Histoprotective Effect of *Salmonella typhimurium* non-Porin Outer Membrane Proteins Against Salmonellosis in Mice

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Abstract

This study was designed to evaluate the histoprotective effect of non-porin outer membrane proteins against *Salmonella* infection in mice. *S. typhimurium* was isolated from stool of infants suffering from diahrria after performing microscopic examination, some biochemical tests and API 20E identification. The outer membrane proteins (OMPs) of *S. typhimurium* was extracted and analyzed by Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE). Results of protein profile of the *S. typhimurium* outer membrane proteins revealed that bands with molecular weight ranged between 33-203 kDa were present. Purification by ion exchange chromatography on DEAE- cellulose was carried out. Results of the purified OMPs by (SDS-PAGE) exhibited that two bands with molecular weights of 33 and 203 KDa were present, then mice treated with non-porin OMPs and infected with *S. typhimurium*. Histopathological study exhibited that infection of mice with *S. typhimurium* showed shortening of intestinal villi with inflammatory cells infiltrate inside the villi and multiple necrosis in the spleen and liver. While mice treated with non-porin OMPs and infected with *S. typhimurium* showed looking like normal appearance of intestinal villi and normal structure appearance of spleen and liver.

Keywords: Salmonellosis, non-Porin, Outer Membrane Proteins.

Introduction

Salmonellosis is an infectious disease of humans and animals caused by organisms of the two species of *Salmonella* (*Salmonella enterica* and *S. bongori*). *Salmonella* causes a serious health problem in developing countries through a wide range of human diseases such as enteric fever, gastroenteritis and bacteremia [1].

People exposed to *Salmonella* bacteria may experience mild to severe diarrhea, abdominal pains, fever, and occasionally vomiting for several days [2].bloodstream infections are infrequent, but can be quite serious, particularly in the very young or elderly. There are 16 million annual cases of typhoid fever, 1.3 billion cases of gastroenteritis and 3 million deaths worldwide due to *Salmonella* [2].

The emergence of resistance to antimicrobial chemotherapeutic agents and the possible reversal of the resistance in *Salmonella* has become a significant issue leading to difficulties in the management of disease for both developed countries [3].There is a need for better and improved new generation vaccines against salmonellosis. The outer membrane proteins (OMPs) of Gram negative bacteria have a role in disease processes as they act at an interface between the host and pathogen [4].

The outer membrane proteins (OMPs) of *Salmonella* are being looked upon as new immunizing agents that can confer protection against typhoid. Four non-porin OMPs (molecular masses 15 KDa, 33 KDa, 37 KDa, and 49 KDa) have been selected from the OM profile of *Salmonella typhimurium* and these proteins confer varying degree of protection against bacterial challenge with experimentally induced murine salmonellosis and also decrease the number of bacteria reaching liver [5].

Materials and Methods

Bacterial isolates

S.typhimurium was supplied by Immunol. LAB. College of Science/ Al-Nahrain Un. That previously isolated from stool of infant suffering from diarrhea and reidentified by performing some biochemical tests and API 20E System.

Extraction of outer membrane proteins from *S.typhimurium*

Extraction of outer membrane proteins from *Salmonella typhimurium* outer surface was carried out according to Nurminen and Kuusi [6 and 7].

Bacterial isolate was grown on brain heart

infusion agar at 37˚C over night, then harvested and washed three times with Tris-Hcl buffer (0.01M at pH 7.8) and centrifuged at 3500 rpm for 10 min. Ten grams of wet weight of bacterial cells were suspended in 100 ml of 2% triton x-100 and left over night, then cells were centrifuged at 5000 rpm for 10 min, after that 100 ml of (2% triton x-100-EDTA 0.01M) was added to pellet and left in incubator at 37˚C for 10 min. Twenty mg of lysozyme was added to the mixture and left in incubator for one hour, then the mixture was centrifuged at 5000 rpm for 20 min. Pellet was washed with 100 ml of Tris (0.01M) –EDTA (0.01M) pH7.8 and centrifuged at 4000 rpm for 20 min, washed with 100 ml of triton x-100 –Mgcl2 pH 7.8, and centrifuged at 6000 rpm for 10 min. Finally pellet was washed with 100 ml Tris-Hcl buffer (1M) at pH 8.8 and centrifuged at 5000 rpm for 15 min and stored in freezer prior to use for further investigation. Protein concentration was determined according to Bradford method [8] and purification was carried out on DEAE-Cellulose. Ten ml of extracted crude OMPs was loaded on ion exchange column, the separated fractions were collected at flow rate 20 ml/hour approximately, 3 ml for each fraction, the washing was achieved by using potassium phosphate buffer (the same buffer used in equilibration), the elution was achieved by the same buffer with gradient concentration of sodium chloride, the flow rate was 20 ml/hour, the protein fractions were measured at wave length 280 nm of the washed and eluted fractions, the volume and protein concentration were also measured. OMPs were detected by Sodium Dodecyl Sulphate-PolyAcryl amid Gel Electrophoresis [9].

