Extraction and Purification of Vibrio Cholerae Enterotoxin and Study its Cytopathic Effect on Some Mice Organs

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Abstract

Vibrio cholerae enterotoxin was extracted by cooling centrifugation and filtration with milipore filter (0.22um) and was purified by using Sephacryl –S- 6 gel filtration,the content of protein was estimated . The results showed protein concentration was 28.5 microgram/ml,the present of enterotoxin was detected by infant suckling mouse method.

.The cytopathic effect of enterotoxin was studied by injecting a number of mice with purified enterotoxin, It was found caused shortening the villi of the intestine at concentration 55 and 45 ug /ml of purified enterotoxin, while the effect on liver showed degenerative change with necrosis at 55 ug/ml of enterotoxin and caused necrosis and infiltration of inflammatory cells at 45 ug /ml, the effect on spleen tissue was represented by widening of white pulp, and reduction or disappearance of red pulp also inflammatory cells wre found i.e. lymphocyte and hypoplasia of white pulp at the two concentrations of the enterotoxin .

Key words: Vibrio cholera, Extraction of enterotoxin, Histopathogenecity.

استخلاص وتنقية السم المعوي لبكتريا Vibrio cholerae ودراسة تاثيراته المرضية في بعض اعضاء الفئران

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الخلاصة

استخلص السم المعوي من بكترياVibrio cholera، تم تنقيته بطريقه الترشيح الهلامي باستخدام هلام Sephacryl –S-6 وقدر كميه البروتين باستخدام طريقه فولن لوري ووجد ان كميه البروتين تساوي ٢٨،٥مايكروغرام /مل و تم التحري عن السم باستخدام الفأر الرضيع،درست التأثيرات النسيجيه للسم المعوي المنقى في عدد من اعضاء الفئران التي حقنت بالسم بتركيزي ٥٥و ٤٥ مايكروغرام /مل ووجد انه سبب قصر في حجم الزغابات للامعاء، اما بالنسبه للتأثيرات في الكبد تمثلت تنكس للخلايا الكبديه وتنخر بتركز ٥٥ مايكروغرام /مل وسبب السم المعوي بتركيز ٤٥مايكروغرام/مل نخر للخلايا الكبديه وارتشاخ للخلايا الالتهابيه، كما سبب توسع في اللب الابيض واختزال اللب الاحمر لنسيج الطحال كما سبب ارتشاح للخلايا الالتهابيه خصوصا الخلايا اللمفاويه.

Introduction

The genus Vibrio consists of gram-negative straight or curved rods, motile by means of a single polar flagellum [1]. Vibrios are capable of both respiratory and fermentative metabolism. O2 is a universal electron acceptor, they do not

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denitrify. Most species are oxidase-positive. In most ways vibrios are related to enteric bacteria, but they share some properties with pseudomonads as well. The Family Vibrionaceae is found in the "Facultatively Anaerobic Gram-negative RodsVibrios are distinguished from pseudomonads by being fermentative as well as oxidative in their metabolism. Of the vibrios that are clinically significant to humans, Vibrio cholerae,the agent of cholera, is the most important[2,3]Vibrios are one of the most common organisms in surface waters of the world. They occur in both marine and freshwater habitats and in associations with aquatic animals. Some species are bioluminescent and live in mutualistic associations with fish and other marine life. Other species are pathogenic for fish, eels, and frogs, as well as other vertebrates and invertebrates [4] V. cholerae produces a number of enzymes and other products which have at times been implicated as participating in the diarrhea producing process [5]. These have been describedin recent reviews [6] V. cholerae produces cholera toxin, the model for enterotoxins, whose action on the mucosal epithelium is responsible for the characteristic diarrhea of the disease cholera. In its extreme manifestation, cholera is one of the most rapidly fatal illnesses known. A healthy person may become hypertensive within an hour of the onset of symptoms and may die within 2-3 hours if no treatment is provided. More commonly, the disease progresses from the first liquid stool to shock in 4-12 hours, with death following in 18 hours to several days[7].The primary symptoms of cholera are profuse, painless diarrhea and vomiting of clear fluid [8,9]. These symptoms usually start suddenly, one to five days after ingestion of the bacteria. The diarrhea is frequently described as "rice water" in nature and may have a fishy odor; an untreated person with cholera may produce 10 to 20 liters (3 to 5 US gal) of diarrhea a day [1] with fatal results. For every symptomatic person, 3 to 100 people get the infection but remain asymptomatic[10]. Cholera has been nicknamed the "blue death" due to a patient's skin turning a bluish-gray hue from extreme loss of fluids.[11,12]

The isolation of cholera enterotoxin in ahighly purified state and the development of means of studying its effect upon isolated viable membranes and cell systems have led to recent studies which have made major contributions to understanding of the biochemical effects of cholera enterotoxin and the relationship of these effects to the disease produced in man by infection with V. cholerae [13]. Although some gaps remain in our knowledge of the mechanism of action of cholera enterotoxin, single hypothesis now appears to explain most of its intestinal and nonintestinal effects, cytotoxic and cytopathic effects .Thusthe following research was designed for purification of enterotoxin and study some cytopathic effect .

