



中國醫藥大學
基礎醫學研究所
碩士學位論文

開發可包覆細菌毒素的奈米載體應用於胃癌的治療

**Development of Nanoparticle-encapsulated bacterial toxin for
the therapeutic effects of gastric cancer cells**

指導教授：賴志河 (Chih-Ho Lai, Ph.D.)

研究生：呂侑倫 (Yu-Lun Lu)

中華民國 100 年 4 月

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致謝

研究生涯的這兩年，我從原本懵懂無知的一個新鮮人，一直到進入實驗室後受到老師們的指導以及學長姐們的指教，才漸漸成長茁壯，到如今我即將從中國醫藥大學畢業，在學期間我真的相當感謝我的指導老師，賴志河老師；他對我的關心無微不至，在生活上關心我的經濟來源，在實驗上老師不只是給了我思考的方向，也給了我許多的支持和鼓勵，常常在實驗上遇到困難時，老師總會用溫柔的眼神看著我叫我不要氣餒再加油再努力，日常生活也會不時的請我們吃飯喝飲料，提振我們的士氣，我覺得能在 CHL lab. 中學習真的是一件很幸福的事情。

CHL lab. 的學長姐們還有基醫的同學們，你們一直給了我很多的幫助跟鼓勵，實驗上遇到不順遂時，你們總會給我很多的建議讓我能遇到困境時順利的渡過，謝謝阿國學長、郁婷、湘湘、宜洵在實驗上給我的幫助；另外 lab. 裡的學弟妹們很感激你們在我研究生涯的兩年中製造了不少的笑料，讓我在實驗室裡的每個日子都充滿歡笑，謝謝畢業的大學部學弟妹，還有家碩、豈睿、昱毅、維凡、力宇、珈琳、宛庭，因為有你們才讓我這兩年過得非常的充實和愉快；此外湯老師家的學長姐們也很謝謝你們給我的幫助，不管是在實驗上或是生活上，大頭學長、小天學長、書寧、振銘感謝你們讓我的研究生涯走得更加順利平穩，還有謝謝學儒，謝謝你願意聽我分享研究生涯的每一天，陪我走過所有不管是歡笑還是淚水的日子，真的很謝謝你。

最後我要特別感謝我的家人，謝謝爸媽對我的支持，感謝您們總是在我背後默默的支持我，給我正面的力量，您們對我的包容與鼓勵都是我完成碩士學業的最大原動力，還有我最愛的阿嬤，謝謝您時不時的關心我的生活，關心我的課業，讓我在台中也感受到家人對我的關懷和溫暖，非常感謝我生命中的每一個人，因為有你們我才能順利的完成學業，謝謝你們。

中文摘要 (Abstract in Chinese)

彎曲桿菌是最常引起人類腹瀉的致病菌之一，它可以在人體中引起許多不同的疾病，包括胃炎、腸胃炎、敗血症和嚴重的神經系統疾病，例如 Guillian-Barré 症候群。在彎曲桿菌的眾多致病因子中，目前以細胞致死腫脹毒素 (cytolethal distending toxin, CDT) 被研究的最為透徹，CDT 會在真核細胞中造成細胞的腫脹，進而誘導細胞週期停滯在 G2 時期最後使得細胞走向凋亡的機制。先前的研究證實 CDT 是由三個次單元所組成，分別為 CdtA、CdtB 和 CdtC，CdtA 和 CdtC 這兩個次單元是扮演著結合到細胞膜表面的功能，而 CdtB 為細胞致死腫脹毒素中負責酵素活性的次單元，CdtB 具有能夠使 DNA 損傷的 DNase I，並且能使細胞的週期暫停。

在本研究中的另一個主角是奈米顆粒，奈米顆粒狹義的定義是其大小必須介於 0.1-100nm 之間，而奈米顆粒在近年來被廣泛的應用在許多不同的領域中，包括了生物醫學，光學和電子領域，正是因為它具有的潛在能力非常可觀，因此在許多的醫學研究中更是利用了它微小以及能夠攜帶藥物或是 siRNA 的能力來做更進一步深入的應用。

本篇實驗中，我們利用奈米顆粒做為替代 CdtA 和 CdtC 這兩個結合次單元來運送 CdtB 進入細胞核中，在實驗中我們裡用了雷射掃描共軛焦顯微鏡 (confocal laser scanning microscopy) 觀察奈米顆粒包覆 CdtB 在細胞中的分布，發現了帶有 CdtB 的奈米顆粒的確有與細胞核重疊的現象產生，而另外在流式細胞儀的分析我們發現了奈米顆粒包覆 CdtB 的確實造成了細胞 DNA 的斷裂，而導致細胞週期停滯在 G2/M 時期，另外在西方轉漬法以及流式細胞儀的分析之下更發現了帶有奈米顆粒包覆 CdtB 最後會導致人類胃上皮細胞走向凋亡的途徑，而且比較細胞處理 CDT 全毒素而言，有更好的毒殺癌細胞之效力，因此本論文也證實奈米顆粒包覆 CdtB 可進一步應用於腫瘤細胞的治療。

Abstract

Campylobacter jejuni (*C. jejuni*) is one of the most common cause of infectious diarrhea world-wide. This bacterium can induce various diseases in human including gastritis, gastroenteritis, septicemia, and serious neurological disorder like Guillian-Barré syndrome. The most important virulence factor of *C. jejuni*—cytolethal distending toxin (CDT), which can cause cell distending and induce cell-cycle arrest as well as apoptosis in eukaryotic cells. Previous studies have been demonstrated that CDT was composed by three subunits, CdtA, CdtB, and CdtC. Among those three subunits, CdtA and CdtC were served as the binding unit which associated with the cell surface. While the catalytic subunit CdtB, which was homology to type I deoxyribonuclease (DNase I) and phosphatidylinositol 3, 4, 5-triphosphate phosphatase enzymes, have the ability to degrade DNA. Translocation of CdtB into nucleus induced cell cycle arrested at G2/M phase. However, none of report explored the molecular mechanisms of CDT in its biological applications.

Nanoparticle is defined as a microscopic particle with at least one dimension less than 100nm. Nanoparticle is widely used in researches, due to its wide variety of potential applications in various areas including biomedical, optical and electronic fields. Current researches indicated that nanoparticle could act as a carrier or encapsulated some drugs or siRNA into the target cell. In our preliminary investigation, we use chitosan/heparin nanoparticles to replace the binding subunit CdtA and CdtC for encapsulation of CdtB subunit and delivery into host cells to achieve the same delivery effect as CdtA-CdtC complex. We developed novel pH-responsive CdtB/chitosan/heparin nanoparticles in the therapeutic effects in gastric cancer cells. The delivery efficiency of CdtB-loaded nanoparticles for tumor

therapy is currently under investigating.



Introduction

Campylobacter spp. is a bacterium that was first recognized as a cause of human gastrointestinal illness in 1975 (1). Previous report indicated *Campylobacter spp.* is one of the major cause of infectious diarrhea world-wide, ahead of *salmonella* and *shigella* (2), and it's the most commonly reported bacterial cause of food-borne illness in the United States (3). *Campylobacter spp.* infection is also an important pre-condition for Guillian-Barré syndrome (4). This bacterium can gain the entry into host intestinal epithelial cells, which was thought to be important for its persistent infection and induction of clinical outcomes both in human and animals (**Table 1**) (5).