Experimental Design

Twenty Albino mice (male and female) were used with (8-10) weeks, and the weight of each was (28-30) grams. They were divided into four groups (each group contain 5 mice); and kept in a separate plastic cage, the animals were hosted at (20- 25) ˚C. [10].

- **Group.1** mice were subcutaneously injected with 1.0ml of normal physiological saline.
- **Group.2** mice were dosed with 100µl ($1x10^9$ cfu/ml) *Salmonella typhimurium.*
- **Group.3** mice were subcutaneously injected with 0.5ml of non porin outer membrane proteins at a concentration (50µg/ml) and 0.5ml of complete Freund´ s adjuvant.
- **Group.4** mice were subcutaneously injected with 0.5ml of non porin outer membrane proteins at a concentration (50 µg/ml) and 0.5ml of complete Freund´ s adjuvant and then orally dosed with (100µl) of *Salmonella typhimurium* ($1x10^9$ cfu/ml).

Mice were sacrificed by cervical dislocation and liver, spleen and intestine were collected. Pieces were taken from previous organs (for histopathological study**)** put in petridishes contain physiological salt solution to remove the fatty tissues and sticky bundles, then the organs were put in tubes containing 10 % formalin for about 16-18 hours for fixation purpose, after that they were transferred into tubes containing 70% ethanol alcohol till the time of the final preparation in which the samples are transferred into 90% ethanol for 6 hours, transferred in 99% alcohol for about 6 hours then put in xylol for 2 hours and sections of about 5µ were obtained using metal knives on a handling rotary microtome, and then the prepared sections were put in a water bath of 43˚C then they were stick in a glass slide in a proper manner. The staining method was performed by using hematotoxilin and eosin [11].

Results

Chemical Characterization of OMPs

Chemical characterization of the crude outer membrane proteins extracted from *S.typhimurium* was performed by estimating the protein concentrations according to Bradford method [8] depending on the standard curve of bovine serum albumin. The result of protein concentration was 6.6 mg/ml.

Detection of *S. typhimurium* outer membrane proteins by SDS-PAG electrophoresis

S. typhimurium outer membrane proteins was analyzed by 10% SDS-PAGE and the lane of proteins band obtained were compared with five marker proteins (Esterase MW=200kDa, γ-globulin MW=150 kDa, transferrin MW=80 kDa, Glutamate Dehydrogenase MW=55 kDa, Trypsine MW=20 kDa), Results of protein profile by SDS-PAGE revealed that five bands with MW ranged between 33-203 kDa Fig.(1).

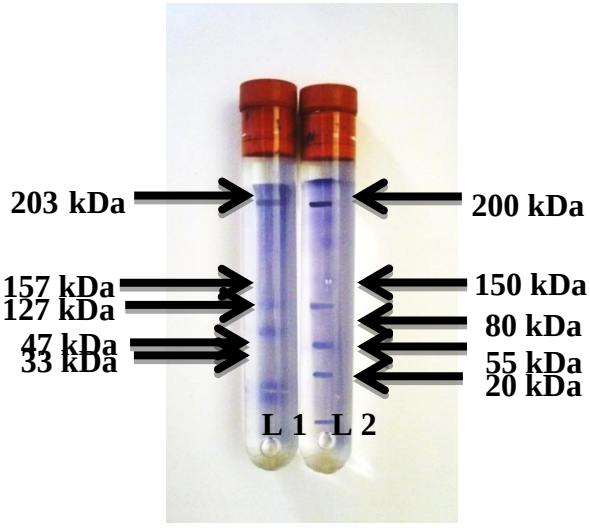

Fig. (1) Protein profile analysis of Salmonella typhimurium outer membrane proteins (crude protein) by 10% SDS-PAGE. (L1): crude OMPs, (L2): marker proteins.

