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Histological response of liposome incorporated lipopolysaccharide of *Salmonella enteric Serovar* Typhimurium in albino mice male

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Abstract

This study was designed to evaluate the role of *Salmonella enterica* serovar Typhimurium as a causative pathogen in hospitalized diarrhoeal patients younger than five years old, extract and purify endotoxin (lipopolysaccharide; LPS) from isolated and identified *S*. Typhimurium, and determine the histological activity of LPS-liposome conjugate in liver and spleen section of albino mice male. The patients (95 cases) were admitted to the Central Pediatric Hospital and Al-Kadhiymiah Pediatric Hospital in Baghdad during the period 24/10/2010 - 30/11/2010, because of severe diarrhoea and fever. Bacterial evaluation of stool samples revealed the identification of two (2.1%) *S*. Typhimurium isolates (S1 and S2). Antibiotic sensitivity test demonstrated that S1 isolate was more resistance than S2 isolate; therefore it was considered more virulent and subjected for extraction and purification of LPS. Chemical characterization of the extracted LPS revealed that the carbohydrate content was 2.34 mg/ml, while the protein concentration was very low (0.52 µg/ml). Partial purification of extracted LPS by using gel-filtration chromatography (Sephacryl 200 S) showed three peaks, and after determination of protein and carbohydrate concentrations for each peak, peak two was observed to have the highest carbohydrate content (25%) and the lowest contaminated protein (0.001%). The LPS of this peak was histologically evaluated in mice at a concentration of 100 µg/ml, alone or in conjugation with a commercially available liposome (LIP). These evaluations revealed that LPS-LIP conjugate was showed in histological section of liver look like normal especially near portal area.

Keywords: Diarrhoea, *Salmonella*, Lipopolysaccharide, Liposome, Histology.

1. Introduction

Salmonella Typhimurium is classified as a facultative, intracellular bacterium by virtue of its ability to survive and multiply within specialized phagocytic cells of the host reticuloendothelial system [1].

Salmonella Typhimurium is one of the non-typhoidal *Salmonella* serovars and being the most common serovar in the USA. Infection always occurs via ingestion of water or food contaminated with animal waste rather than human waste [2]. The emergence of multidrug-resistant *S*. Typhimurium DT104 has been associated with outbreaks related to food contamination and resulted in increased rates of hospitalization [3]. Therefore, *S*. Typhimurium continues to represent a major public health problem worldwide, and vaccine development has been an important target for researches in salmonellosis. The prevention of salmonellosis by vaccination has been the subject of many investigations, and despite this, the mechanism of protective immunity against *Salmonella* infections remains a controversial subject [4].

Lipopolysaccharides have a complex structure in Gram-negative bacteria and is composed of three parts including: complex part named lipid A (the source of LPS is on the membrane and has immunologic property), polysaccharide (is composed of 10 to 15 sugar and has a special role in penetration membrane) and specific O side chain (O side chain composed of repetitive units of 7 sugar and has a special role in bacteria antigenicity) as shown in figure 1-1 [5]. The LPS combination is the most surface part of the cell wall of Gram-negative bacteria and is concerned as their endotoxin, and causes the severe reactions in immune system and highly poisonous for animals [6]. Endotoxins of Gram-negative bacteria are attached to cell wall of bacteria and cannot separate from it unless the bacteriolysis is done and the bacterium is decomposed. When LPS is degraded, the toxic part will be attached to lipid A. Polysaccharides are the formers of surface antigens of bacteria and are called Antigen O, and LPS molecule is attached to outer membrane of cell wall by hydrophobic bands. The place of LPS synthesis is in the cytoplasm membrane and after synthesis is transferred to its final location whiles it is ready [7]. Several studies have demonstrated that the lipopolysaccharide (LPS) of the *S*. Typhimurium is a key component associated with bacterial virulence, but in terms of vaccine potential, the LPS itself is not very immunogenic, and as a result, attempts have been made at synthesizing a vaccine which incorporates *Salmonella* LPS antigenic determinants, but is devoid of the toxic properties inherent in the lipid A moiety of the LPS, through the covalent attachment to carrier molecules, such as phospholipid bilayered vesicles (liposomes) [8]. Liposomes have received a considerable amount of attention as carriers for the delivery of a wide variety of biologically active substances to cells and tissues *in vitro* and *in vivo*, and they have been employed as immunological adjuvants for the enhancement or modulation of immune responses, especially CMI, to various antigens [9].