Characteristics and epidemiology of *Campylobacter jejuni*

Campylobacter spp. is a Gram-negative bacterium which is curved, rod-shaped, and non-spore forming bacteria. Apart from the genus *Campylobacter*, the group also contains *Arcobacter* and *Helicobacter*. *Arcobacter* are closely related to *Campylobacter*, and can cause intestinal infection in human, too. *Helicobacter pylori* is well known as major causative pathogen for gastritis and peptic ulcer disease (6). The natural habitat for *Campylobacter spp.* is the intestine of birds and warm-blooded animals including sea gulls and other wild animals or poultry. In addition, different types of *Campylobacter spp.* can infected with different hosts. For example, *Campylobacter jejuni* can cause disease not only in human but also in beast animal and pets. Furthermore, *Campylobacter fetus* was found can infect of beast animals such as cattle and sheep. It has also been reported that this bacteria can induce various types of clinical outcomes in human including gastritis, gastrointestinal, septicemia, and serious neurological disease like Guillian-Barré syndrome (7).

Campylobacter jejuni is a microaerophilic pathogen, which requires an environment containing a reduced concentration of 5-7% oxygen and 10% carbon dioxide with optimal growth temperature 37-42°C. The width and length of the cells varies from 0.2-0.5µm and 1.5-1.6 µm, respectively. The motility of *C. jejuni* is mediated by single polar flagellum. They do not metabolize sugars and use intermediates of the TCA cycle as source of energy (8). When *C. jejuni* exposed in some special environment such as high oxygen-containing or other improper environment may leads *C.jejuni* to become coccoid form, that will trigger *C. jejuni* to enter viable but none-culturable (VNC) state (9). The routes of *Campylobacter* transmission include person-to-person, fecal-oral, unpasteurized raw milk and waterborne (such as contaminated water). Consumption of improperly cooked, contaminated foodstuffs or food products which from infected animals also can transmits of *Campylobacter* organisms to human (Figure 1) (10).

Virulence factors of *Campylobacter jejuni*

Most of the bacteria can infect human or animals and cause severe disease. The mechanisms they use to infect host is usually linked to their virulence factors. The virulence mechanisms is contributing to the pathogenesis of the disease. In general, the virulence factors of *C. jejuni* used including:

(1) Flagella

Flagella is the most important factor associated with bacterium motility, and it's also playing an essential role in *C. jejunis*' adhesion and colonization of the host intestinal epithelial cells (11). *C. jejunis*' flagella was controlled by two important gene, *flaA* and *flaB*, the major component of the flagella is flagellin, which was encoded by *flaA* and *flaB* (12). *C. jejuni* have one or both ends unsheated single flagella, which can help *C. jejuni* attaching to intestinal

epithelial cells, thus promote the invasion of *C. jejuni*. Based on 1999 Wassenaar's research, the authors suggested that the ability of *C. jejuni*'s adhesion and colonization has obvious decrease when *flaA* and *flaB* gene were mutated (13). Thus, indicating that flagella may play an important role for bacterial-induced pathogenesis of host.

(2) Enterotoxin

Enterotoxin is a protein toxin which is secreted by several bacterial microorganisms. This toxin is a small molecule with heat stable and water-soluble characters. It comprises of two subunits: subunit A and subunit B. The larger subunit A has enzymatic activity, and the smaller subunit B is a pentamer, which is receptor binding-related. After toxin binding to the receptor, the active subunit A will be transported into the host cell and promotes signaling responses, subsequently increase the level of intracellular cyclic AMP, resulted in ions and water effluxed from epithelial cells. This may cause watery diarrhea, and has been demonstrated by exposed cultured Chinese hamster ovary (CHO) or rounding of mouse adrenal tumor cell (Y-1) (14).

(3) Lipopolysaccharide (LPS)

Lipopolysaccharide is a major component in the outer membrane of gram-negative bacteria cell wall. LPS classified as an endotoxin which can improve pathogens infection to the host cells and trigger a severe immune response. This toxin consisted of a hydrophobic domain lipid A and a hydrophilic domain polysaccharide. The lipid A is composed by a long-chain fatty acid which is an important factor for induction of inflammatory responses. The polysaccharide domain consists of two regions, one core domain and one O-antigen. The core structure has a high reservation formed by a hexose include a octose. In addition, the O-antigen has a high variability which has the different

components in different species (15). After microorganisms infection of the host cells, LPS can secret from the bacterial cell wall and induce serious disease such as sepsis, fever and eventually shock (13).

(4) Cytotoxin

C. jejuni can produce several cytotoxins which were reported and summarizes in an article by *Wassenaar* in 1997 (14) including: (1) 70-kDa cytotoxin, (2) cytolethal distending toxin (CDT), (3) Shiga-like toxin, (4) hemolytic cytotoxin, and (5) hepatotoxin. Cytolethal distending toxin and hemolytic cytotoxin are well investigated in recent years. First introduce the hemolytic cytotoxin, in 1990 *Arimi S.M.* found that *C. jejuni* can secret hemolysin which has the ability to destruct the erythrocytes, current research indicating this behavior was cause by the phospholipase A and siderophore-binding protein which can hydrolysis the cell membrane and then disintegrate the cell membrane, finally destruction of the cells (16,17).

Cytolethal distending toxin (CDT)

Several virulence factors in *Campylobacter* were found to contribute the bacteria survival and establishment of disease in the host. Our current study is focusing on one of *C. jejuni's* virulence factor—cytolethal distending toxin (CDT). CDT was first published in 1988 by *Johnson* and *Loir*, who indicated this toxin secreted by *E. coli* and different from heat-labile toxin, heat-stable toxin, verotoxin, and hemolysin (18). CDT can cause Chinese hamster ovary cells (CHO cells) to be obviously distending. At the same time, CDT has been found in *C. jejuni* (19), and then CDT was continually found in other gram-negative bacteria such as *Actinobacillus actinomycetemcomitans* (20,21), *shigella dysenteriae* (22), *haemophilus ducreyi* (23), and subsets of *Helicobacter* species (24,25), Interesting, all of these bacteria are

belong to diarrheal disease- causing enteropathogens.

CDT is a tripartite protein toxin encode by three genes- *cdtA*, *cdtB* and *cdtC*, with molecular weights of 30, 28 and 21 kDa, respectively (26), CDT holotoxin appears to function as an AB₂ toxin in which CdtA and CdtC serve as the binding units which associate with the cell surface and delivering of CdtB into the target cells. CdtB is the activate unit which can enter the host cell and induce genotoxic effects (27,28). Indeed, a wide range of CDT can induce genotoxic effects including CHO cells, HeLa cells, Hep-2 cell (human epidermoid cancer cells), Vero cell (African green monkey kidney cell), and CaCO-2 (human kerationcyte cell line). (18-20). Previously study also shown that lymphocytes are the most sensitive cells upon CDT treatment (29). CDT not only can cause cell distending but also can induce chromatin condensation and cleavage (30). Previous study described the CDT can mediate secretion of interleukin-8 (IL-8) from intestinal epithelial cells (31,32) as well as induce cell cycle arrest at G2/M phase (20,33,34), Since CDT toxicity is associated with cell cycle arrest, subsequently may cause activation of the apoptotic cascade (35,36).

CdtA and CdtC are not only required for the toxin to associate with cells but also necessary to localize the toxin to lipid membrane microdomains (29,37). These two subunits increase the ability of CdtB to associate with host cell and greatly enhanced intoxication (38,39). The catalytic subunit CdtB has homology to type I deoxyribonuclease (DNase I) and phosphatidylinositol 3,4,5-triphosphate phosphatase enzymes which was found can degrade DNA (28,40). Upon CdtB entering into host cells, CdtA and CdtC remain associated with the cell surface. The translocation of CdtB into the nucleus subsequently induces cell cycle arrest at the G2/M phase. Thus, resulting in cellular distension and ultimately cell death (41). In 1998, *Whitehouse* indicate that *C. jejuni* can trigger cells to accumulate the inactivated, phosphorylated form of cell-division cycle 2(CDC2) and thus to become irreversibly block in the

G2/M phase of the cell cycle (42). However, CdtB without binding subunit CdtA and CdtC, this is not able to cause cell cycle arrest (43,44). Further study demonstrated that mutation of the conserved sites which is necessary for CdtB catalytic activity will prevent the induction of double-strand breaks as well as chromatin disruption. (30) According to the previous studies, we thus considered CdtB could play an important role in CDT holotoxin in the induction of genotoxic effects of host cells.