Detection of outer membrane protein purity

Partial purification of *S.typhimurium* OMPs were carried out by using ion exchange chromatography on DEAE cellulose (Fig.(2)), and 55 fractions of elution were collected and subjected for protein profile analysis.

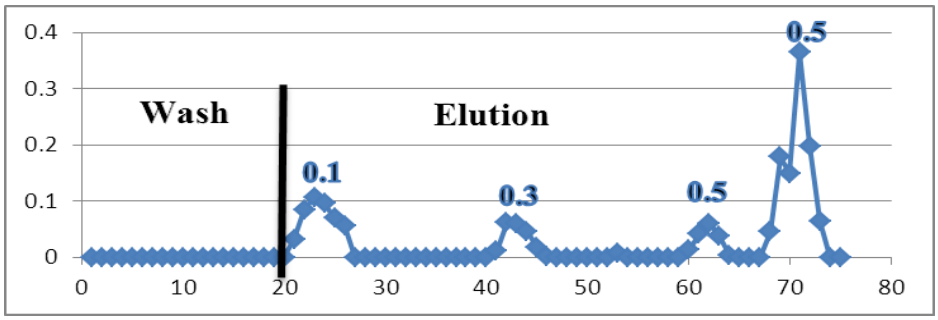

Fig. (2) Ion exchange chromatography of outer membrane protein of S. typhimurium on DEAE-Cellulose column with a flow rate of 3ml/min.

After partial purification of *S.typhimurium* OMPs by using ion exchange chromatography on DEAE cellulose, Chemical analysis of the partially purified OMPs was carried out and involved determination of protein concentration. The result indicated that the protein concentration was 0.450 mg/ml. The analysis of protein profile of OMPs by SDS-PAGE gave two bands 33 and 203 kDa (Fig.(3)).

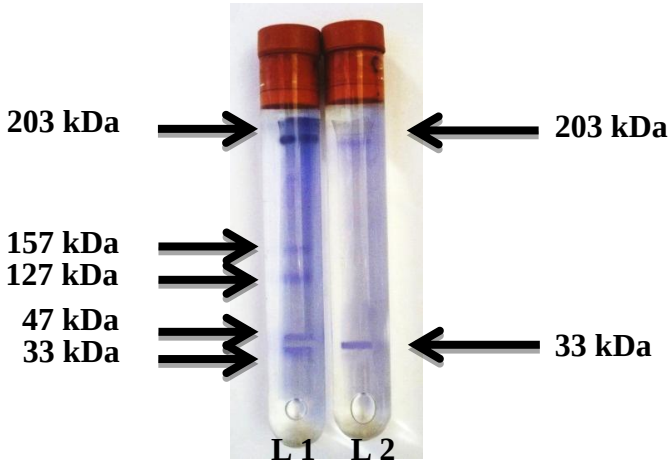

Fig.(3) Polyacrylamide gel electrophoresis of purified outer membrane protein of S. typhimurium after ion exchange on DEAE-cellulose. (L1): crude OMPs, (L2): Partial purified OMPs.

Histopathological effects on intestine, spleen and liver of mice

Different histopathological changes were observed in intestine, spleen and liver for four groups of mice. For the ease of presentation, under each picture, the histopathological profile is given.

- Intestine section of mice (negative control) showing presence of payer's patch in the jejunum (Fig.(4)).
- Spleen section of mice (negative control) showing normal structure appearance with presence of white pulp and red pulp (Fig.(5)).
- Liver section of mice (negative control) showing normal looking appearance of parenchymal hepatic tissue with portal area and central vein (Fig.(6)).

