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Experimental study on the role of purified LPS of *E.coli* O111:B4 in preventing mammary gland infection in mice

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Abstract

Mice were selected in this study as a model to induce *E.coli* mastitis and sttudying the role of purified LPS in performed the protection against induce *E.coli* infection of mammary glands in mice. After LD50 of purified antigen were determined (250 µg/ml of purified LPS). The experimental study was included 56 mouse which divided into two groups and the LPS group was included 26 mouse, while the control group was included 30 mouse. The mouse in LPS group was immunized by: 2.5 µg of LPS /gm of mice and by sterile PBS (pH=7.2) in control group. In all groups, the route of inoculation was subcutaneous, and these doses repeated at 15 days, 27 days after first inoculation as boostered doses. At 34 days post the first inoculation the humoral and cellular immune response were assesement by many particular tests to determine the activity of their antigen in provided protection against challenge dose by virulent *E.coli* O111:B4 which gave intramammary, and histopathogical study was carried out in challenged mouse. The study was reported that LPS was possessed high and significant efficacy and potent ability to rise the humoral and cellular immune response and reduced significantly the severity of mammary gland infection in mouse with lowest histopathological change in mammary tissue when compared with control group as a good role of LPS in reduced severity of mammary gland infection when compared with control group.

Keywords: *E. coli*, Mastitis, Mice.
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دراسة تجريبية لتقييم فعالية منقى متعدد السكريد الشحمي لجرثومة الاشيريشيا القولونية ذات الضرب المصلي O111:B4 في منع حدوث التهاب الغدد اللبنية في الفئران

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الخلاصة

في الدراسة التجريبية والتي اعتمدت إناث الفئران كبديل مختبري عن الأبقار في استحداث التهاب الضرع ودراسة تأثير المستضد المنقى من جرثومة الاشيريشيا القولونية في توفير الحماية ضد الإصابة بجرعة التحدي بجرثومة الاشريشيا القولونية في استحداث التهاب الغدد اللبنية في الفئران، حيث تم تحديد الجرعة نصف المميتةلمتعدد السكريدالشحمي في الدراسة التجريبية وكانت 250 مايكروغرام /مل لمنقى متعدد السكريد الشحمي. استخدمت في الدراسة التجريبية 56 فأر مقسمة على مجموعتان وبواقع 26 فأر للمجموعة الاولى و 30 فأر لمجموعة السيطرة. وتـــم التمنيع بالمستخلص المنقى من متعدد السكريد الشحمي في المجموعة الاولى (2.5 مايكروغرام /غم من وزن الفأر) اما مجموعة السيطرة فقد اعطيت داري الفوسفات المتعادل المعقم وكانت طريقة الحقن ووقته ثابتة في كلتا المجاموعتين حيث كان التمنيع تحت الجلد في اليوم الأول للدراسة ثم تعاد نفس الجرعة في اليوم الخامس عشر من بداية التجربة وتعطى جرعة ثالثة (جرعة تقوية) في اليوم السابع والعشرين من بداية التجربة وفي اليوم الرابع والثلاثين من بداية التجربة تم تقيم الحالة المناعية الخلطية والخلوية في منع أو التقليل من تأثير جرعة التحدي والتي أعطيت مباشرة داخل الضرع (جراثيم من

الاشريشيا القولونية ذات الضرب المصلي *E.coli*O111:B4 حية وفعالة). وقد آخذت عينات من الضرع المصاب لغرض العزل الجرثومي، وعد الخلايا الحية وكذلك إيجاد معامل وزن الغدة اللبنية إلي وزن الجسم الكلي إضافة إلى دراسة التغييرات النسيجية المرضية فيه. وظهر من نتائج تقييم المعايير المناعية والدراسة النسجية المرضية الكـــــفاءة العالية والمميزة لمتعدد السكريد الشحمي النقي في رفع مستويات المناعية الخلطية والخلوية والتقليل من شدة التهاب الغدة اللبنية المستحدث في الفئران مع تغيرات نسجية مرضية طفيفة مقارنة مع مجموعة السيطرة.