Characteristics of nanoparticles

Nanoparticles (NPs) are defined as microscopic particles with at least one dimension less than 100nm, which with a positive surface charge and were stable at pH 1.2-2.5 (45,46). Recently, nanoparticle is widely used in a lot of research, due to a wide variety of potential applications in areas including biomedical, optical and electronic fields. Several previous studies have been described that the mechanisms of nanoparticles in the cancer therapy (47-51). Moreover, nano-drug carriers have been widely used in biology and medicine. Current researches indicated that nanoparticles could be a carrier or encapsulated some drugs or siRNA into the target cell (50,52,53). The nanoparticles consist of chitosan and heparin can protect the drug from destruction by gastric acids and increase the ability to adhere to the gastric cells (54). Nanoparticles containing a positive surface charge which can penetrate into the gastric epithelial cell through the mucus layer and become unstable and disintegrates because of its pH sensitivity. When delivering into cells, the carried drug will release from the nanoparticles, therefore, to achieve the complete therapy (54).

Chitosan is a hydrophilic biopolymer with a polycationic, non-toxin, biodegradable polysaccharide and mucoadhesive polymer. The property of chitosan is safe for human and stable in natural environment (55,56). The other component in nanoparticles is heparin, which is a polyanionic mucopolysaccharide with low

molecular weight around 15kDa. Recently, heparin is becoming a famous anticoagulant which have the ability to stimulate gastric ulcer recover, and it's also associated with mucosal regeneration, proliferation, and angiogenesis (57-59).

Gastric cancer is the second most common cause of cancer-related death in the world. In 1994, *H. pylori* is declared as a group I carcinogen for gastric cancer, and the 4th leading cause of cancer death in Taiwan (60). The correlation of *H. pylori* etiology and gastric cancer was virtually certain. In addition, inherited predisposition, environmental factors, smoking, drunk, diet, as well as pressure were thought to be associated with gastric cancer. Because of the high prevalence of gastric cancer in Asian has been became a severe issue worldwide. Development of effective therapeutic methods and low manufacture costs for the eradication of gastric cancer is urgently required.

Combination with these features, in the present study we utilized the nanoparticle to substitute the CDT binding subunit (CdtA and CdtC) for delivering the activity subunit (CdtB) into host cells. Our current study indicated that nanoparticle-loaded CdtB harboring activity to induce the genotoxic effects in human gastric adenocarcinoma cells.

Materials and methods

Antibodies

Antibodies specific for Bcl-2 family including Bax, Bak and Bcl-2 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-caspase-3 and anti-Poly(ADP-ribose) polymerase (PARP) were purchased from BioLegend (San Diego, CA), anti-caspase-9, phospho-ATM, phospho-H2A.X, phospho-Chk2 was purchased from Cell Signaling (Danvers, MA).

Bacterial strain and culture

C. jejuni strain 7729 was isolated from patients' feces, identified, and stored at the Chang Gung Memorial Hospital (Taoyuan, Taiwan) (61). The bacterial strain was grown on Brucella blood agar plates (Becton Dickinson, Franklin Lakes, NJ) supplemented with 10% sheep blood and 1.5% agar in a microaerophilic atmosphere at 37°C for 1 to 2 days.

In vitro cell culture system

The AGS cell line (ATCC CRL 1739; human gastric adenocarcinoma cell) purchased from Food Industry Research and Development Institute. The cell were cultured in F12 medium (Invitrogen, CA, USA) supplemented with 10% Fetal bovin serum (HyClone, Logan, UT, USA), penicillin (100U/mL), and streptomycin (100µg/mL) (GIBCO BRL) were add in culture medium after nanoparticle treatment and maintained under 37°C under 5% CO₂ condition.

Construction and protein purification of CDT subunits

Recombinant His-tagged CDT subunits were cloned following standard protocols. DNA fragments of *cdtA*, *cdtB*, and *cdtC* were derived from PCR amplification of *C. jejuni* 7729 genomic DNA. The forward and reverse oligonucleotide primers were *cdtA*-F (CATGCCATGGCTTGGTTCTTCTAAATT TG-AAAATGT) and *cdtA*-R (CCGCTCGAGTCGTACCTCTCCTTGGCGATATA) for PCR amplification of the *cdtA* sequence; *cdtB*-F (CATGCCATGGCTAATTTAGAA-AATTTTAATGTTG GC) and *cdtB*-R (CCGCTCGAGAAATTTTCTAAAATTTACTGGAAA) for *cdtB* sequence; *cdtC*-F (CATGCCATGGCTACTCCTACTGGAGATTGAAAGA) and *cdtC*-R (CCGCTCGAGTT CTAAAGGGGTAGCACTG) for *cdtC* sequence. Each *cdt* fragment was inserted into pET21d (Invitrogen, CA, USA) using *Nco*I and *Xho*I. Briefly, *cdtA* was amplified using primers *cdtA*-F and *cdtA*-R by PCR at 95°C 10 min for one cycle; 35 cycles at 95°C for 1 min, 55°C for 1min, and 72°C for 2 min; and a final extension 72°C for 20 min. The *Nco*I/*Xho*I fragment was then ligated into pET21d to create the CdtA expression plasmid. Similar protocols were used to obtain the CdtB and CdtC expression plasmids from *C. jejuni* 7729 using primer pairs: *cdtB*-F and -R, and *cdtC*-F and -R, respectively. The PCR program used to amplify *cdtB* and *cdtC* were the same as *cdtA*. The nucleotide sequence of each *cdt* constructs were verified using the ABI Prism Dye Terminator Cycle Sequencing Ready Reaction kit (Perkin-Elmer Corp, Norwalk, CT) in an automated DNA sequencer (model 377-96; Perkin-Elmer Corp). Sequence analysis was performed by the University of Wisconsin Genetics Computer Group (Madison, WI) package. *E. coli* BL21-DE3 cells harboring either *cdtA*, *cdtB*, or *cdtC* expression plasmid was induced at OD₆₀₀ of 0.8 by 0.5 mM of isopropyl β-D-thiogalactopyranoside (IPTG) at 37°C for 3 h. The expressed His-tagged CdtA, CdtB, and CdtC fusion proteins were

purified by metal affinity chromatography (Clontech, Palo-Alto, CA) and assessed by SDS-PAGE.

Generation of anti-serum against each CDT subunit

Each purified CDT subunit (1 μ g) was used to immunize a 6-week-old BALB/C mouse. All of the mice were purchased from the National Laboratory Animal Center (Taipei, Taiwan). The mice were immunized at weeks 0, 2, 4, 6, 8, 10, and 12, and the titer of the antiserum was detected at weeks 7, 9, 11, and 13. Mice were maintained in the animal center of China Medical University (Taichung, Taiwan). All procedures were performed according to the “Guide for the Care and Use of Laboratory Animals” (National Research Council, USA) and were approved by the animal experiment committee of China Medical University (Taichung, Taiwan). The titers of antibodies against the CDT subunits in the serum were determined by Enzyme-linked immunosorbent assay (ELISA). Ninety-six-well plates were coated with 500 ng of purified recombinant CDT subunits and blocked with 2% BSA in TBS (0.1 M Tris-HCl pH 7.5, 0.03 M NaCl). Serial dilutions of the antiserum (1:1,000, 1:2,000, 1:4,000, 1:8,000, and 1:16,000) in TBS-Tween 20 were added to each well. Bound antibody was detected by HRP-conjugated secondary antibodies (Invitrogen, CA, USA) and quantified by measuring the optical density at 450 nm after development with the TMB substrate buffer system (Kirkegaard & Perry Laboratories, Gaithersburg, MD).