Materials and Methods

Patients

Patients of present study were hospitalized children and infants (95 cases: 60 males and 35 females) under the age of five years (40 days – 2.5 years). They were admitted to the Central

Pediatric Hospital and Al-Kadhiymiah Pediatric Hospital in Baghdad during the period 24/10/2010 - 30/11/2010, because of severe diarrhoea and fever.

Stool Sample Collection

A stool sample was taken from each pediatric patient upon admission to the hospital and before taking any medication using a disposable swab applicator, which was transferred to a test tube containing 10 ml sterilized peptone water.

Isolation and Identification of S. Typhimurium

After 2-3 hours, the sample was incubated at 37 °C with a total time of approximately 24 hours. After incubation, 5 ml of cultured stool sample were transferred to 250ml flask containing 50 ml tetra-thionate broth, and incubated at 37 °C for 24 hours. After incubation a loopful from each flask was streaked on SS agar (selective medium) plates, and incubated at 37 °C for 24 hours. The suspected colonies were subjected to further identifications, which included Gram stain, catalase test, indole test, methyl red test, Vogas-Proskauer test, citrate utilization test, Kligler iron test and glucose fermentation test [10]. The *Salmonella* was further identified by APi 20E system, while *S*. Typhimurium was identified by serotyping using a commercially available kit (BioRad Company, USA). Antibiotic sensitivity was also assessed for the isolated and identified *S*. Typhimurium.

Extraction and Purification of Lipopolysaccharide

The LPS was extracted from the bacterial outer membrane of one isolate by a method given by [11]. The extracted LPS was partially purified by gel filtration using Sephacryl 200 S gel, which was washed and suspended in 0.025 M of PBS (pH 7.2), degassed by using vacuum pump and then poured with care to avoid bubbles into a column with a dimension of 75×2 cm. The final volume of the column was 235.5 cm^3. The column was equilibrated with 0.025 M of PBS (pH 7.2), and the flow rate was 75 ml/hour.

The recovery of LPS was according to [29], in which 5 ml of crude LPS was applied gently to the column, and flow rate was approximately 75 ml/hour. Five milliliters fractions were collected, and absorption was read at 280 nm for protein determination [12]. Determination of carbohydrate content in the collected fractions was made by phenol-sulphuric acid method, by transferring 0.5 ml from each fraction in a sterile tube, then 0.5 ml of 5% phenol and 2.5 ml of sulphuric acid were added to each tube, and cooled in ice bath. Then, the absorbance was measured at 490 nm [13]. Protein concentration was determined according to [14]. The peak with the highest carbohydrate content and lowest contaminated protein was immunologically evaluated in albino male mice at a concentration of 100 µg/ml, alone or in conjugation with a commercially available liposome (LIP; Promega, USA).

Experimental Design

The mice were distributed into eight groups (negative controls; NC, positive controls; PC, complete Freund's adjuvant; CFA, heat-killed bacteria; HKB, formalin-killed bacteria; FKB, LPS, LIP and LPS-LIP conjugate). Each mouse was injected intraperitoneally (IP) with 0.1 ml of the respective solution in day 1, and a further dose in day 8. These mice were considered as pre-challenged groups, and they were dissected for laboratory evaluations in day 15. Further similar groups were challenged with 0.1 ml live bacteria in day 15 (5 x 10^4 cell/ ml), and they were dissected for laboratory evaluations in day 29 (post-challenged groups) [13]. The laboratory evaluation include histological examination of liver and spleen.