Introduction

Lipopolysaccharide (LPS) is the major and an integral part of the outer membrane of the cell wall of gram-negative bacteria, and by weight LPS makes up about 10 % of cell wall of gram –negative bacteria and that a single *E.coli* cell contains approximately 3.5 $\times 10^6$ LPS molecules (1).

Lipopolysaccharides are complex amphilic molecules with a molecular weight of about 10 KDa, that vary widely in chemical composition between and among bacterial species (2).

LPS produce the same range of biological effects in animal host, and the toxicity of LPS is associated with lipid A and immunogenicity is associated with polysaccharide component (3).

Lipopolysaccharides can stimulate the immune response of the host, is thought that LPS released into the blood stream by lysing gram-negative bacteria is first bound by certain plasma proteins identified as LPS –binding protein. The LPS-binding protein complex interacts with CD_{14} receptors on monocytes and other types of receptors on endothelial cells (4).

In monocytes (macrophages) three types of events are triggered during their interaction with LPS: production of cytokines, activation of the complement cascade and activation of the coagulation cascade (5).

The LPS interacts with LPS-binding protein and CD_{14} which in turn promotes the ability of the Toll-like receptor TLR_4 (aPRR) on macrophages to respond to the LPS and release of various pro-inflammatory cytokines, and these inflammatory mediators bind on target cells via specific receptors and initiate inflammation (6). LPS also act as a B –cell mitogen stimulating the polyclonal differentiation and multiplication of B-cells and the secretion of immunoglobulins, especially IgG and IgM (7).

The aim of this experimental study is to evaluate the immune response of the host to prophylactic dose of LPS.

Materials and methods

Isolate

E.coli O111:B4 which isolated from mastitic cow, identified according to (**8**) and serotyped by Lab. of Public Health Center-Baghdad.

LPS

Extracted according to (**9**) and purified by gel filtration Chromatography according to (**10**).

Determination LD50 of LPS

According to (11), serial concentrations of LPS were prepared and given intraperitonealy into 6 mice which may be stayed under observation for 5 days and then the dead and survival animals were counted. Serial concentrations of antigens:LPS:125, 250, 375, 500 µg /ml

Experimental design

56 lactating mice (white mouse) their weight (22-30gm)divided into two groups,the LPS group included 26 mice and the control group) had 30 mice: Group I- (LPS group) -Included 26 female mice which inoculated with purified LPS as 2.5 µg /gm of mouse s/c at dose 0.2 ml / mouse at day zero, then repeated after 15 days and gave booster dose at 27days. Group II- (control group) -Included 30 mice, (26) mice of them were inoculated with PBS 0.2 ml / mouse s/c at dose 0.2 ml / mouse at day zero, then repeated after 15 days and gave booster dose at 27days. While, another (4 mice)were challenged with *E.coli* as 10-20 cfu intramammary as a positive control at day 34 after the experiment was started.

After 34 days of first inoculation

Blood was collected from four mice in each group to separate serum for passive haemagglutionation test (PHA)and ouchterlony test. Delayed hypersensitivity test (DTH)was made in four mice in each group.

Abdominal mammary glands (L4,R4) of four animals of each group were challenged injecting 10-20 cfu /mammary gland directly, and then the mice may be euthanized and examined mammary glands (L4,R4) grossly and weight it, bacterial isolation from it, bacterial counting, finally histopatholgical examination of challenged mammary glands.

Peritoneal fluid was collected from five mice of each group after 72 hrs from liquid parafin I/P injection for MIF test.

Immunological tests

Passive haemagglutination test (PHA):(12), Delayed type hypersensitivity test DTH (skin test):(13), Macrophage migration inhibition factor (MIF)test:according to (14).

Bacterial challenged with *E.coli* by direct intramammary injection

Preparation of bacterial suspension

Brain heart infusion broth was inoculated with frozen *E.coli* O_{111}:B_4 and incubated 37 C° /18-24 hrs, and the broth culture was cultured on brain heart infusion agar 37 C° /18-24 hrs and their were harvested via PBS (pH=7.2) and centrifuged 3000rpm for 15min at 7 C°, and washed via PBS and centrifuged 2000 rpm /5 minutes at 7 C°, after that the pellet was resuspended with sterile PBS (pH=7.2) and the suspension was diluted and adjusted to $1x10^8$ cfu/ml.