Protein concentration and quantification

Each recombinant protein was concentrated using centrifugal filter (Millipore, MA, USA) 4000 rpm at 4°C. After centrifuge for 2 h, the purified protein was harvested and added PMSF (0.2 mM) to prevent protein degradation. The

concentration of each CDT subunit was then quantified using Pierce BCA Protein Assay Kit (Thermo, IL, USA) following by manufacturer's manual protocol.

Preparing of chitosan/heparin nanoparticles

The nanoparticles were prepared by a simple ionic gelation method with magnetic stirring at room temperature. In brief, aqueous heparin (1.0 mg/mL, 2 mL, pH 7.4) will add by flush mixing with a pipette tip into aqueous chitosan at various concentrations (0.3, 0.6, 0.9, 1.2 or 1.5 mg/mL, 10 mL, pH 6.0). The nanoparticles produced will be collected by ultracentrifugation at 15000 rpm for 50 min. The supernatant will be discarded and the nanoparticles will be resuspended in deionized water for further studies. The size distribution and zeta potential of the particles in deionized water, will then be determined with a Zetasizer (Malvern Instruments Ltd., Worcestershire, UK) (62,63) and the morphology of the prepared nanoparticles will be examined by transmission electron microscopy examination. The nanoparticle suspension will be placed onto a 400 mesh copper grid coated with carbon. About 2 min after deposition, the grid will be tapped with a filter paper to remove surface water and positively stained with an alkaline bismuth solution (64).

Preparing of CdtB-loaded nanoparticles

To study the loading efficiency of CdtB from test samples (chitosan/heparin nanoparticles), the cdt-encapsulated nanoparticle system will be prepared. The 0.1 mL of CdtB at various known concentrations (24.0, 12.0, 6.0, and 3.0 by mg/mL) was premixed with a heparin solution (2.0 mg/mL 0.1 mL) and added to a chitosan solution (1.2 mg/mL, 1.0 mL) under magnetic stirring as described before (n = 5). To determine the loading efficiency, the CdtB-loaded nanoparticles will be collected by ultracentrifugation at 15,000 rpm, 4°C for 50 min, and the concentration of free CdtB in

the supernatant will be determined by Elisa analysis. The CdtB loading efficiency of nanoparticles will be determined as described in the literature and calculated from the following equation (65,66):

$$\text{Loading efficiency} = \frac{\text{Total amount of protein} - \text{free protein}}{\text{Total amount of protein}} \times 100\%$$

Western blot analysis

AGS cells were treated with CDT holotoxin or NP-CdtB for the indicated periods, the cells were washed with PBS and added 1mL lysis buffer for 10 min at 4°C. Cell lysates were then collected and subjected to Western blot analysis. The samples were then resolved by 12% SDS-PAGE and transferred onto polyvinylidene difluoride membranes (Millipore, MA, USA). The membranes were incubated with primary antibodies, and then incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies (Invitrogen, CA, USA). The proteins of interest were visualized using the ECL Western Blotting Detection Reagents (GE Healthcare, Piscataway, NJ) and detected using X-ray film (Kodak, Rochester, NY).

Cell viability assay

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay was used to test the cytotoxicity of CDT holotoxin or NP -CdtB in AGS cells. AGS cell were seeding in 96 well culture plate (4×10^4 /200 μ l/well) overnight allow the cell to attach to the wells, the medium was replaced with HBSS solution (containing 50mM glucose, pH 6.5) that contained various concentrations of NP-CdtB, after 2 hours remove the NP -CdtB, wash twist with PBS. The cell incubated in new medium for 24 hours, then remove the medium, add the new medium containing MTT (final

concentration 5mg/mL) (Sigma-Aldrich) solution incubate for 2-4 hours allow the ability of viable cells to reduce MTT to formazan, and then dump off the liquid add 100ml 2-propanol and HCl mixture, after pipetting completely the optical density was read with the BioRad spectrophotometer at a wavelength 570nm.

***In vitro* cellular uptake and CLSM visualization**

AGS cells were seeding on 12-well plates and treated for specific times with NP-CdtB at a concentration of 0.5 mg/mL. After incubation, the cells were washed three times with PBS and solubilized with 1 mL of 0.5% Triton X-100 in 0.2 M NaOH. The cell-associated test samples were quantified by analyzing the cell lysates in a microplate spectrofluorometer.

To track the internalization of the NP-CdtB, the cells were seeded onto glass coverslips at a density 3×10^5 cells/cm² and incubated for two days. The test samples (0.5 mg/mL) were then added to the cells for specific times. After incubation, the test samples were aspirated. The cells were then washed three times with PBS before they were fixed in 3.7% paraformaldehyde. The cells were washed again three times with PBS and permeabilized with 0.2% Triton X-100 for 15 min at 37 °C. The washes were repeated and the cells were stained with DAPI, which specifically bind to the nucleus. The stained cells were examined with excitation at 340, 488 and 543 nm, under a CLSM (Confocal laser scanning microscopy). The images were superimposed with the LCS Lite software (version 2.0).

Flow cytometry analysis

AGS cells treated with CDT holotoxin or NP-CdtB were analyzed by flow cytometry. Cells were pretreated with HBSS for 1 h, washed, and exposed to CDT holotoxin or NP-CdtB subunit for an additional various time points. Cells were

harvested and fixed with ice-cold 70% ethanol for 1 h. Cells were then stained with 20 $\mu\text{g/ml}$ propidium iodide (Sigma-Aldrich) containing 1 mg/ml RNase (Sigma-Aldrich) for 1 h. The stained cells were analyzed with an FACScalibur flow cytometer (Becton-Dickinson, San Jose, CA). The data were collected using 10,000 cells from each sample, and analyzed using Cell Quest software WinMDI (Verity Software House, Topsham, ME). All samples were examined in triplicate from at least three independent experiments. The data represent one of three independent experiments.

Contrast microscopy

In this study the contrast microscopy was utilized to observe the cell morphology after treatment of cells with CDT holotoxin or NP-CdtB. AGS cells were seeded at 4×10^5 in the 6 well plates for 48 h. AGS cells were rinsed three times with pre-warmed HBSS solution (Invitrogen, CA, USA, containing 50mM glucose, pH6.5) and incubated for another 30 min at 37°C. The cells were then incubated with HBSS (containing 50mM glucose, pH6.5) containing NP-CdtB at 37°C. After 2 h incubation, the culture supernatant was carefully removed and replaced with fresh culture medium containing penicillin (100U/mL), and streptomycin (100 $\mu\text{g/mL}$) (AMRESCO, Solon OH, USA) for 24-72 hours. The cell morphology was observed by contrast microscope (Carl Zeiss, Göttingen, Germany). The images were captured and analyzed by the Axiovision software (Carl Zeiss).

Determination of the mitochondrial membrane potential

AGS cells were first seeding in 6-well culture dishes and treated with CDT holotoxin or NP-CdtB for 24 hours. The mitochondrial membrane potential ($\Delta\Psi_m$) was assessed using a fluorometric probe JC-1 (Calbiochem, CA, USA), with a positive charge of a mitochondrial-specific fluorophore, indicated by a fluorescence

emission shift from green (525 nm) to red (610 nm). After incubation, cells were stained with JC-1 (5 µg/ml) for 30 min at 37°C. Samples were analyzed by FACScan using an argon laser (488 nm). Mitochondrial depolarization is specifically indicated by a decrease in the red to green fluorescence intensity ratio and analyzed by a FACScan and the Cellquest program (Becton Dickinson; Lincoln Park, NJ, USA).