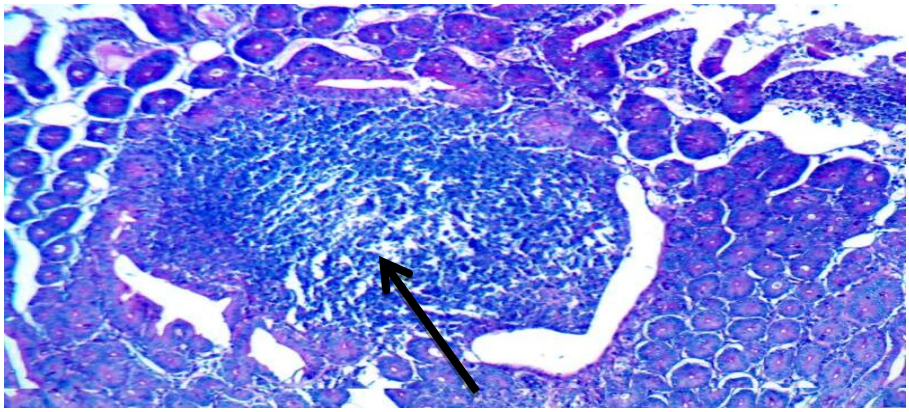

Fig.(4) Intestine section of mice (negative control) showing presence of payer's patch in the jejunum (→) (H and E; 200X).

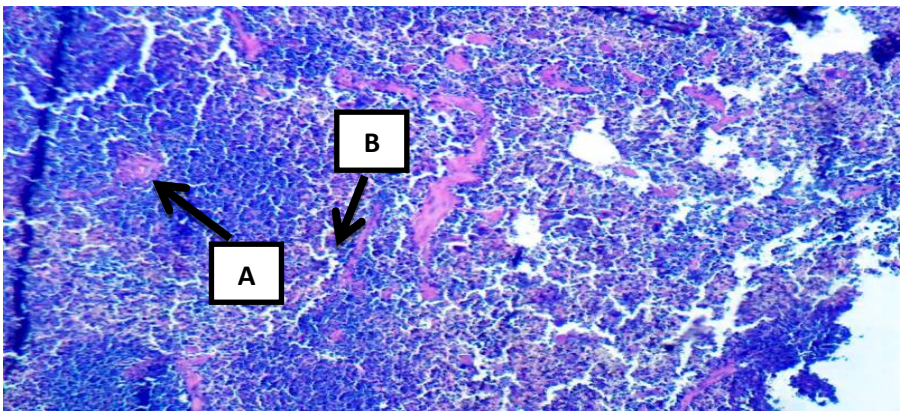

Fig. (5) Spleen section of mice (negative control) showing normal structure appearance with presence of white pulp (A) and red pulp (B) (H and E; 200X).

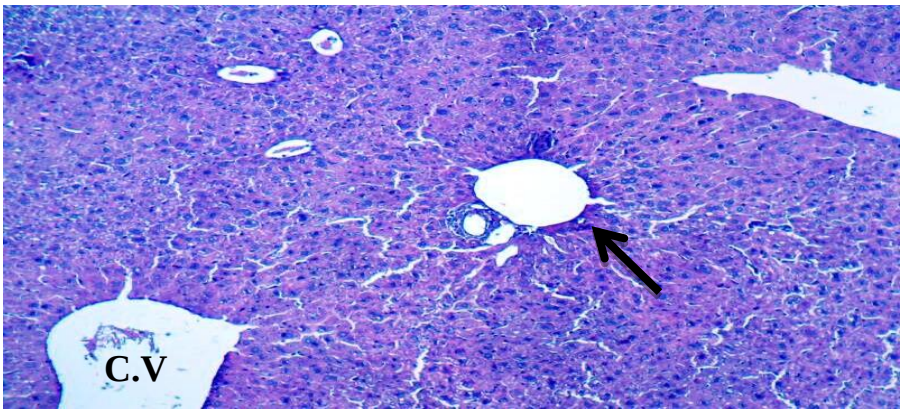

Fig.(6) Liver section of mice (negative control) showing normal looking appearance of parenchymal hepatic tissue with portal area (→) and central vein (H and E; 200X).

- Intestine section of mice infected with *Salmonella typhimurium* showing shortening of intestinal villi with infiltrate of inflammatory cells inside the villi (Fig.(7)).
- Spleen section of mice infected with *Salmonella typhimurium* showing certain necrotic areas of parenchymal spleening tissue and widening of white pulp (Fig.(8)).
- Liver section of mice infected with *Salmonella typhimurium* showing dispersed necrotic cells with inflammatory cells infiltrate (Fig.(9)).