Histopathological Examination of Liver and Spleen

The liver and spleen were fixed in 10% formalin, [15]. was followed to prepare sections for Histopathological examinations. The procedure is outlined as the following:

- **Washing:** The sample was placed in 70% ethanol overnight.
- **Dehydration:** The sample was dehydrated with ascending concentrations (70, 80, 90 and 99%) of ethanol. There were two hours for each concentration.
- **Clearing:** The sample was placed in xylene for two hours.
- **Infiltration**: The sample was first placed in paraffin-xylene (1:1) for 30 minutes at 57-58 ºC, and then in paraffin alone for 2 hours at 60-70 ºC.
- **Embedding**: The sample was embedded in pure paraffin wax (melting temperature: 60-70 ºC) and left to solidified at room temperature.
- **Sectioning**: The paraffin block was sectioned (rotary microtome) at a thickness of 5 microns, and then the sections were transferred to a slide covered with Mayer's albumin. The section of tissue was placed in a water bath (35-40 ºC) for few seconds.
- **Staining**: The slide was first placed in xylene for 15-20 minutes, descending concentrations (100, 90, 80 and 70%) of ethanol (two minutes for each concentration) and finally distilled water. After that, the slide was stained with haematoxylin for 10-20 minutes and then washed with distilled water for 5 minutes. Then, the slide was placed in acidic alcohol for one minutes and washed with distilled water. After washing, the slide was placed in eosin stain for 10-15 seconds, and then in ascending concentrations (70, 80, 90 and 99%) of ethanol (two minutes for each concentration). Finally, the slide was cleared with xylene for 10 minute.
- **Mounting**: The slide was mounted with a Canada balsam and covered with a cover slip. Then, the slide was examined microscopically to inspect the Histopathological changes.

Statistical Analysis

They data were statistically analyzed using the statistical package SPSS (Statistical Package for Social Sciences) version 13.0. The investigated parameters were presented in as mean ± standard error (S.E.), and differences between means were assessed by ANOVA (analysis of variance), followed by LSD (least significant difference) or Duncan test. The difference was considered significant when the probability (P) value was ≤ 0.05, 0.01 or 0.001 [16].

Results and Discussion

Frequency of *S*. Typhimurium in Stool Samples

Out of 95 stool samples, *S*. Typhimurium was isolated and identified from two samples only (2.1%), which were belong to two children; the first was at age 25 months, while the second was 4 months older (S1 and S2 isolates). Both children had severe diarrhoea that was associated with fever.

Enteric infection with *Salmonella* spp. is an important cause of children diarrhoeal disease worldwide, but the frequency of

children with the infection shows variations between studies. A study from Bolivia of 133 consecutive children less than 5 years old presenting with bloody diarrhoea revealed bacterial aetiology in 41%, and out of this percentage, *Salmonella* spp. accounted for 4% [17]. However, a more recent study from Vietnam, no *Salmonella* infection was reported in 587 children with diarrhoea under the age 24 months, although other potential pathogens were identified in 67.3% of children with diarrhoea, including Gram negative bacteria [18]. Accordingly, understanding the burden of pathogen specific diarrhoeal disease and the variation by region is important for planning effective control programs for the overall reduction of diarrhoea disease among persons of all ages, especially in children under the age of 5 years.