Serial dilution were made with PBS to approximately 400 cfu/ml and kept on ice until injection (the actual number of cfu injected between 10-20 cfu of *E.coli* O_{111}:B_4 in 0.05 ml / intramammary injection).

Prepared female mice for challenge

20 lactating white - mice 15-20 days after parturition. Pups were removed from mother for 4 hrs and allowed to suck for 30 minutes before challenge of two abdominal mammary glands (R4,L4). The 30 minutes of nursing was performed to elongate the teats and facilitated procedure.

Intramammary injection of mice was carried out as described by (15)

Abdominal surface was disinfected and the challenged dose injected directly into two abdominal mammary gland (L_4,R_4) by insulin syringe with needle gauge 30 (amplifying lens and miniforcepes were used), and after injection, mice disinfectant again and the two injectable mammary gland sealed with parafilm tape to prevent pups to suck these teat.

After 3 days and 7 days, mice were scarified and firstly, any general clinical sings or local (mammary gland) clinical sings were recorded.

Then abdominal skin was cut with scissors to expose the mammary glands, and any macroscopic lesions on challenged mammary gland were recorded.

Remove challenged mammary glands, weighted, then homogenized with sterile PBS for pour-plate to count *E.coli*, and some bacterial suspension was cultured on MacConky agar for detecting the colony morphology and examined by gram stain.

Challenge mammary gland was fixated with 10 % formalin solution for histopathological examination.

Histopathological examination

According to (16).

Statistical Analysis

Statistical analysis were conducted to determine the statistical differences among different groups using ready – made statistical design statistical package for social science (SPSS).

Results

Immunological tests

Humornal immune response

Passive haemagglutination test (PHA)

Immunized group was revealed humoral response. Table (1) was showed that means of antibodies titrations were increased significantly ($P \leq 0.05$) within LPS group (88 ±48) when compared with Control group (1±1.1).

Table (1): Means of antibodies titration of immunized and control groups by PHA test.

Groups	*LPS*	Control
Abs Titer	a	b
Mean ±SD	88± 48	1±1.1

Same letters = no significant differences $P \geq 0.05$, the different letters= significant differences $P \leq 0.05$.

Cellular immune response

Delayed type hypersensivity skin test (DTH)

Cellular immune response was recorded in immunized group as in table (2) was showed highly significant increase of skin thickness of right footpad of mice after 24 hrs and became at peak after 48 hrs ($P \leq 0.05$) in LPS (0.32 ± 2.160) when compared with control group.

Macrophage Migration Inhibition Factor (MIF) test

A significant decreasing of MIF indices ($P \leq 0.05$) were showed in immunized group with different antigens concentrations as a responsive reaction (Table 3), (photo 1).

The high response was showed with concentrated Ag (1mg /ml) in LPS group (0.013 ± 0.025) when compared with control groups. While by adding pHA was not revealed any responsive reaction (photo 2).

Table (2): Means of skin thickness (millimeters)of immunized and control groups in DTH test.

Groups	Zero hrs Mean ±SD	24 hrs Mean ±SD	48 hrs Mean ±SD	72 hrs Mean ±SD
LPS	Aa 0.19 ±9.250	Ab 0.28 ±2.944	Ac 0.32 ±2.160	Ad 0.24 ±2.944
Control	Aa 0.19 ±4.625	Ba 0.21 ±8.165	Ba 0.21 ±8.165	Ba 0.192 ±5.0

Small letters =differences inside the same group, capital letters =differences between the different groups, the same letters = no significant differences $P \geq 0.05$ The different letters = significant differences $P \leq 0.05$.

Table (3): MIF indices in immunized and control groups.

Groups	Concetrated Ag 1mg/ml Mean ±SD	Ag 0.1 mg /ml Mean ±SD	Ag 0.01 mg/ ml Mean ±SD	pHA 10mcg /ml Mean ±SD
LPS	Aa 0.013 ± 0.025	Ab 0.05± 0.012	Ac 0.11 ± 0.025	0
Control	Ba 0.98 ± 0.057	Ba 0.97 ± 0.012	Bb 0.94 ±0.017	0

Small letters =differences inside the same group, capital letters =differences between the different groups, he same letters = no significant differences P≥0.05 The different letters = significant differences P≤0.05.