Measurements of reactive oxygen species (ROS)

In order to determine the quantify of ROS produced by the AGS cells, the H₂O₂ concentration within the AGS cells were measured by 10 µM 2,7-dichlorodihydrofluorescein diacetate (H₂DCFDA; Molecular Probes Inc., Eugene, OR, USA) for 30 min. The fluorescence intensities were obtained by recording the FITC fluorescence. AGS cells were washed twice with cold PBS and incubated in cold PBS containing 10 µM H₂DCFDA at 37°C after 30 mins, cells were collected and analyzed by a FACScan and the Cellquest program (Becton Dickinson; Lincoln Park, NJ, USA).

Statistical analysis

The *in vitro* statistical analysis between two samples was performed using Student's *t*-test. *In vitro* statistical comparisons of more than two groups were performed using one-way analysis of variance (ANOVA) test. The statistical software was the SPSS program (version 12.0 for Windows, SPSS Inc., Chicago, Illinois, USA). In all cases, $p < 0.05$ was considered as significant.

Results

1. Preparation of chitosan/heparin nanoparticles

Nanoparticles were prepared by the ionic gelation of positively charged chitosan with negatively charged heparin. As shown in **Table 2**, chitosan:heparin in distinct chitosan concentration (0.3, 0.6, 0.9, 1.2, 1.5 mg/ml; 12.0 ml) and heparin (1.0 mg/ml; 2.0 ml) formed complexes on the nanometer scale. The mean particle sizes of the prepared nanoparticles were in ranges of 200–300 nm, with positive zeta potentials, depending on the relative concentrations of chitosan and heparin used. The amount of positively charged chitosan significantly exceeded that of negatively charged heparin, because some of the excessive chitosan molecules were entangled on the surfaces of the nanoparticles produced. A chitosan concentration was 1.2 mg/ml and appeared to enlarge the particle size (273.1 ± 10.6 nm) with a significantly zeta potential of 37.4 ± 1.9 mV (**Table 2**). Therefore, the nanoparticles prepared with this specific composition were used for our further study.

2. Preparation of CdtB-loaded nanoparticles

CdtB loaded in chitosan/heparin nanoparticles were prepared by the ionic gelation of negatively charged protein mixed heparin, and then added into positively charged chitosan. As shown in **Table 3**, the chitosan (1.2 mg/ml, 1.0 ml) and the CdtB:heparin in distinct compositions (12.0:1.0, 6.0:1.0, 3.0:1.0, and 1.5:1.0 by mg/ml, 0.2 ml) had mean size ranges of 2800–300 nm, with different positive zeta potentials, depending on the relative concentrations of protein used. In addition, the polydispersity index of nanoparticles measured by a dynamic light scattering analyzer revealed a narrower distribution (polydispersity indices: 0.31 ± 0.08), when the CdtB:heparin composition

at 1.5:1.0 (mg/ml) was used (**Figure 2**). The result obtained by the transmission electron microscopy examination showed that the morphology of the prepared CdtB-loaded in chitosan/heparin nanoparticles remained spherical and smooth shaped (**Figure 2**).

3. Expression and characterization of recombinant CdtB

Since CdtB played a crucial role in the cytotoxicity of cells, we thus constructed *C. jejuni cdtB* and ligated into pET21d for CdtB protein expression. Recombinant CdtB was then purified and analyzed by SDS-PAGE (**Figure 3**). The purified recombinant CdtB was readily detected by western blot using monoclonal anti-His (**Figure 3**). Western blotting was carried out to determine whether polyclonal antibodies generated against each subunit could recognize the CdtB subunit when assembled and associated with cells. As shown in **Figure 3C**, the individual recombinant CdtB proteins were recognized by the respective polyclonal CdtB antiserum at 28 kDa. .

4. Confocal images of cells with internalized FITC-CdtB solution or FITC-CdtB/Cy3-chitosan/heparin nanoparticles

CdtB subunit was found to be an activity subunit of CDT holotoxin which was delivery by the binding subunits CdtA and CdtC. We next used confocal microscopy to visualize the delivery efficiency of NP-CdtB. AGS cells were incubated with FITC-cojugated NP-CdtB and FITC-CdtB at 37°C for the indicated times. The cells were then stained with 4',6-diamidino-2-phenylindole (DAPI) to visualize the nucleus. As shown in **Figure 4** (1st row), no CdtB fluorescence could be detected on the plasma membrane upon cells treated with only FITC-conjugated CdtB. When AGS cells were treated with NP-CdtB at 37°C for 2 h, CdtB (green) was co-localized with Cy3-chitosan around the plasma membrane (**Figure 4**, 2nd row). After treatment of cells with

FITC-conjugated CdtB for 4 h, NP-CdtB was found localized in the nucleus (**Figure 4**, 3rd row). The results from confocal microscopy suggests that NP-CdtB has activity to localized into the nucleus.

5. Detection of NP-CdtB by western blot analysis

A previous study by Lin *et. al.* suggested that chitosan/heparin nanoparticle was an efficient carrier for antibiotic drug delivery (54). We thought to investigate whether the nanoparticle have ability to deliver CdtB into the AGS cells. To detect the efficiency of CdtB or NP-CdtB into the AGS cells, we treated the AGS cells with CDT holotoxin (200 nM) and NP-CdtB (200 nM) for various incubation periods (1, 2, 4, 6, 8 hours) and detected by the western blot analysis using an anti-CdtB serum. As show in **Figure 5**, after treatment of cells with NP-CdtB, the level of CdtB was increase at 4 hours and then begin to decrease at 6 to 24 hours. However, the level of CdtB was increase at 8 hours when treated of cells with CDT holotoxin (**Figure 5B**). Thus, the data suggests that the delivery efficiency of NP-CdtB is higher than CDT holotoxin.

6. Treatment of cells with NP-CdtB induces cell cycle arrested at G2/M

A previous research indicated that CDT not only causes cell distending but also induces chromatin condensation and cleavage, as well as induce cell cycle arrest at G2/M phase (42). It has also been suggested that CDT toxicity is associated with cell cycle arrest and eventually cell death resulting from activation of the apoptotic cascade (23). We next thought to detect whether NP-CdtB has ability to induce G2/M arrest and cell death in AGS cells. As shown in Figure 6A, NP-CdtB induced G2/M arrest shown gradually increased in a time-dependent manner. The statistical analysis showed that G2/M arrest was significantly increased after AGS cells were treatment

with NP-CdtB for 2 hours as compare with the negative control (only AGS and NP). The results from this study demonstrated that NP-CdtB not only delivery into the cell nucleus but also have ability to induce cell cycle arrest at G2/M phase.

7. Observation of cell morphology

CDT holotoxin can induced genotoxic effect in AGS cells including cell cycle arrest at G2/M phase and leading to cell change the morphology finally cause cell apoptosis, in this data we detection weather the NP-CdtB can cause cell death, the data was shown in **Figure 6**, to further determine the effects of CDT holotoxin or NP-CdtB, we observed the cell morphology by using the contrast microscopy. This data indicated the cell detachment was observed the AGS cell exposed to the CDT holotoxin or NP-CdtB at 24 hours (**Figure 7**), then the cell was detached more after cell was incubated with CDT holotoxin or NP-CdtB for 72 hours. Otherwise, the normal cell or the cell treat with nanoparticle only was not significant effect in this data. As the results, the AGS cell was dead by the effects of CDT holotoxin or NP-CdtB, but not by nanoparticle treatment only.

8. NP-CdtB induced apoptosis in AGS cells

CDT toxicity is associated with cell cycle arrest and eventually cause cell death through the activation of apoptotic cascade (35,36). Our preliminary data showed that cell cycle was arrested at G2/M phase after treatment of cells with NP-CdtB by using flow cytometry analysis (**Figure 7**). We next detected the cell viability after cell was treatment with NP-CdtB for 24 hours by using MTT assay. As shown in **Figure 8**, cell viability was decrease with a dose-dependent manner when AGS cells were treated with variety concentrations (20nM, 200nM, 500nM and 1000nM) of NP-CdtB for 24 hours. We also detected sub-G1 population using flow cytometry to confirm the our

observation. When AGS cells were treated with NP-CdtB for different incubation times (24, 48, 72 hours), the population of the sub-G1 shown increased in a time-dependent manner (**Figure 9A**). Our results showed that NP-CdtB-induced cell apoptosis at 24-72 h with a significant increase when compare to control groups (**Figure 9B**).

