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The Differences in Bioactivity between Crude and Purified Cholera Toxin from Local Isolate of *Vibrio cholera*

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Summary

In this investigate Cholera toxin was purified from the local clinical isolate designated as *V. cholerae* S . Few steps were employed for purification of CT including concentration of the protein, back extraction, and gel filtration. The bioactivity of CT reflected that crude and purified toxin had lethal activity on Balb/c mice in which a dose of 2.5µg/ ml of purified toxin caused mice death with symptoms such as muscle cramps and tachycardia while erythematous and induration was seen at intradermal I. d injection of skin of Guinea pig. Histopathological examination for liver, intestine, adrenal gland and spleen of white mice showed that liver tissue was more affected compared with spleen, intestine and adrenal gland tissues.

الاختلافات في الفعالية الحيوية بين ذيفان الكوليرا الخام والنقي من بكتريا *Vibrio* المحلية *cholerae*

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الخلاصة

في هذا البحث تمت تنقية ذيفان الكوليرا من العزلة المحلية المرضية والمسماة *Vibrio cholera* S واعتمدت بضع خطوات لتنقية ذيفان الكوليرا شملت تركيز البرزتين والاستخلاص المعاكس والترشيح الهلامي. اشارت الفعالية البايولوجية امتلاك الذيفان النقي والخام لفعالية قاتلة للفئران البيضاء balb/C mice وسبب استعمال جرعة 2.5 مايكرورام من الذفان النقي موت للفئران مع ظهور علامات مثل تشنج العضلات وتسارع القلب بينما ادى الحقن تحت الجلد الى حصول احمرار واودمة للمناطق المحقونة. اظهر الفحص النسيجي للكبد والامعاء والغدة الادرينالية والطحال للفئران البيضاء ان انسجة الكبد هي الاكثر تأثرا من الانسجة الاخرى.

Introduction

Many emerging and reemerging bacterial pathogens synthesize toxins that serve as primary virulence factors. Epidemic cholera caused by O1 and O139 which both produce and secrete cholera toxin (CT).

The pathogenesis of cholera is a complex process and involves a number of factors which help the pathogen to reach and colonize the epithelium of the small intestine and produce a variety of extracellular products that have deleterious effects on euokaryotic cells which is considered as a main difference between pathogenic and nonpathogenic isolates of the same species (1). Sack *et al*. (2) mentioned that *V. cholerae* produces one of the most profound physiologic responses known to occur in any human infection without invading the body and without bringing about significant structural changes in any tissue. The first step in *V. cholerae* O1 infection as a result from ingestion of the organism, depends on the size of inoculums and the susceptibility of the person who has been exposed. The incubation period for infection can be as short as 12 hr or as long as 72 hr (3). The inoculums size required to establish infection is relatively large and this may be due in part to the fact that *V. cholerae* O1 is highly acid labile, thus gastric acidity itself is a powerful natural resistance mechanism, and vibrios administered with food are much more likely to cause infection with inocula of 108-1011 organism produced diarrhea. However, when gastric acid was neutralized by

administering sodium bicarbonate the infectious dose of *V. cholerae* O1 ranging from 104-106 CFU (4).

The exotoxin is secreted by gram negative and gram positive bacteria during exponential growth. The production of toxin is generally specific to a particular bacterial species that produces the disease associated with toxin, and usually virulent strains of the bacterium produce the toxin which is consider as the major determinant of the virulence, while non-virulent strains do not. Exotoxins resemble enzymes in a number of ways. They are denatured by heat, acid and proteolytic enzymes which have a biological activity and exhibit specificity in mode of action and substrate utility (5).). CT is a polymeric protein consisting of two major domains A and B, in which B region speculated to be a polymer which is the excess portion actually having the fate to be a constitutional part of the toxin. It is composed of five identical non- covalently associated peptide chains of molecular weight 11500 dalton, the B subunit serve to bind the holotoxin to the GM1[gal(B1-3) gal Nae(B1-4) NeuAc(α2-3) gal(B1-4)glc(B1-1) ceramide a glycosphingolid found ubiquitously on the surface of mammalian cells, and being present on such diverse cell types as ovarian and neural cell as well as intestine, the pentasaccharide moiety of GM1 is exposed at the cell surface, anchored into the outer leaflet of the plasma membrane by insertion of the ceramide tail. It could also bind to other ganglioside such as GM3, GM3-NGN, GM2, GD1a, GD1b, GT,and GA1 but with low affinity compared with GM1. The neuramindase enzyme can increase the number of receptors by acting upon higher order, the ganglioside to convert them to GM1. The binding of the toxin appears to require that at least two of the five B-subunit interact with GM1(6,7).