Extraction and Partial Purification of Lipopolysaccharide

Antibiotic sensitivity test demonstrated that S1 isolate was more resistance than S2 isolate; therefore it was considered more virulent and subjected for further manipulations, which included extraction of LPS from the bacterial outer membrane. Chemical characterization of the extracted LPS revealed that the carbohydrate content was 2.34 mg/ml, while the protein concentration was very low (0.52 µg/ml). Partial purification of extracted LPS by using gel-filtration chromatography (Sephacryl S200) showed three peaks, and after determination of protein and carbohydrate concentrations for each peak, peak two was observed to have the highest carbohydrate content (25%) and the lowest contaminated protein (0.001%) (Figure 1 and Table 1)

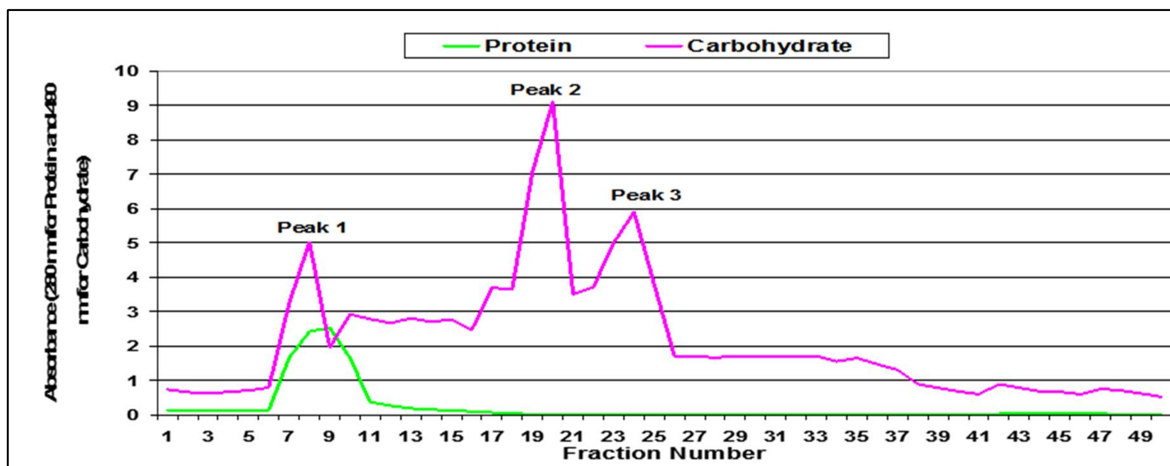

Fig 1: Gel-filtration chromatography for lipopolysaccharide partial purification from *S*. Typhimurium (S1 isolate) by using Sephacryl S200, 75×2 cm column equilibrated and eluted with 0.025 M PBS pH 7.2 with a flow rate of 75 ml/hour.

Table 1: Carbohydrate and protein contents of crude and partially purified lipopolysaccharide.

Lipopolysaccharide		Carbohydrate (%)	Protein (%)
Crude		17.2	6.3
Partially Purified	Peak 1	11.0	3.0
	Peak 2	25.0	0.001
	Peak 3	12.0	0.001

Most studies agree that percentage yield of carbohydrates may vary widely following endotoxin purification. The first group obtained 12-18% carbohydrate from partially purified endotoxin [19], while other investigators reported a less percentage range, which was 16.2-24.8% [20]. In addition, 33.3% was obtained by a further group of investigators [21]. These differences can be attributed to the types of bacterial species from which LPS was extracted, method of extraction and purification process.

The present finding (carbohydrate yield of 25%) differs from that recorded by [22], who demonstrated that the carbohydrate percentage in partially purified LPS of local *P. aeruginosa* isolate was 15%, and a further contradicting finding was also recorded by [19], in which 12-18% range was observed. However, an agreement was reached with further studies. The first demonstrated a yield of 16-24% [23], and the same finding was reported by [20], in which the carbohydrate percentage in purified LPS was 16.2-24.8%.

In table 1, we can notice that the protein percentage in partially purified LPS was 3% in peak 1 and 0.001% in peaks 2 and 3. The percentage of peak 1 differs from the percentage recorded by [22], who recorded that the protein percentage in partially purified LPS from *P. aeruginosa* was 2%. It also differs from the percentage recorded by [24], who stated that the percentage of protein bound to the purified LPS was 4.3%. However, an agreement with [25] is reached, because they found that the percentage was less than 0.1%. Furthermore, [20] recorded that the percentage of contaminated protein was very little, so they neglected such finding.