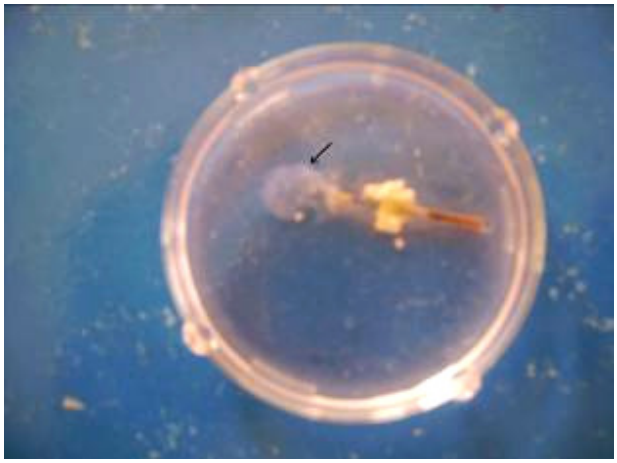

Photo (1): High MIF responsive with concentrated LPS antigens, smaller macrophages migration area.

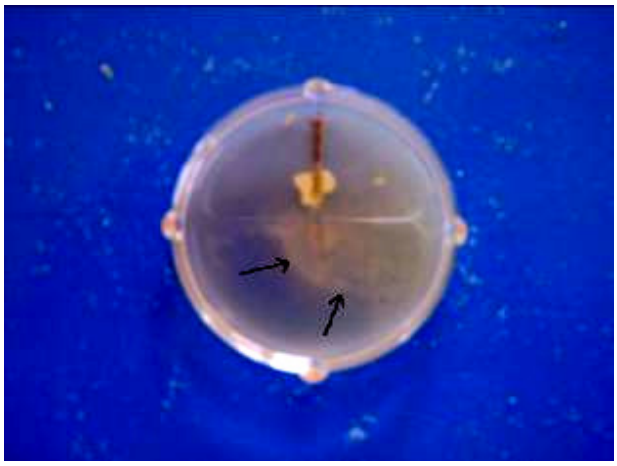

Photo (2): No responsive MIF with PHA antigen, complete macrophages migration outside capillary tube and bigger migration area.

Challenge by virulent *E.coli* O111:B4

Table (4) was showed the clinical signs as the rough coat and illness (depression) in control group as sever signs, whereas, illness was observed on mice in LPS group during the first 24 hrs after challenge only, and these fact was confirmed by macroscopic (postmorteum) examination of challenged mammary glands which revealed sever congestion of mammary gland vessels and odematous swelling signs of challenged mammary gland especially in control group (photo-3), whereas, these macroscopic lesions were not observed in LPS group.

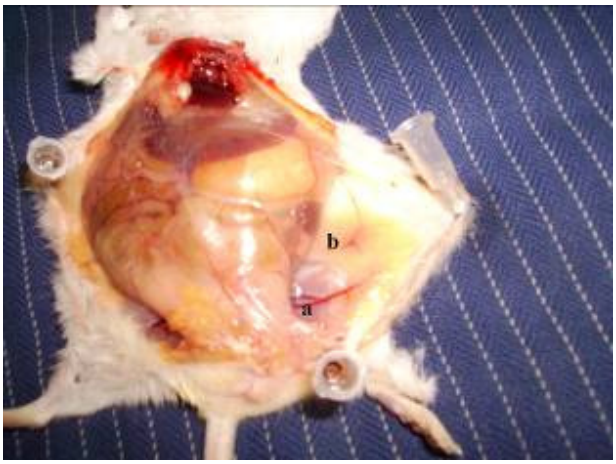

Photo (3):Macroscopic changes in challenged mammary gland in control group:sever blood congestion (a) and odematous swelling in mammary gland (b).

Index of mammary gland weight

The mean of mammary gland weight (index) was decreased significantly in immunized group (P ≤0.05) and in negative control group when compared with positive control group, Table (5).

Bacterial (*E.coli*) count

Table (6) showed the significant decrease (P ≤ 0.05) of number of *E.coli* that isolated from challenged mammary gland after 3 days of challenge with 10-20 cfu/ mouse in immunized group when compared with control group. The lowest *E.coli* numbers were enumerated in LPS group ($2\times10^2 \pm 0.816$).