A- subunit of CT consists of two covalently linked polypeptide A1 and A2 which nicked and subsequent activation by protease activity leading to enzymatic activation of A1 subunit which causes activation of adenylate cyclase, after A1 reaches adenylate cyclase, the A1 peptide catalyzes the transfer of ADP- ribose from NAD to an arginin residues on Gsα, the ADP- ribosyl transferase activity of CT, which $\longrightarrow$ catalysed the reaction NAD+ Gsα [ADP-ribosyl+ Gsα]+ nicotinamide+ H+ (4).

The effects of CT on the cell morphology were studied by Donta *et al*. (8) On Y1 mouse adrenal tumor cells in which the production of D4 , 3 Keto steroids induced by CT. It is accompanied by changes the cellular flattened shape to a round cells that are mediated by Adenyl Cyclase stimulation (9) while Guerrant *et al*. (1974) found that CT induces characteristic changes in the shape of Chinese hamster ovary (CHO) cells, from Oval to spindle forms and the cells are sensitive to 10pg of the toxin. Likewise, CT causes the increase of cyclic Adenosyn Mono Phosphate (cAMP) level in S49 mouse lymphosarcoma cells leading to inhibit the growth of the cells (11). Any one reviewing research papers in Iraq so far, would notice the absence of studying the differences of bioactivity between crude preparation of cell free extract containing CT and purified extract compared with other researches. For this reason this paper came to study different bioactivity InVivo for crude and purified CT.

Materials and Methods

The isolation and identification of *Vibrio cholerae:* Fifteen clinical isolates were collected from diarrheal stool samples by Central Health Laboratory, Baghdad, Ministry of Health. The characterization was confirmed in this study by performing morphological characterization, Microscopic examination biochemical test, and serotyping to characterize *V. cholerae* following Elliot *et al*. (12); and WHO (13).

Production and Extraction of CT:

The production of CT from *V.cholerae* isolates was done using overnight culture of *V. cholerae* grown on 20 ml of production medium (AKI pH 8.5 supplemented with 0.2% of Asparagine and Glucose) which was centrifuged at 5000 rpm for 15 minute to prepare free cell extract. Concentration at 80 % salt saturation was done for free cell extract using Ammonium Sulfate salt. Finally desalting of concentrated extract was achieved by Sephadex G25 (14).

Measuring of CT

Quantitative measurement of CT was determined by measuring the erythemal activity EA using Guinea pig (15). Toxin Units was calculated (depends on its definition) in that each 5-8 mm of EA is equivalent to 1 Toxin Unit (TU) of enterotoxin.

TU/ml= (EA mm ÷ 5) ×10; Specific toxin activity was calculated as the ratio of TU/ml divided by the protein concentration .

Determination of protein concentration:

Protein concentration was measured by dye binding Bradford method using Bovine Serum Albumin for preparing the standard curve (16).

Purification of CT

purification of CT was achieved after extraction, concentration, back extraction between 80% - 20% of salt saturation and gel filtration through Sephadex G100 to obtain purify protein following Stellwagen (1990) and Al-Khafaji (2010).

The biological activity of CT:

Crude preparation and purified CT was used to determine the toxin *Invivo* bioactivity as bellow: Mice lethality assay: Two animal of Balb /c mice were injected intraperitonially (I. p) with 1.25, 2.5, 5 µg/ ml of toxin extracts for mice lethality assay. Mice were watched for 24 hour for the appearance of symptoms or death (18).

Erythemal activity: Guinea pig was injected with 0.1 ml of crude extract and purified CT intradermally (I. d) in the shaved dorsal area after dividing it into 12-18 equal square. Diameter of erythema and induration area resulted from injected toxin extract was measured and the animal was kept under observation for 72 hour. Erythemal activity (EA) was obtained and 4-8 mm of erythemal effect was considered as a positive reaction for enterotoxin (18).

