAC	+
3	ADO	-	34	dTAG	+
4	PYrA	-	35	dTRE	+
5	IARL	-	36	CIT	-
7	dCEL	-	37	MNT	-
9	BGAL	+	39	5KG	+
10	H ₂ S	-	40	ILATK	-
11	BNAG	-	41	AGLU	-
12	AGLTP	-	42	SUCT	-
13	dGLU	+	43	NAGA	-
14	GGT	-	44	AGAL	+
15	OFF	+	45	PHOS	-
17	BGLU	-	46	GlyA	-
18	dMAL	+	47	ODC	-
19	dMAN	+	48	LDC	+
20	dMNE	+	53	IHISO	-
21	BXYL	-	56	CMT	+
22	BAlap	-	57	BGUR	+
23	Pro A	-	58	O ₁₂₉ R	-
26	Lip	-	59	GGAA	-
27	PLE	-	61	IMLTa	-
29	TYrA	+	62	ELLM	-
31	URE	-	64	ILATa	-
32	dSOR	+			

The purity of extracted LPS was shown in Table (2) and these results were consistent with finding of (Benjamin *et al.*, 2017 ; Kota *et al.*, 2019) they explained the possession of fatty acids in the cell walls of *E. coli* (FAs) and they showed that fatty acids consist of six saturated fatty acids

($C_{12:0}$, $C_{14:0}$, $C_{16:1}$ and $C_{18:0}$), three unsaturated FA (16:1) and two isomers of the high ratio of those FAs was $C_{16:0}$, this similar to what we got as the percentage of FAs consisting of 16 carbon atoms equal to 42.48 which is represented by the compound Hexadecanoic acid .

Table 2: The compounds of *E.coli* LPS by GC/MS technique

	Name	Structure	Percentage
1	2-Butoxyethylacetate		32.22
2	5-Isopropenyl-2-Methyl-2-Cyclohexane		4.42
3	Pentadecane		3.45
4	Triacontane-7- bromo		4.19
5	Hexadecanoic acid, Methyl ester		4.19
6	n- Hexadecanoic acid		42.48
7	Heneicosane		3.80
8	Methyl stearate		5.25

Polysaccharides were also detected in samples of LPS which had an important role in stimulating the innate immunity and production of cytokines such as TNF- α and IL-6, (Table 3) illustrate were significant differences for the three concentrations (50,100 and 200) $\mu\text{g/ml}$ of LPS and induction of TNF- α and IL-6, the results showed an increase in the concentrations of both cytokines compared to control ,these results were consistent with the finding (Mingfunflu *et al.*, 2008 ; Yasuaki *et al.*, 2004) as they confirmed our results about the concentrations of TNF- α and IL-6 in serum after they recorded an increase in their levels after 7 days of intraperitoneal injection of LPS and were (34.89 \pm 8.7) and (45.67 \pm 8.6) pg/ml for TNF- α IL-6 respectively . As well as the values of IL-6 in previous studies were (2000 pg/ml) when animals injected with a single dose of LPS (Felix *et al.*, 2019). The results that we obtained caused by induction of much less suppressive effects on both humeral and cell- mediated immune responses. In addition we also observed that single LPS injection induced significant levels of mRNA expression of TNF α and interferon gamma in thymus 1 h after in injection, whereas in double LPS injection, the levels of these cytokines were dramatically reduced 1hr after the second LPS injection, this phenomenon is described as a late phase tolerance when a second LPS injection (Shannon *et al.*, 2019). Endotoxin tolerance can be induced by TLR ligands other than LPS and it had been reported to induce endotoxin that may underlie the ability of TRL ligands to augment cellular antimicrobial functions is metabolic reprogramming which rapidly change to support specific innate immune cell needs. Macrophages and dendritic cells are known to shift their metabolic profile to one that glycolytic process over oxidative metabolism (Sumanta *et al.*, 2009; Surabh *et al.*, 2019)