The differences in protein and carbohydrate percentages in the purified LPS may be related to differences between the bacterial strains and their content of LPS, differences in the methods used for extraction and purification of LPS and experiment circumstances. The results also showed that there were no nucleic acids in the partially purified LPS. It was also observed that the carbohydrate percentage in the partial purified LPS (25% in peak 2) was higher than that of the crude LPS (17.2%), and a similar observation was made for the protein (3.0 and 0.001 *vs*. 6.3%). Both observations suggest the efficiency of the applied method of purification by gel filtration.

Histopathological Evaluation of Liver and Spleen

Different Histopathological changes were observed in liver and spleen of pre- (day 15) and post-challenged (day 29) groups of mice, but such changes were subjected to the type of group and if it was pre- or post-challenged. For the ease of presentation, under each picture, the Histopathological profile is given:

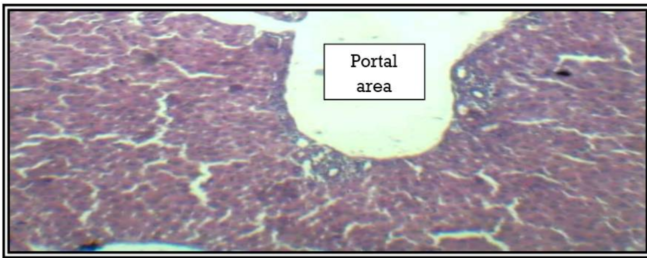

Fig 1: Histological section of mouse liver (pre-challenged negative control) showing normal looking hepatocytes (H and E; 200X).

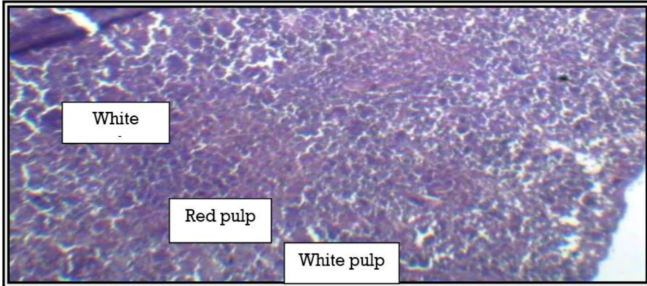

Fig 2: Histological section of mouse spleen (pre-challenged negative control) showing normal structure appearance of white and red pulp of the lymphoid parenchyma tissue (Han E; 200X)

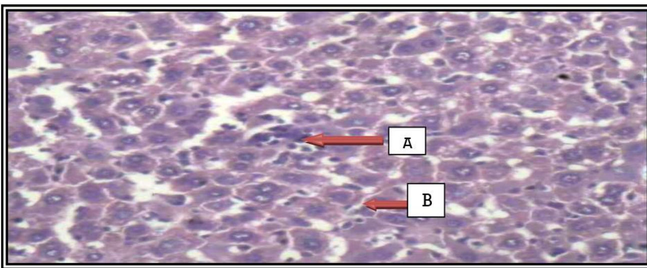

Fig 3: Histological section of mouse liver (pre-challenged positive control) showing degenerative change and necrosis (A) with inflammatory cells infiltration (B) (H and E; 250X).

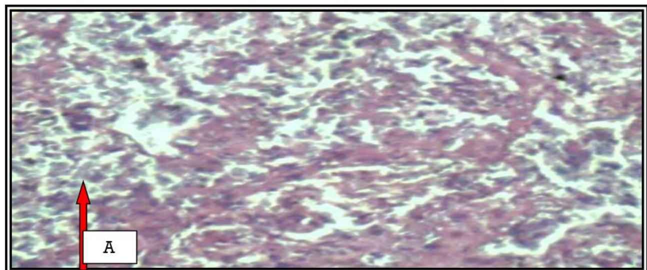

Fig 4: Histological section of mouse spleen (pre-challenged positive control) showing diffuse hyperplasia of the lymphoid parenchymal tissue(A) (H and E; 200X).