Materials and Methods:

1-bacterial strains:

V. cholerae (NAG) isolate was obtained from central health labortaory /Baghdad,whcich was diagnosed previously and rediagnosed by inoculated in TCBS, and identified by Api 20 E kit [14].

2- Production and Extraction of Enterotoxin:

Enterotoxin of V.cholera was produced by inoculation 500 ml of brain heart infusion broth with V. cholera isolate and incubated at 37 C for 24 hr.Enterotoxin was harvested by cooling centrifuge at 6000 r.p.m. for 30 min. and filtrate by using Millipore filter (0.22 um) [15].

3- detection of Enterotoxin activity

Enterotoxin activity was detected using infants suckling mouse method according to [16,17].

4- Purification of Enterotoxin:

Enterotoxin was purified according to[18] by using Sephacryl S- 6 gel filtration, and the amount of protein was estimated by [19] and standard curve of bovine serum albumin,.

5- Study cytopathic effect of enterotoxin:

Two groups of six 3 mice (**6**weeks old) were injected intraperitonialy with **1**ml of stock partial purified toxins and from each two fold dilution prepared from this stock **(1/10,1/20,)** which represent 55and 45 ug /ml concentration of entertoxin and less then LD50 value(which was determined in previous our study) to ensure survival of animals,using normal saline for dilution, **2**mice were injected with 1ml normal saline as a control .After **5** days, all mice which wewe injected were examined, The survived mice were killed, and dissected taking intestine, liver and spleen to study the histopathological changes. The tissues were prepared according to Humson [20]. The organs were fixed by 10% formalin (10 ml formalin + 90 ml 0.9% NaCl), then washed by tap water for several min. passing through a serial concentrations of alcohol (50%, 70%, 80%, 90% and 100%) for 2 hr. in each concentration, then cleared by xylol, saturated with paraffin at 60C° for 3 hr.,

embedded in pure paraffin; the blocks were cut into sections with 5 µm in thickness by using microtome. These sections were held on glass slides using Myer's albumin; they were left for drying at 37C°. Haematoxylin stain was used for 5-10 min., washed by tap water then with acidic alcohol then washed by tap water. After that Eosin stain was used for 15-30 sec. and then washed by D.W. Serial concentrations of alcohol were then used (70%, 90%, and 100%) for 2 min. in each concentration, cleared by xylol for 10 min.; then Canada balsam was used, covered by cover slide and examined by light microscope.

Results and discussion:

Identification of V. cholerae isolate:

The isolate was identifed as V.cholerae according colonies were appearance yellow in TCBS agar, and the result of Api 20 E [21].

Extraction and purification of enterotoxin:

V. cholera enterotoxin was extracted by centrifugation, filtration and purified with gel filtration chromatography by Sephacryl S-6, the eluted fractions of the last step contained two protein peaks one of them was a large peak the other was a small peak figure 1. The large peak (fractionation tubes from 13-17) with protein concentration was 28.5 ug/m, and zero carbohydrate. In eariler study on Vibrio enterotoxin was found the amount of protein in the Purified enterotoxin 85 to 92%protein and contains no carbohydrate and less than 1% lipid [22].

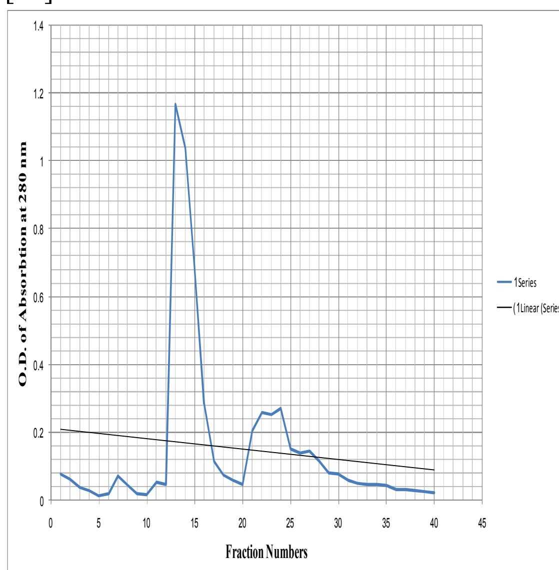

Figure 1- Gel filtration for partial purification of enterotoxin from V.cholera by Sephacryl S 200 column (2X60 cm), the gel was equilibrated with 0.1 M PBS pH 7 the flow rate was 0.5 ml/minute.volume of each fractions was 5 ml.