Histopathological examination: Also The differences in histopathology examination of different mice organ was studied after I.P injection of crude and purified CT.

Balb/c mice were injected I.p by 0.05 and 0.1 ml of purified CT mice were sacrified after 24 hr and small pieces from liver, adrenal gland, spleen and small intestine were taken and kept at normal formaline 10%. Histopath was done for each organ comparing with histopath for normal organs. The organ sections were stained by Hematoxiline/ Eosin stain at Laboratory of histopathology / Medical College / Baghdad University

Microscopic examination was carried out and the differences between toxin- treated samples and normal samples were determined and slides were photographed.

Results

The study confirmed the presence of five clinical isolates which are resemblant to *V. cholerae* from all the suspected isolates taken from Central Health Labortory/ Baghdad/ Ministry of Health.

Overnight cultures of the five clinical isolates, grown on TCBS medium with yellow color, gave 3-4 mm diameter of circular, smooth, glistening and slightly flattened appearance of colonies (figure 1).

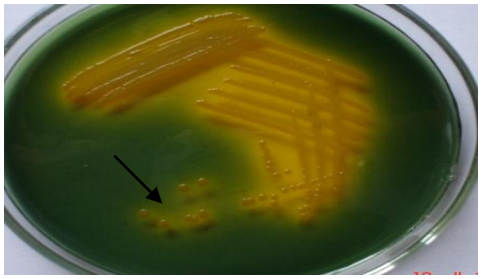


Figure (1) The shape of colonies of *V. cholerae* on TCBS agar

Whereas they appeared as pale or slightly pink often resembling colonies of late or slow lactose fermenting organisms, with 1-3 mm diameter on MacConkey agar. They appeared as offwhite color with a dot in their center on the TSA.

Microscopic examination showed that the bacteria were gram-negative non-spore forming, slightly curved rods arranged as single or double of bacteria. The isolate gave positive reaction with oxidase test , string test which confirmed by making a string like of DNA when a large loopfull of growth was suspended in a drop of 0.5% aqueous solution deoxycholate with some variation of string length. Also gave positive results with cholera red reaction, fermented glucose not lactose appeared as red surface and yellow bottom of KIA slant with no gas and H2S formation.

The recent purification scheme used in CT purification proved that specific activity increased through the purification steps in that salt fractionation by back extraction method led to increasing specific activity from only 62.5 to 117.2 and purification field reached to 1.4. This is due to the exclude of the contaminants from the crude extract. Desalting of the crude concentrated extract with the use of Sephadex G25 led also to increase both specific activity and purification field but CT yield however, highly decreased to reach about only 12% because of the sample dilution that reached to 2.5 fold of dilution factor. The most important advantage was that the specific activity of CT highly increased with gel filtration step on Sephadex G100 to reach 613.4 corresponding approximately 100-fold the increase in specific activity. The estimation of purification field revealed an increment which reached to 9.8 fold; CT could be purified with high specific activity by only few steps as presented in table (1).

Table (1) The purification table of CT

***Sample diluted 1:5**

Purification step	Volume (ml)	Protein concentration (mg/ml)	Total protein (mg)	EA (mm)	(TU/ml)	Specific toxicity (unit/mg)	Purification field	Yield %
Culture extract	500	0.16	80	5	10	62.5	1	100
20%-80% saturation	20	3.4	68	30*	300	88.2	1.4	30
Desalting	45	0.8	36	12*	120	150	2.4	12
Gel filtration	30	0.326	9.78	20*	200	613.4	9.8	20

The results obtained in this research showed that 2.5 µg of purified CT extract caused mice death before 18 hour compared with 5 µg/ml of crude preparation of CT toxin caused the same effect,

It is found that purified CT as crude extract caused muscle cramps, heartcardia, and other symptoms were observed on mice after I.p injection. The results of this research showed that purified CT like- crude extract caused erythemal effect and induration in the injected site of Guinea pig skin, remained even after more than one week, but with no pronounced bleeding detected with injected crude extract toxin figure (2).