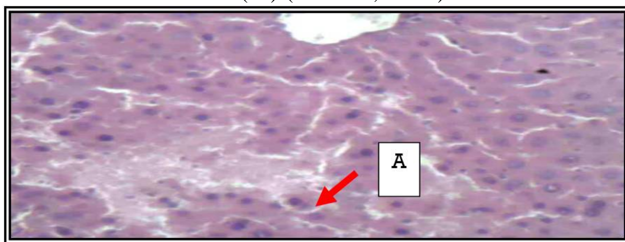

Fig 5: Histological section of mouse liver (pre-challenged lipopolysaccharide+lipsome conjugate group) showing certain necrosis area (A) of hepatic paranchymal tissue (H and E; 200X).

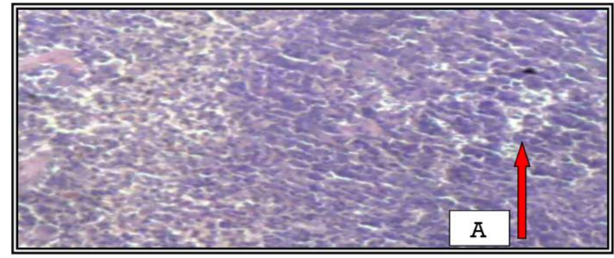

Fig 6: Histological section of mouse spleen (pre-challenged lipopolysaccharide+lipsome conjugate group) showing widening of white pulp (A) and follicular lymphoid hyperplasia (H and E; 200X).

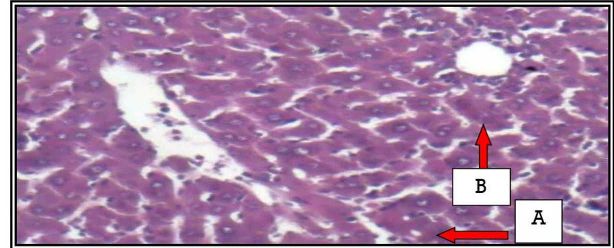

Fig 7: Histological section of mouse liver (post-challenged negative control group) showing mild degenerative changes, dilatation of sinusoid (A) and mild inflammatory cells infiltration(B) (H and E; 200X).

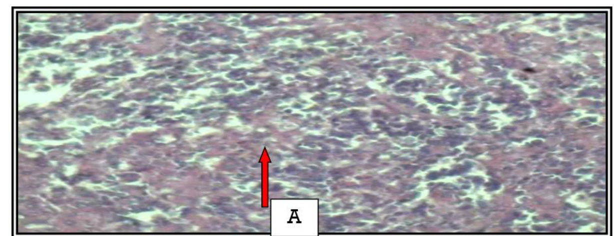

Fig 8: Histological section of mouse spleen (post-challenged negative control group) showing follicular lymphoid hyperplasia with mild infiltration of inflammatory cells in red pulp(A) (H and 200x).

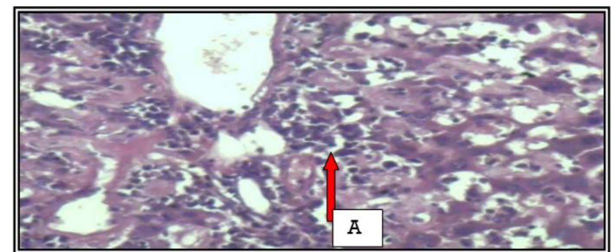

Fig 9: Histological section of mouse liver (post-challenged positive control group) showing necrosis of hepatocytes with heavy inflammatory cells infiltration (A) (H and E; 200X)