It has been reported that several molecules were involved in the apoptosis pathway, including the Bcl-2 families, caspase families, p53 (67-69). The Bcl-2 families function as an anti-apoptosis which included Bcl-2 and Bcl-xl. On the other hand, the Bax, Bak, Bad, and Bid were molecules that involved in pro-apoptotic. We next assessed whether the cell apoptosis induced by NP-CdtB in the AGS cells was associated with an anti-apoptosis family. As show in **Figure 10**, the levels of the Bax and Bak were increased after treatment of cells with CDT holotoxin or NP-CdtB when compared with control groups (only AGS cell and only nanoparticle). Additonally, the expression of anti-apoptosis molecular, Bcl-2, was decreased. This result suggested that NP-CdtB has the ability to delivery CdtB subunit enter the AGS cell nucleus and then activate the apoptotic cascade. The results from this study suggested that NP-CdtB has ability to delivery into the host cell nucleus and thus induced cell cycle arrest at G2/M phase and cell apoptosis (**Figure 11**).

Discussion

Campylobacter jejuni is one of the major cause of infection diarrhea world-wide as well as the most commonly reported bacterial cause of food-borne illness in the United States (3), *C. jejuni* was found can cause disease in different hosts including human, beast animal, and pets. This bacterium can also induce several types of diseases in human including gastritis, gastroenteritis, septicemia and serious neurological disease like Guillian-Barré syndrome. Recently, the infection of *C. jejuni* was became a seriously problem around the world.

The cytolethal distending toxin (CDT) is one of the important virulence factors secreted by *C. jejuni*. CDT also contributes to survival and establishment of disease in the host cells. One of the most important function of CDT is to induce cell distending and lead to a severe genotoxin effects on host (19). The earlier study indicated that CDT is a family of heat-labile protein cytotoxin found in various gram-negative bacteria pathogens of human (70,71). CDT is typically compose by three subunits, including CdtA, CdtB and CdtC (72). CDT holotoxin function as an AB₂ toxin in which CdtA and CdtC serve as a binding subunits, and CdtB plays an important role in destruction of the host DNA. Once CDT holotoxin get into the host cell from the plasma membrane, CdtB using an endoplasmic pathway through Golgi and ER, after which CdtB translocated into the nucleus (73). The catalytic subunit CdtB has DNase-I like activity, whereas CdtA and CdtC serve as the binding subunit which associate with the cell surface and delivering of CdtB into the target cell lead to cell apoptosis (74). The research in 2001 by Lara-Tejero *et al.* indicated that Cdt activity requires the function of three subunits, when applied individually, purified CdtA, CdtB or CdtC doesn't exhibit toxic activity in the host cells (75). Moreover, several

groups have shown that CdtA-CdtC complex can inhibit subsequent intoxication by holotoxin (76,77). The results from these studies suggested that the binding subunits CdtA and CdtC play a crucial role in the association with the host cell membrane. Thus we attempt to utilize other substances to substitute as the binding subunits and achieve the same results.

Nanoparticle is a microscopic particle which contains biodegradable and safety used *in vivo*. Nanoparticle has a widely potential application in several fields. Recent years, nanoparticle was commonly used in cancer therapy due to its ability to conjugate and inject some bacterial toxins as well as delivery of drugs into target cells which may induce the toxin effects (51). In 2007 Townsend *et al.* who used nanoparticle to directly deliver therapies to neurons in the central nervous system, which is protected by blood-brain barrier (BBB). However, nanoparticle has ability to bypass the blood-brain barrier (BBB) because of its small volume, (50). Due to the widely utilized of nanoparticle, the major object of this study is to develop a novel nanoparticle encapsulated bacterial toxin for gastric cancer therapy.

The acidic environment in gastric probability cause drug decomposition or loss of their function. Thus, this is one of the problems we consider in this study: the nanoparticle used in this study was consisted of chitosan and heparin. Chitosan has structural characteristics similar to glycosaminoglycans which is non-toxin and biodegradable (78). Heparin is a well-know anticoagulant and has low-molecular-weight about 15kDa. Heparin harbors a polyanionic mucopolysaccharide which has ability to bind to cell receptors and associate with mucosal regeneration, angiogenesis, and proliferation (59,79), Recently, polymer nanoparticle was wide application in target therapy in which serve as a carrier for drug delivery. Those two components has a pH sensitivity characteristic that are stable at pH 1.2-2.5 but unstable and break apart at pH 7.0. A previous study also indicated

that the drug was release by nanoparticle at pH 1.2 (35%) and at pH6.8 (80%). This result acknowledges that the nanoparticle can protect drugs from destruction by gastric acids (54).

The current study created a system which has ability to encapsulate CdtB and deliver CdtB into the AGS cells. This system was substituted CDT holotoxin for nanoparticle to achieved the same results. The nanoparticle-encapsulated drug for delivery into the cells must be smaller than 200 nm to be taken up by epithelial cells (54,80). **Figure 2** shows the size of NP-CdtB is between 200nm and 300nm. This is not in the range from previous study. Thus, we first determined whether the NP-CdtB have ability to enter the AGS cells. As show in **Figure 4**, the images from CLSM indicated that upon treatment of cells with NP-CdtB for 2 hours, NP-CdtB was located in intercellular spaces and cell cytoplasm. When changes the fresh medium and incubation for further 4 hours, NP-CdtB was found co-localized with the cell nucleus. This result suggests that nanoparticle play an important role in the delivery of CdtB subunit into the cell nucleus, In addition, the western blot results from **Figure 5** also indicated the maximum of NP-CdtB get into the AGS cell is around 4 hours. This is better than 8 hours after CDT holotoxin treatment. Thus, the results from these studies suggest that NP-CdtB has better efficiency then CDT holotoxin in the delivery of CdtB into the AGS cells.

Several groups have been utilized nanoparticle as a delivery system which has been approved can translate *in vivo* for cancer therapy (50,51,81). In this study we used the bacterial toxin CDT to destroy the gastric cancer cells. CdtB function as a phosphatidylinositol-3,4,5-triphosphate phosphates which is analogous to the tumor suppressor phosphatases, PTEN and SHIP 1 (40,82). CdtB cause cell cycle arrest at G2/M phase on host cell was reported in 1997 (33). In our current study, as shown in **Figure 6**, the cell cycle analysis indicated that NP-CdtB not only has ability to deliver

into the host cells, but also cause cell cycle arrest at G2/M phase (83). The data from these studies also indicated that nanoparticle cannot cause cell cycle arrest in AGS cells.

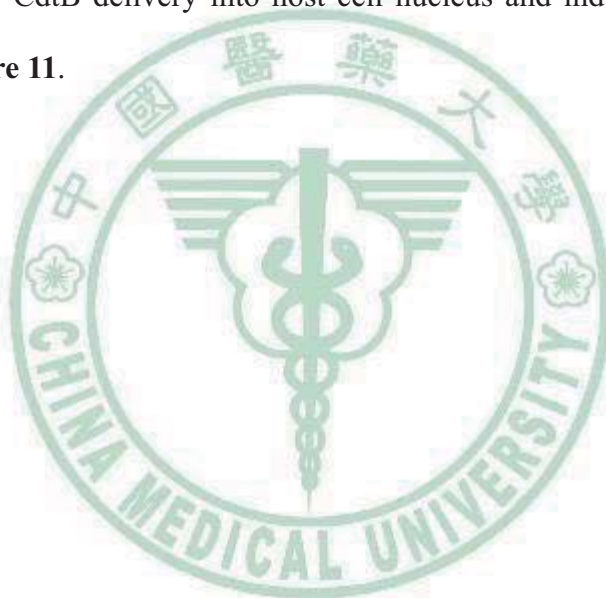
Upon cells treated with CDT holotoxin and NP-CdtB for 24 hours, the cell viability was significantly decrease in a dose-dependent manner (**Figure 8**). In addition, CDT holotoxin cause cell cycle arrest at G2/M phase then induced cell death via the apoptosis pathway. We thus detected the cell apoptosis after treatment of cells with NP-CdtB. The population of apoptotic cells was graduated increased with a time-dependent manner.