Result of cytopathic effect of Vibrio cholera enterotoxin on mice organs:

During injection of mice with 55ug/ml of enterotoxin and 45ug /ml which represent less concentration of LD50, observed increase permeability of the blood vascule and watery diarrhea for mice, the section which taken from intestine of mice injected with both concentration of enterotoxins revealed shortness of the intestinal villi,and hyperplasia figure 2,3 in comparison with the control which reavaled normal structure of intestine figure 4.

While the effect on liver showed degenerative change with necrosis using a concentration of 55 ug/ml of enterotoxin and caused necrosis and infiltration on coc. 45 ug/ml figure 5, 6 with normal of tissue in control liver figure 7.

The effect on spleen tissue was represented by widening of white pulp, and reduction or disappearance of red pulp also inflammatory cells were found i.e. lymphocyte and hypoplasia of white pulp figure 8 compared with control spleen which showed normal structure figure 9.

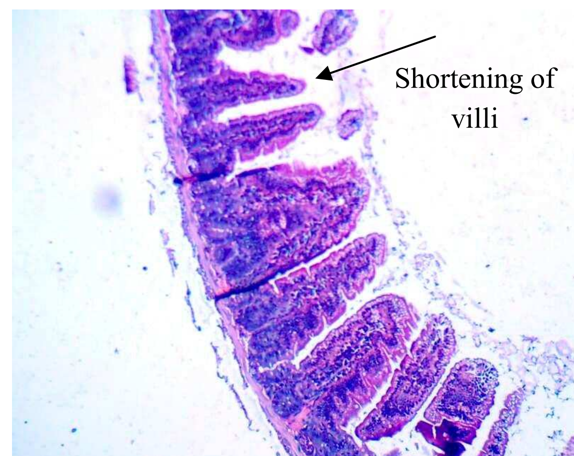

Figure 2- Cytopathic change of mice intestine which were injected with 55 ug/ml of enterotoxin

Figure 3- Cytopathic change of mice intestine which were injected with 45 ug/ml of enterotoxin showing shortening of villi (200x)

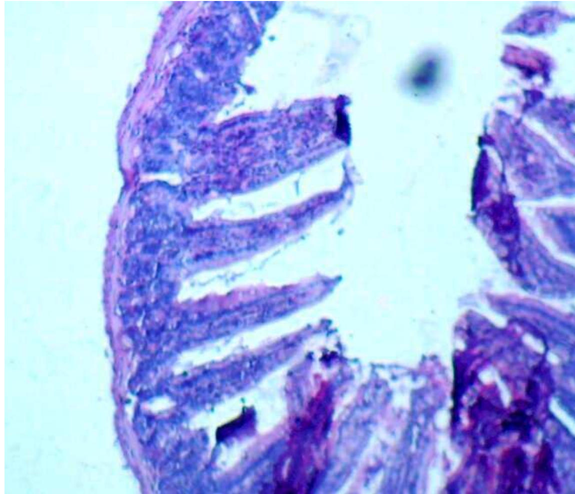

Figure 4- Normal structure of mice intestine which were injected with normal saline (200 x)

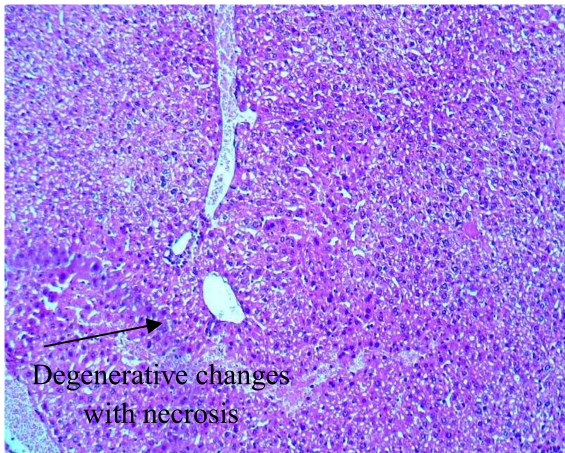

Figure 5- Cytopathic change of mice liver which injected with 55 ug/ml of enterotoxin showing degenerative changes with necrosis (200x)

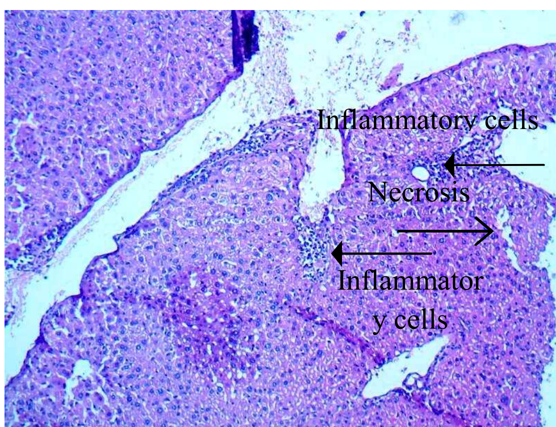

Figure 6- Cytopathic change of mice liver which were injected with 45 ug/ml of enterotoxin showing inflammatory cells (➔) and necrosis(→) (200x).