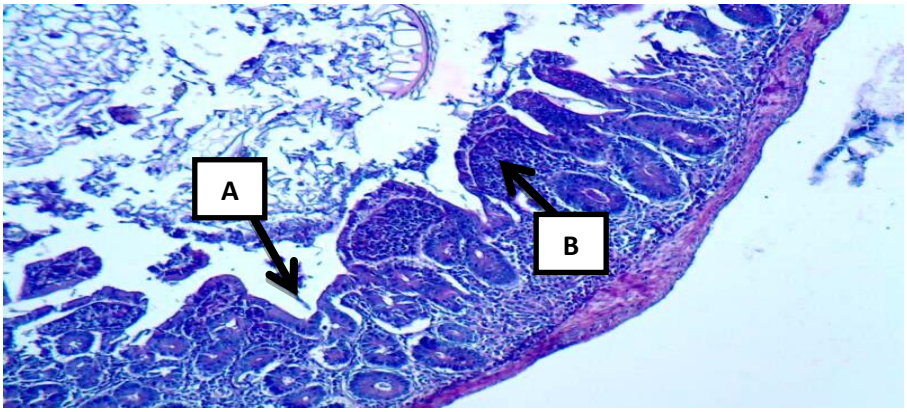

Fig. (7) Intestine section of mice infected with Salmonella typhimurium showing shortening of intestinal villi (A) with infiltrate of inflammatory cells inside the villi (B) (H and E; 200X).

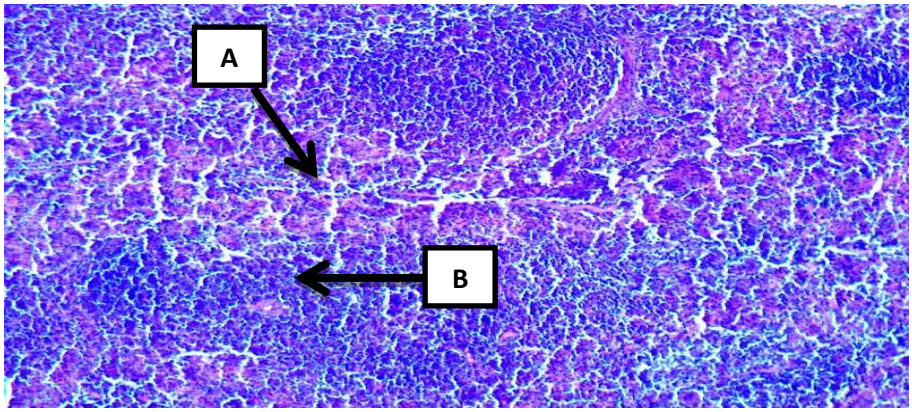

Fig.(8) Spleen section of mice infected with Salmonella typhimurium showing certain necrotic areas of parenchymal spleening tissue (A) and widening of white pulp (B) (H and E; 200X).

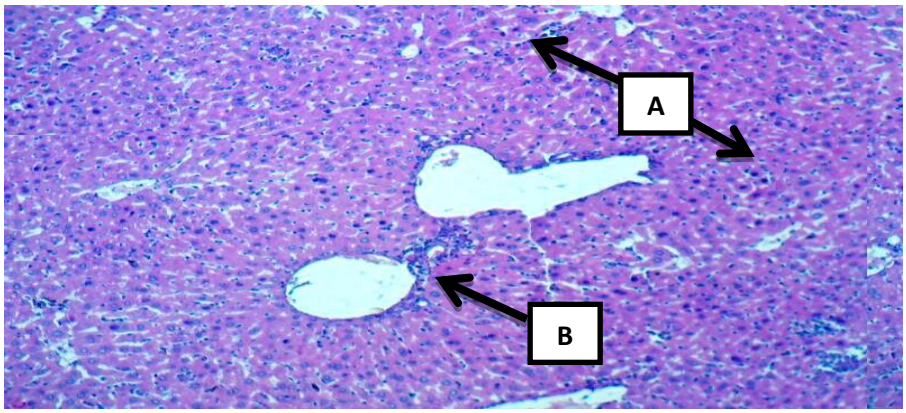

Fig. (9) Liver section of mice infected with Salmonella typhimurium showing dispersed necrotic cells (A) with inflammatory cells infiltrate (B) (H and E; 200X).