CDT holotoxin can be produced by some Gram-negative bacteria, which may associate with double-stranded DNA breaks resulting in cell cycle arrest at G2/M phase and leading to cell apoptosis via the mitochondrial-dependent apoptosis pathway (84). Our study by western blot analysis suggests that the expression level of the Bax and Bak was increase after AGS cells treated with Cdt holotoxin or nona-CdtB. This result demonstrated that the NP-CdtB have the ability in the activation of the apoptotic cascade (**Figure 10**). Most important, our study also found that the caspase families are not involved in NP-CdtB-induced apoptosis pathway (data not show).

Because of the previously research indicated that cell exposure to CDT holotoxin can triggers activation of checkpoint responses that lead to cell DNA damage, cell cycle arrest as well as cell apoptosis. The key molecules involved in DNA double strand breaks was refered to Ataxia telangiectasia mutated (ATM) kinase. A previous study suggest that CDT holotoxin can trigger full activation of ATM and subsequent phosphorylation of its downstream effectors such as DNA repair complex (H2AX), checkpoint kinase (chk2) and the tumor suppressor gene (p53) (85). Thus, we suppose that NP-CdtB-induced cell apoptosis may through the ATM-dependent DNA damage

checkpoint responses. However whether those molecules involved in the NP-CdtB-induced apoptosis of AGS cells required further investigation.

In conclusion, we developed a novel nanoparticle which carried heparin and chitosan for encapsulation of bacterial toxin CdtB. We also proposed that nanoparticle can successfully replaced CdtA and CdtC to deliver CdtB subunit into the AGS cells. In this study we can demonstrate that NP-CdtB can induced cell cycle arrest at G2/M phase and finally led to cell apoptosis in AGS cells. This system we created have a higher efficiently to delivery CdtB into the human gastric adenocarcinoma cells.. The mechanism of NP-CdtB delivery into host cell nucleus and induce cell apoptosis is described in **Figure 11**.



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Tables

Table 1. The species of Campylobacter and the related diseases.

Species	Reservoir Host	Human Disease	Frequency
<i>C. jejuni</i> subsp. <i>jejuni</i>	Poultry, pigs, bulls, dogs, cats, rabbits, birds, minks	Gastroenteritis, septicemia, meningitis, spontaneous abortion, proctitis, Guillain-Barré syndrome	Common
<i>C. jejuni</i> subsp. <i>doylei</i>	Humans	Gastroenteritis, gastritis, septicemia	Uncommon
<i>C. coli</i> subsp. <i>fetus</i>	Pigs, poultry, bulls, sheep, birds	Gastroenteritis, septicemia, insects, abortion, spontaneous, meningitis	Uncommon
<i>C. upsaliensis</i>	Dogs, cats	Gastroenteritis, septicemia, abscesses	Uncommon
<i>C. fetus</i> subsp. <i>fetus</i>	Cattle, sheep	Septicemia, gastroenteritis, spontaneous abortion, meningitis	Uncommon
<i>C. fetus</i> subsp. <i>venereal</i>	Cattle	Septicemia	Uncommon

Adapted from On (5)

Table 2. Particle sizes and zeta potentials of nanoparticles prepared with different chitosan and heparin concentrations in deionized water (n = 5).

Chitosan Concentration (mg/mL)	Heparin Concentration (mg/mL)	Mean Particle Size (nm)	Zeta Potential (mV)
0.3	1.0	●	●
0.6	1.0	208.4 ± 10.6	30.4 ± 1.5
0.9	1.0	248.5 ± 8.7	33.6 ± 0.8
1.2	1.0	273.1 ± 10.6	37.4 ± 1.9
1.5	1.0	298.1 ± 9.6	39.4 ± 21.4

● Precipitation of aggregates was observed.

Table 3. Particle sizes and zeta potentials of the prepared bacterial toxin-loaded nanoparticles in deionized water (n = 5).

Protein:Heparin Concentration (mg/mL)	Chitosan Concentration (mg/mL)	Mean Particle Size (nm)	Zeta Potential (mV)
12.0:1.0	1.2	2788.1 ± 300.9	14.8 ± 5.9
6.0:1.0	1.2	983.5 ± 163.9	20.8 ± 3.1
3.0:1.0	1.2	414.3 ± 8.3	29.9 ± 1.7
1.5:1.0	1.2	312.4 ± 15.7	32.3 ± 0.7

Figures

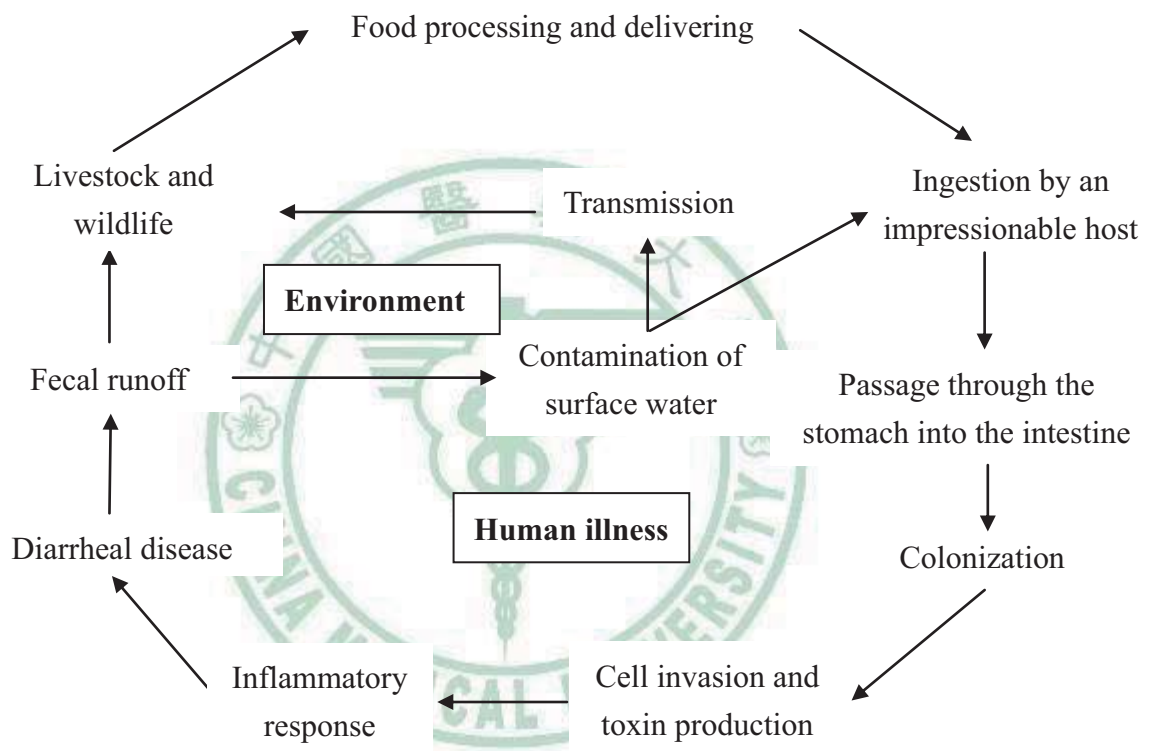


Figure 1. The infection routes of *Campylobacter jejuni*. (Adapted from Konkel (86))