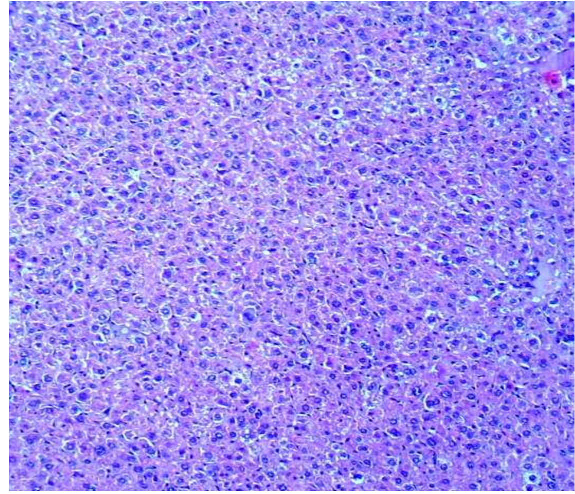

Figure 7- Normal structure of mice liver which injected with normal saline (200 x).

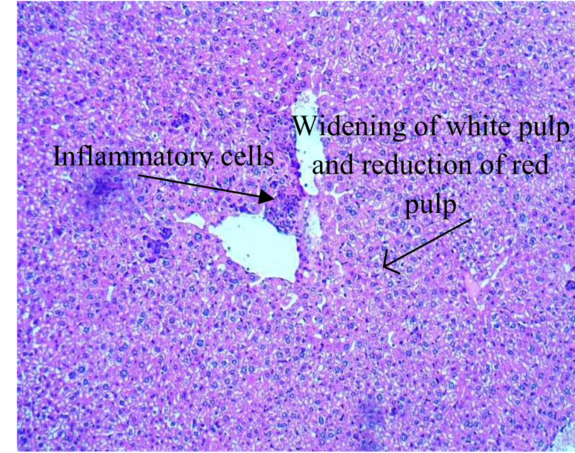

Figure 8- Cytopathic change of mice spleen which were injected with 55 ug/ml of enterotoxin showing inflammatory cells in addition to widening of white pulp and reduction of red pulp (200x)

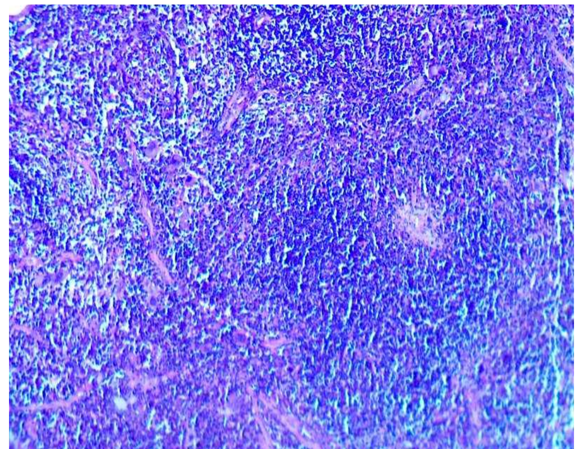

Figure 9- Normal structure of mice spleen which were injected with normal saline (200 x).

The results reflects the cytotoxic and cytopathic effect of vibrio enterotoxin this was in agreement with the result of [23] who examined sections of the ileum treated with V.

cholerae enterotoxin showed damage of the intestinal villi, lacteals, presence of blood cells, and rupture of intestinal capillaries. Another study shows the effect of Culture supernatant of V. cholerae O54 TV113 exhibited alteration in the morphology of CHO cells manifested as cell shrinkage with intact cell boundaries and finally cell death[24]. Owen et. al., showed, when viable classical vibrio supernatant were inoculated into the intestinal lumen pof non immune rabbits, they were phagocytized and infiltrate by M cells over peyers patches lymphoid follicles, carried in vesicles through the epithelium, and discharge among the underlying lymphocytes and macrophages, electron microscope reveled the disruptive effect of O139 strains and filtrate suspension on the apical membrane of the epithelial cells, even though it proliferants and colonized the mucosal surface of the rabbit small intestine [25].

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