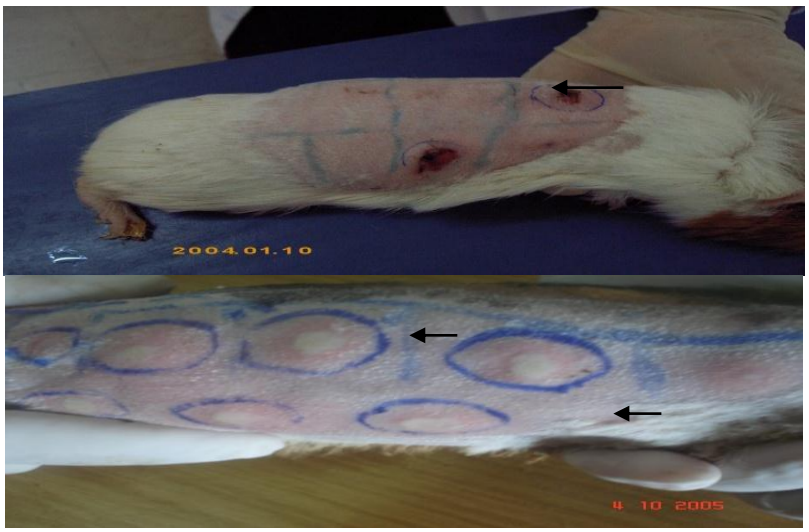


Figure (2) Permiability factor in Guinea pig skin I. d injected by CT (purified CT) top and (crude extract of CT) below

In the present trial the purified and crude preparation of CT was administrated intraperitonealy to Balb/c mice and after 24 hour mice were sacrificed and the CT effects on different tissues were screened compared with control mice tissues which administrated normal saline.

The liver tissue was examined and histopathological lesion of liver showed vaculated of hepatocyte indicated the hydrobic to moderate degeneration, slightly loosed of the tissue architecture with dialated of some sinusoids as in figure (3). The examination of slide on power 50X showed congestion of central vein.

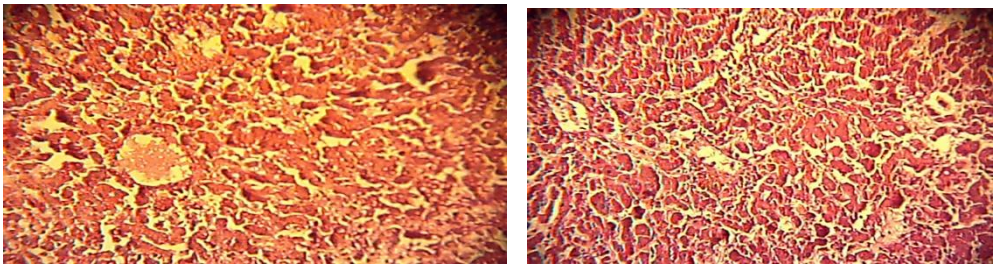
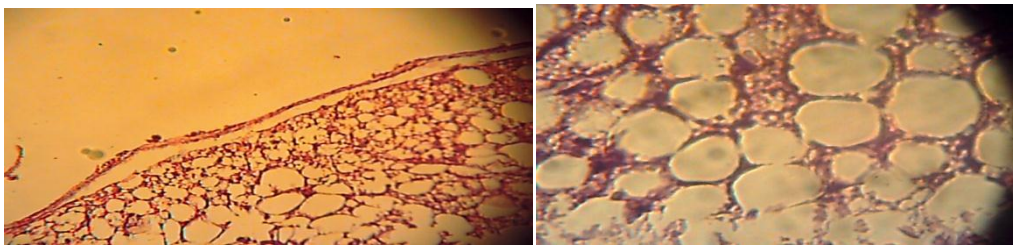


Figure (3) liver tissue of the mouse after treated with purified CT (magnification 50X)

Microscopic examination of adrenal gland tissue showed that purified CT as crude preparation caused flattening of the lining cells with sluffing and detached in capsule. Big vacule appeared with hydrobic degeneration of the zona tissue. Medulla suffered from hydrobic degeneration, congestion of blood vessel with haemorhage in some area figure (4).