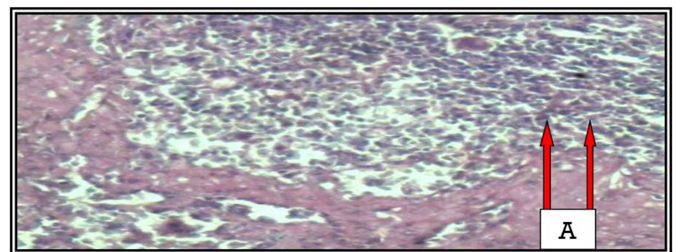

Fig 10: Histological section of mouse spleen (post-challenged positive control group) showing degenerative changes and necrosis of the lymphoid paranchymal tissue (H and E; 200X).

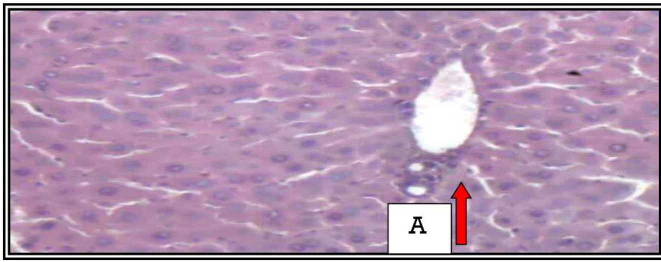

Fig 11: Histological section of mouse liver (post challenge lipopolysaccharide liposome conjugate group) showing look-like normal hepatic tissue appearance especially near portal area ((A) (H and E; 200X).

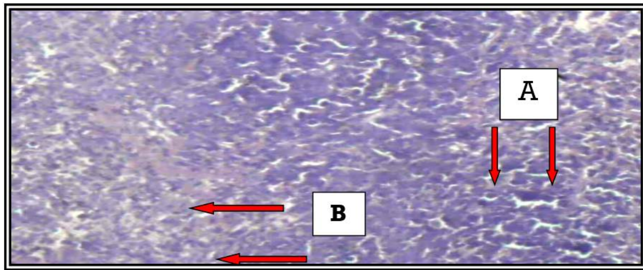

Fig 12: Histological section of mouse spleen (post-challenged lipopolysaccharide liposome conjugate group) showing follicular hyperplasia, in which widening of white pulp (A) and reduction in red pulp are observed (B) (H and E; 200X).

Mice infected with *S. typhimurium* showed hyperplasia in intestinal mucosa and multiple necrosis in the liver and spleen. These results agreed with many researchers who mentioned that *S. typhimurium* penetrates the intestinal epithelium, enters M cells that overlying the Peyer's patches, translocate very rapidly to other organs, and invade the macrophages in liver and spleen [26].

Another study indicated that *S. typhimurium* oral infection leads to systemic disease similar to typhoid results in inflammation and immune response in the intestine and gastrointestinal lymphoid tissue including peyer's patches and mesenteric lymph nodes [27].

The most important finding of evaluating histopatholigical changes in mice of different groups in the present study, is post-challenged mice vaccinated with LPS+LIP, in which the liver histological section showed look-like normal hepatic tissue appearance especially near the portal area; a finding that confirms the other laboratory evaluations (phagocytosis, delayed hypersensitivity reactions and bacterial isolation). The reduced load of liver bacteria and the enhanced humoral and cellular immunity could have contributed to the observed re-normalling the histopathological profile of liver tissues. In agreement with [28] demonstrated that bacterial dissemination to the blood and, subsequently, to the reticuloendothelial system and other organs was diminished in vaccinated mice, and as a consequence, fewer histopathological lesions were evident in the liver of mice. This led the authors to hypothesize that more effective early inactivation of *S*. Typhimurium might be responsible for the increase in mean survival time of mice. This is also raised the question of which immune mechanisms contribute to the enhanced host defence, and their answer was in favour of enhanced phagocytic and killing activity of neutrophils.

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