- Intestine section of mice treated with non-porin OMPs showing normal looking appearance of intestinal mucosal tissue (Fig.(10)).
- Spleen section of mice treated with non-porin OMPs showing diffuse hyperplasia of parenchymal lymphoid tissue with reactive collagen fibers (Fig.(11)).
- Liver section of mice treated with non-porin OMPs showing normal looking appearance of hepatocyte cells (Fig.(12)).

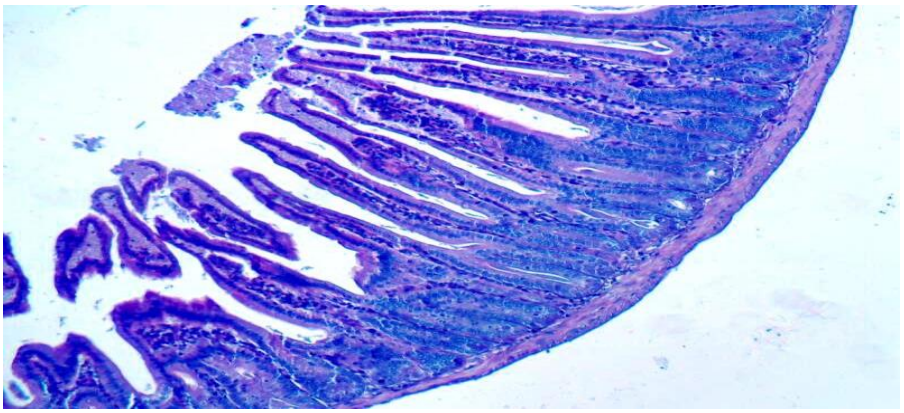

Fig. (10) Intestine section of mice treated with non-porin OMPs showing normal looking appearance of intestinal mucosal tissue (H and E; 200X).

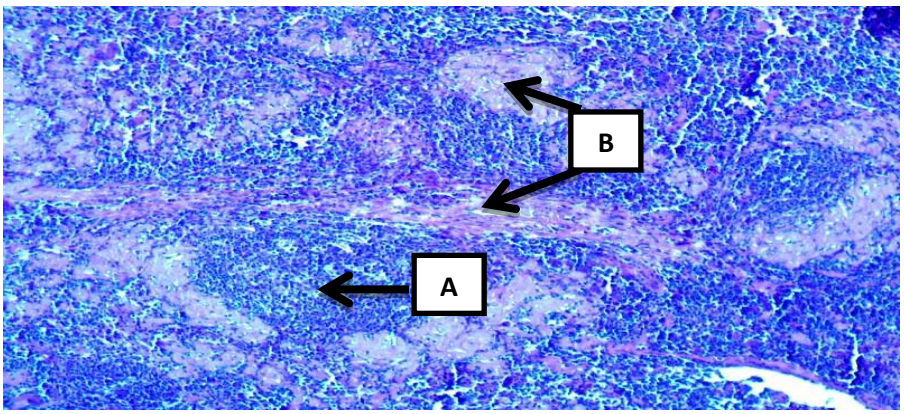

Fig. (11) Spleen section of mice treated with non-porin OMPs showing diffuse hyperplasia of parenchymal lymphoid tissue (A) with reactive collagen fibers (B) (H and E; 200X).