A **B**

Figure (4) Adrenal gland tissue of the mouse after treated with purified CT (A- magnification 50X, B- magnification 100X).

Small intestine suffered from damage with diffused sluffing in mucosa, some depress was located in the lumen of intestine, normal appearance of muscularis mucosae with its duodenal glands and submucosa (Figure 5).

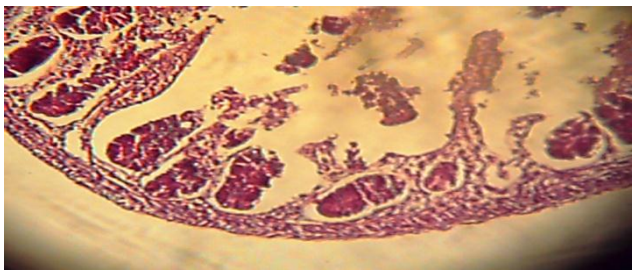


Figure (5) Small intestine tissue of the mouse after treated with purified CT (magnification 50X)

While the spleen tissue not markedly changed except of some or slightly distend or dialated of sinusoide with moderate degenerative, no other changes were observed at white or red pulp.

The investigation results showed that liver highly affected by CT while spleen apparently not changed.

Discussion

TCBS agar is a selective medium used extensively in *V. cholerae* isolation, and the comma shape or vibriod shape distinguishes this bacterium from other gram- negative bacilli as described by WHO (13). The two tests of string and cholera red tests are considered as the most important diagnostic tests during the primary identification of *V. cholerae* (19) also in agreement with Elliot *et al*. (12) and Otawa (19). The permeability factor (PF) was used extensively for the quantitative measurement of CT and present estimation of CT was based on the modification of measuring the Erythemal Activity (EA) which happened during skin injection of Guinea pigs. The modification of measuring EA based on the omitting the Evan's blue dye which may possess some toxicity and may cause allergic effect to animal as used by Al-Shakhely (15). Gel filtration chromatography was used by other researchers as a step in CT purification from *V. cholerae* and its related LT enterotoxin from *E. coli* and enterotoxin from *Clostridium perifringens* (15,20,21). and this may be due to the sequester of some biological effects of CT by other proteins exist in crude preparation. Also Finklestein (22) demonstrated the role of haemolysin isolated from El Tor *V. cholerae* is rapidly lethal for mice and toxic for cultured heart cells. Other studies determined a readily detectable hormone like effects of purified CT on a variety of cultured eukaryotic cell systems *in vitro* and the observation that the toxin stimulated steroidogenesis and alter the morphology of mouse adrenal tumor cell line (8). which may be the result from the removable of the other proteins or toxins which exist in crude preparation which cause bleeding . Richardson (18) and Finklestein (4) are mentioned that one of CT effect on various animals a characteristic delayed (maximum response at 24 hours), sustained (visible up to one week or more) erythematous, edematous induration associated with a localized alteration of vascular permeability responses appeared after intradermal inoculation of relatively minute amount of CT reached to only 30 pg. The results of this research are in agreement with the conclusion of Finklestein (4) in the appearance of a broad spectrum of CT activity in cells and tissues that it never contacts in nature. In each the first step of cell intoxication, the B- pentamer of the toxin binds specifically to the branched penta saccharide moiety of ganglioside GM1 in the target cell. After the binding the toxin caused a characteristically delayed, but sustained, activation of adenylate cyclase which occurred rapidly after 15 min required for the generation of A1 peptide the active compound of CT, increased the level of cAMP saw, and it may cause additional, readily recognizable, morphologic alterations of certain culture cell line. On the other hand, the action of CT as a transduction signal which is followed by a complex series of events in which CT enters the apical endosome and moves through multiple vesicular compartments of intestinal epithelium before it activates adenylate cyclase with no effect of low intravesicular pH on CT association with its membrane receptor GM1, or on subsequent processing/ signal transduction events. Also both ADP- ribosylation factors (ARFs) and ADP-ribosylation factor like (ARLs) shown to serve allosteric activation of CT via phospholipids

and GTP- dependent manner and these proteins expressed by all tissue except brain with some variation of their expression level (23,24,25).

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