Table (4): Systemic and local signs in immunized and control groups after challenged with virulent ***E.coli*** O111:B4.

Group	Animal no.	Challenge													
		general sings						Local sings(mammary gland)							
		Rough coat			Illness			Swelling			Redness			Postmortem exam	
		24 hrs	48 hrs	72 hrs	24 hrs	48 hrs	72 hrs	24 hrs	48 hrs	72 hrs	24 hrs	48 hrs	72 hrs	Vessels congestion	Odematous Swelling
LPS	1	+	+		+			+			±				
	2	+			+			+							
	3	+	+		+	+		+			+	+			
	4	+			+			+						+	+
Contr.	1	+	++	++	+	+	++	+	+	++	++	++	+	+++	+++
	2	+	++	++	+	+	++	+	+	+	++	+		+++	++
	3	+	++	++	+	+	++	+	+	+	++	+	+	+++	++
	4	+	++	++	+	+	++	+	+	++	++	++	+	+++	+++

Mild = +, Moderate = + +, Sever = + + +.

Table (5): Means of index of mammary gland weight (gram) in immunized and control groups.

Groups	LPS Mean±SD	Positive Control Mean±SD	Negetive Control Mean±SD
Mammary weight index	a 0.0022 ± 0.0326	b 0.01 ± 0.0027	a 0.0009 ± 0.021

Table (6): *E.coli* count in immunized and control groups.

Groups	LPS Mean ±SD	Control Mean ±SD
Bacterial count	a $2 \pm 0.816 \times 10^2$	d $25 \pm 1.825 \times 10^2$

The same letters = no significant differences P≥0.05, the different letters = significant differences P ≤0.05.

Results of histopathological examination

The histopathological changes in the mammary gland tissues after 72 hrs from challenged by virulent *E.coli* strain in control group were revealed highly infiltration of neutrophils intra–acini and intracellular tissue with desequamation of lining cells of acini and sever congestion the blood vessels, as well as hyperplasia of epithelial cells of milk acini, photo (4).

While, the histopathological changes at 7 days post challenge were characterized by macrophage infiltrations inside acini with desequamation of epithelial cells of ducts, and the granular foci of neutrophils and macrophages were present which conformed tumor like lesion to nearest of congested vessels.

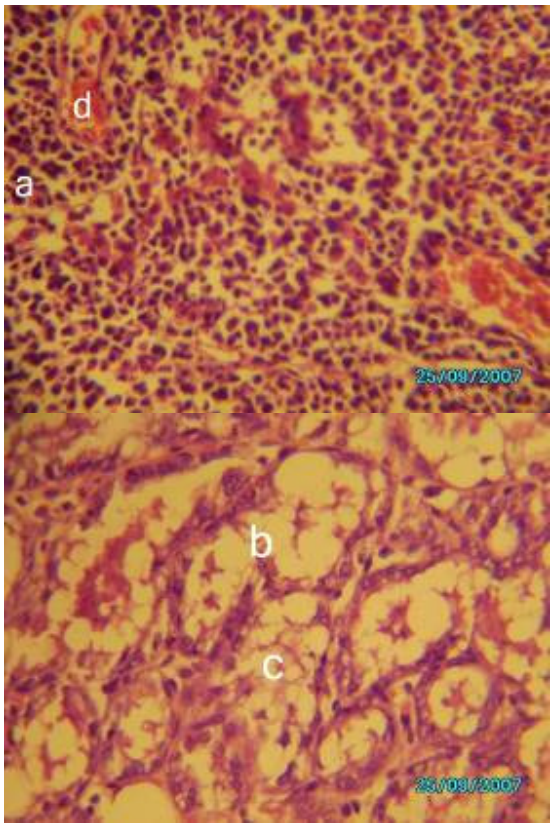

Photo (4): Massive neutrophil infiltration (a), desequamation of epithelial cells of acini(b), hyperplasia of epithelial cells of acini (c) and blood vessels congestion(d) in control group, 72 hrs post challenged.(H & E X 40).

Also the supramammary lymph node was infiltrated with plasma cells and macrophages and different degree of necrosis inside lymph node were appeared, photo (5).