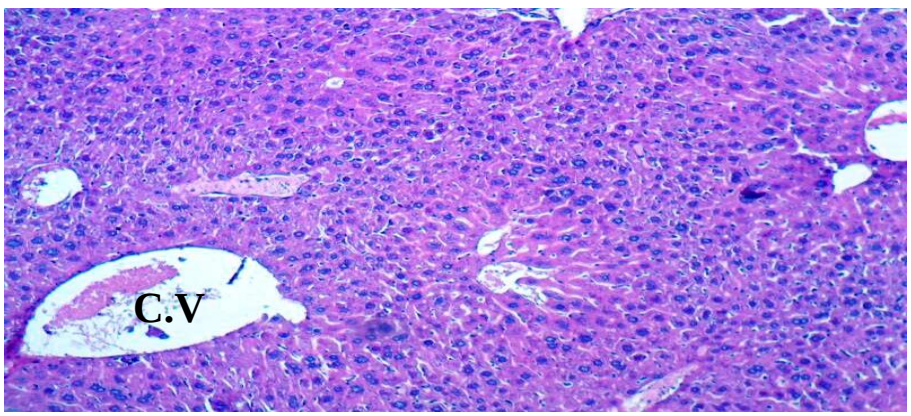

Fig. (12) Liver section of mice treated with non-porin OMPs showing normal looking appearance of hepatocyte cells (H and E; 200X).

- Intestine section of mice treated with non-porin OMPs and infected with *Salmonella typhimurium* showing looking like normal appearance of intestinal villi (Fig.(13)).
- Spleen section of mice treated with non-porin OMPs and infected with *Salmonella typhimurium* showing lymphoid folicular hyperplasia with presence of megakaryocyte (Fig.(14)).
- Liver section of mice treated with non-porin OMPs and infected with *Salmonella typhimurium* showing normal structure with slight hypertrophy appearance of hepatic cell(Fig.(15)).

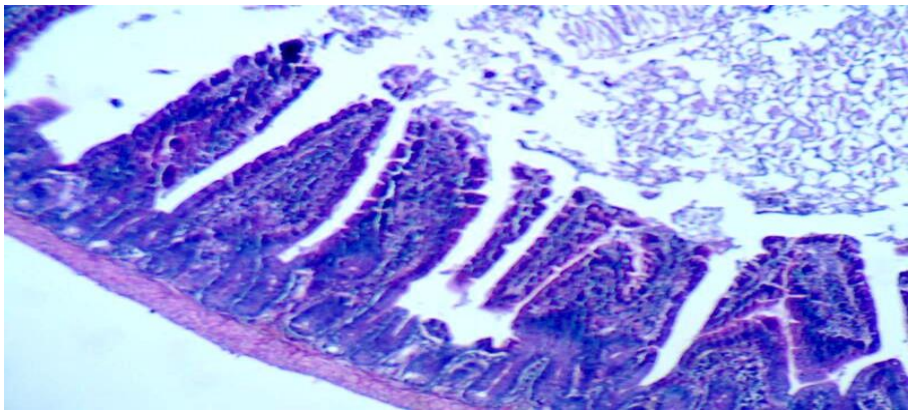

Fig. (13) Intestine section of mice treated with non-porin OMPs and infected with Salmonella typhimurium showing looking like normal appearance of intestinal villi (H and E; 200X).

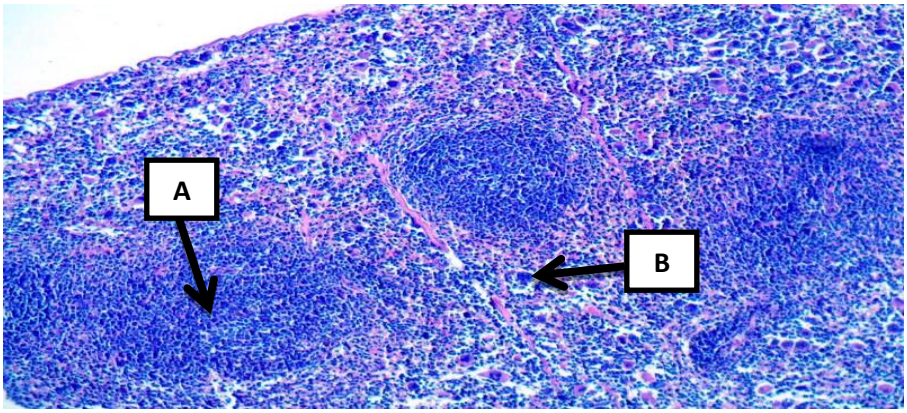

Fig.(14) Spleen section of mice treated with non-porin OMPs and infected with Salmonella typhimurium showing lymphoid folicular hyperplasia (A) with presence of megakaryocyte (B) (H and E; 200X).