Table 3: The concentrations of TNF- α and IL-6 in serum of rats that injected by LPS

Concentration of LPS $\mu\text{g/ml}$	Concentration of TNF- α pg/ml	Concentration of IL-6 pg/ml
Control	0.000 \pm 0.000 A	0.000 \pm 0.000 A
50	125.566 \pm 1.826 B	37.733 \pm 4.747 B
100	133.566 \pm 1.149 C	51.333 \pm 4.475 C
200	150.766 \pm 1.954 D	63.000 \pm 1.982 D

-Similar letters in one column indicate that there were no significant differences between the treatment and control groups at $p > 0.05$

- Different letters in one column that there were no significant differences between the treatment and control groups at $p > 0.05$

CONCLUION

All Gram-negative bacteria have the most important structure in their cell wall, which is the LPS, which consists of three main parts which are O antigen, core oligosaccharide and lipid A. The LPS stimulate the immune system and produce many cytokines that have been studied (TNF- α and IL-6) where it was estimated after simulating it inside the animal bodies under studies with different concentrations of LPS where their level were differed, thus, concentrations of bacterial LPS can be injected with counted doses as a vaccine against many bacterial diseases.

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تحديد مستويات الانترلوكين-6 والعامل التنخري الورمي -الفا في مصول الحيوانات المختبرية المحقونة بتركيزات مختلفة من متعدد السكريات الدهني المعزول من الاشريكية القولونية

الملخص

استخلص متعدد السكريات الدهني من بكتريا الاشريكية القولونية وتم تحضير ثلاث تراكيز منه وهي 50، 100 و200 مايكروغرام /100 غرام من وزن الجسم. حسب الجرعة لكل تركيز حسب وزن جسم الحيوان المختبري قيد الدراسة حيث استخدمت ثلاث مجاميع من الجرذان نوع Swiss Albino Rats بعمر 2-3 شهر، حققت بثلاث جرعات من متعدد السكريات الدهني داخل الغشاء البريتوني كل 48 ساعة واتبعت بجرعتين معزتين خلال اسبوعين بعد ذلك تم سحب الدم وفصله للحصول على المصل، باستخدام تقنية الاليزا قدرت مستويات TNF- α حيث كانت مساوية الى 1.826 ± 125.566 ، 1.149 ± 133.566 و 1.982 ± 150.766 بيكوكرام للتراكيز 50، 100 و200 مايكروكرام / 100 غرام من وزن الجسم على التوالي. وكانت مستويات IL-6 مساوية الى 4.747 ± 37.733 ، 4.475 ± 51.333 و 1.982 ± 63.000 بيكوكرام للتراكيز الثلاثة على التوالي. وقد تبين من هذه الدراسة ان التعرض لجرعات مختلفة من متعدد السكريات الدهني ادى الى ارتفاع مستويات الساييتوكينات قيد الدراسة مقارنة بالسيطرة وان مستويات ال TNF- α كانت اعلى من مستويات ال IL-6 في اجسام الحيوانات قيد الدراسة. ان عينات السيطرة لم يتم حقنها بمتعدد السكريات الدهني لذلك عند تسقيط قيم الكثافة الضوئية التي تم تسجيلها خلال خطوات عمل فحص الاليزا عند تسقيطها على المنحنى القياسي لكل من IL-6 TNF-a كانت النتيجة صفر وبما انه يجب الاعتماد على المنحنى القياسي كان يجب تسقيط النتائج كلها ولذلك كانت قيم عينات السيطرة تساوي صفر لأنها لم تعامل باي تركيز من تراكيز ال LPS وانما حققت حيوانات السيطرة بالمحلول الملحي المعقم.

الكلمات الدالة: السموم الداخلية البكتيرية، جدار الخلية البكتيرية، الجهاز المناعي، البكتريا السالبة لصبغة كرام، البلمع الكبير.