In immunized groups the histopathological changes in mammary tissue were appeared as very little changes in LPS group simple neutrophil infiltration inside acini with different degree of blood congestion in mammary glands at 72 hrs post challenge (photo 6).

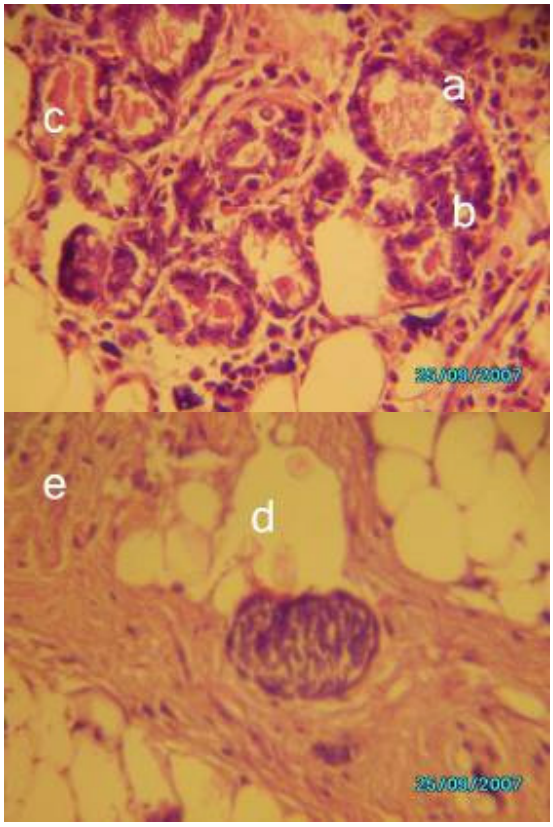

Photo (5): Macrophages infiltration inside acini (a), granular foci of neutrophils and macrophages (b), sever blood vessels congestion (c) area of necrosis in supramammary lymph node (d) and inflammed cell infltration (e) (in control group, 7 days post challenged. (H & E X40).

Disscusion

Experimental study

Mice have been used in present study because the mammary gland of mouse has only one streak canal and is functionally independent from the others, which seems to be similar to bovine udder, as indicated by (15) ;(17) and (18) whom they used the mouse as a model for mastitis in experimental study. The advantages for using mouse model are lower management efforts and lower cost when compared with dairy cows.

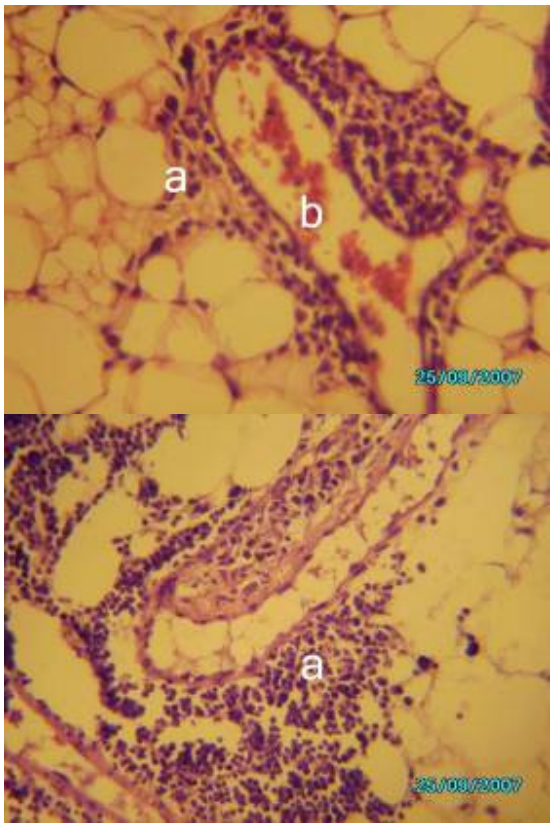

Photo (6): Mild infiltration of neutrophils between acini (a) & simple blood vessels congestion (b) (in LPS group, 72 hrs post challenged. (H & E X 40).

Humoral immune response

Passive haemagglutination test (PHA) was revealed higher mean of antibody titer in LPS group. A comparative reading of table (1) is showing the antibody titer in LPS groups was increased significantly than in control group, therefore we concluded that LPS has a pivotal role in enhancement of antibody producing as reported by (19).