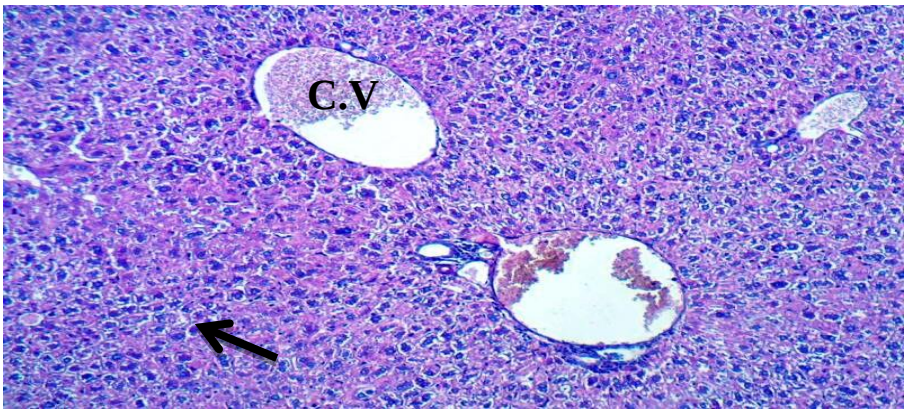

Fig. (15) Liver section of mice treated with non-porin OMPs and infected with Salmonella typhimurium showing normal structure with slight hypertrophy appearance of hepatic cell (→) (H and E; 200X).

Discussion

Mice infected with *S. typhimurium* showed shortening of intestinal villi with inflammatory cells infiltrate inside the villi and multiple necrosis in the spleen and liver. 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 [12, 13 and 14].

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 [15].

Mice treated with non-porin OMPs of *S. typhimurium* and infected with *S. typhimurium* showed looking like normal appearance of intestinal villi and normal structure appearance of spleen and liver. These results come in accordance with [16] who observed that the OMPs are good immunogens in the induction of protective immunity against the *Salmonella* infections. This protective effect could be attributed to OMPs ability to activate dendritic cells (DCs) and enhance Th1 polarization. It is known that DCs can capture degraded bacteria or protein of bacteria and present their antigens on major histocompatibility complex (MHC) class molecules to T cells. As a result, an adaptive immune response that specifically targets bacteria-derived antigens is initiated.

Conclusions

Outer membrane proteins with MW 33 and 203 kDa alleviate the histopathic effect of *S.typhimurium* on intestine, spleen and liver.

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الخلاصة

أجريت الدراسة الحالية لتقييم التأثير الوقائي للبروتينات اللابورينية الموجودة في الغلاف الخارجي لبكتيريا *Salmonella typhimurium* ضد امراض السالمونيلا في الفئران. استخلصت البروتينات اللابورينية الموجودة في الغلاف الخارجي للبكتيريا وبعدها حللت محتويات هذه البروتينات باستخدام طريقة الترحيل الكهربائي ، بعد إجراء بعض الاختبارات الكيمو حيوية على البكتيريا و فحصها مجهريا و تأكيد نتائج التشخيص باستخدام نظام العدة التشخيصية للعائلة المعوية API 20 E .اظهرت نتائج هذا الترحيل وجود مجموعة من الحزم تتراوح أوزانها الجزيئية بين (٢٠٣-٣٣) كيلو دالتون. نقيت هذه البروتينات باستخدام تقنية التبادل الأيوني عن طريق المبادل DEAE-Cellulose ، تم التعرف على نقاوة البروتين باستخدام طريقة الترحيل الكهربائي، وكانت نتائج هذا الترحيل هو الحصول على حزمتين ذات وزن جزيئي ٣٣ و ٢٠٣ كيلو دالتون.عوملت الفئران مع البروتينات اللابورينية الموجودة في الغلاف الخارجي للبكتيريا. اظهرت نتائج الفحص النسيجي ان اصابة الفئران بالبكتريا تسبب في قصر الزغابات المعوية مع ارتشاح الخلاية الالتهابية داخل هذه الزغابات مع تنخر في الكبد والطحال بينما ادت معاملة الفئرات بالبروتينات اللابورينية بعد اصابتها بالكتريا الى معادلة التأثير السمي للبكتريا.