LPS was an excellent mitogen for B-lymphocyte as well as activated macrophage to secrete IL-1 which in turn enhancement Th2 to release IL-4 and IL-5 to provoke B-lymphocyte to proliferation and differentiation to plasma cell and producing antibodies (20).

Cellular Immune response

All tests of cellular immune response were revealed that LPS as a potent antigen which stimulated immune response. In delayed type hypersensitivity skin test, Table (2) was showed the mean thickness of skin of padfoot in LPS group

which increased significantly after 48 hrs than others, this result was similar to reports by (21) and (19).

DTH test depend on ability and activity of Tdh cells to recognize antigen and secrete IL-1 which enhanced proliferation and differeniation of other T-cell into Th-cells which secrete IL-2 as a chemoatractive factor to attract macrophage around the area of activated T-cell which also secrete INF- δ that enhancing the cytolytic activity of accumulated macrophages leading into skin thickness (22).

MIF indices in table (3) were showed that antigens with different concentration were revealed responsiveness reaction when compared with standard means of migration that applicated by (23), and that was in agreement with many reports which appeared that macrophage migration inhibition indices could be induced by different type of antigens as (24);(25) and (26).

We recorded that concentrated LPS was inducing higher migration inhibition which in turn lead to best lowest and significant MIF index than other in immunized groups and these result was in similarity to concluded reports of (27) and (19) which they showed that LPS could be induced higher responsiveness of MIF index as a good cellular immune response stimulator.

LPS has a particular ability to stimulate anterior pituitary gland to release migration inhibitor factor as well as activeated T-cell and macrophage which inresulting to give a significant MIF index than other antigens (28).

Intramammary challenge of mice by *E.coli* O111:B4

Mouse mastitis has been induced in control group by direct injection of *E.coli* O111:B4 into mammary gland (R4,L4) with ultrafine needle which allowed for safe penetration into gland without noticeable damage to the mammary gland of mice.

Clinical signs, indices of mammary gland weight and histopathological changes, which all attributed to severity and ability of *E.coli* to grow and multiply and induce inflammation of mammary gland.

The best protective status with less significant changes in parameters of challenged mice was performed by LPS which induce higher humoral and cellular immune response, thereby prophylactic dose of LPS reduced isolated *E.coli* count with variant degrees of clinical signs (non to mild illness)was observed within only 24hrs post challenge and the mammary weight index approximate as in negative control group with microscopic lesions were limited by mild neutrophils infiltration, blood vessels congestion and no changes were noticeable in infected mammary gland after 7 days post inoculation.

All results were refered to the important role of toxic dose of LPS of *E.coli* to induce mammary gland infection which conducted in control group.

Elucidation of mechanism by which *E.coli* induce mastitis in mouse as that *E.coli* after inoclated to mammary gland,it grown and multiply in rich media (milk) and liberated LPS which in turn stimulate milk leukocytes to activate the proenzyme (matrix metalloproteinase MMP) by removal of their NH2-terminal domain,and that MMP destroyed basal membrane and all interstitial tissue proteins as in blood vessels and lead to increase vascular permeability as well as chemoattractants, and the result was elevated recruitment of blood derived neutophils and monocytes to migrate into mammary tissue (that explain of high infltration of neutrophils and macrophage as well as blood vssels congstion in histopath section) and these lymphocyte produced many cytokines especially IL-1 and TNF-α which they were induced programmed cell death in endothelial cells of mammary gland (apoptosis) (that exhibit sever desequamation of epithelial cells which lining duct and acini) and at the same time mammary cell proliferation increased to serve as a mechanism to ameliorate tissue damage (hyerplasia could be noticeable in histopathological examination) (29);(30) and (18).

The reduction in number of *E.coli* in mammary glands of LPS group was occurred due to that CD14-LPS complexes activate epithelial cells of mammary gland by binding to Toll like receptor –4 (Toll-LR4) on epithelial cells, lead them to secrete IL-8 (a potent chemoattractant of neutrophils) and in resultant early recruitment of neutrophils is crucial to clearance of *E.coli* from the mammary gland (31).

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