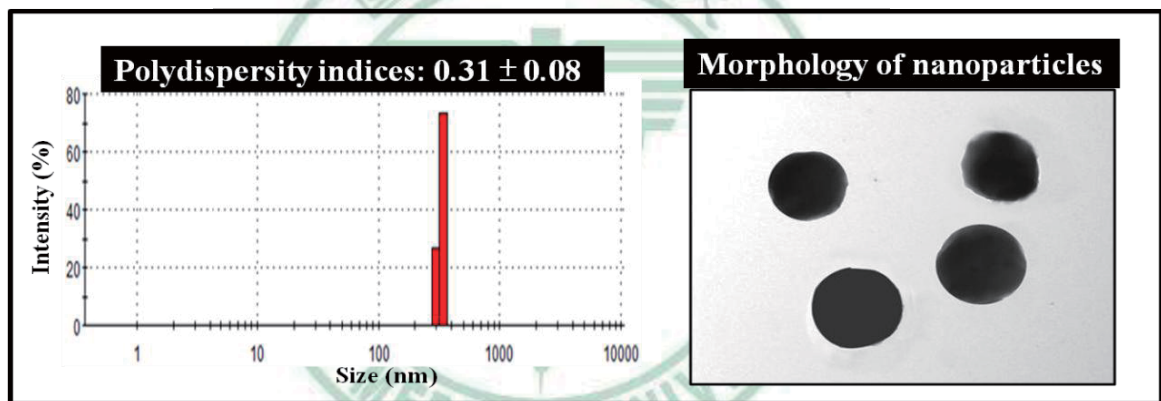


Figure 2. The polydispersity index (left panel) and transmission electron microscopy examination (right panel) of CdtB-loaded chitosan/heparin nanoparticles.

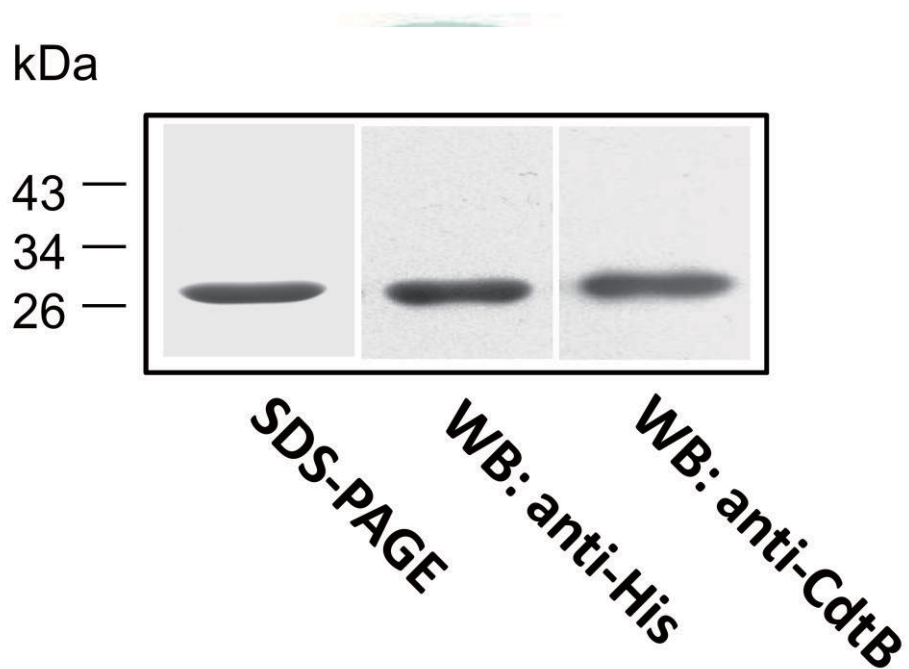


Figure 3. Determination of CdtB by using SDS-PAGE and western blotting. CdtB was first constructed from *C. jejuni* and ligated into pET21d. The molecular weight was determined by using SDS-PAGE (A) and western blot analysis against His-tag (B) and CdtB (C). The molecular weight marker was denoted at the left side.

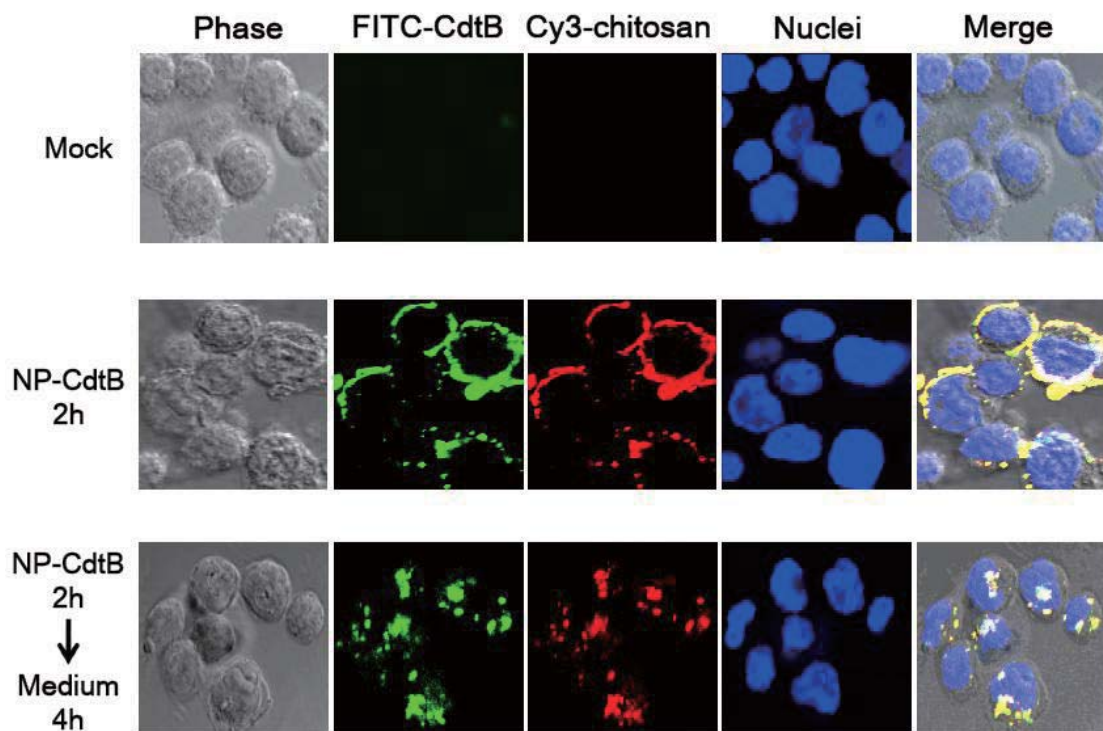
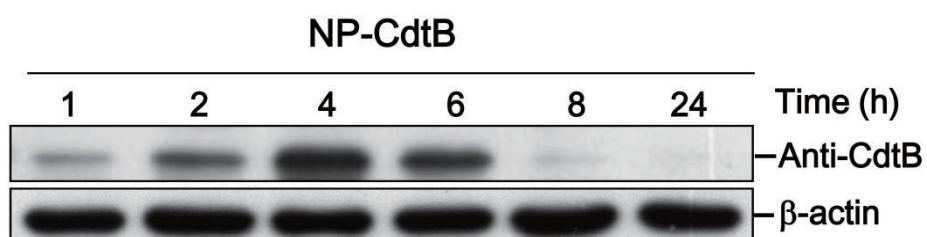


Figure 4. Confocal microscopy analysis of Nanoparticle -CdtB delivery.

Cells were pre-treated with HBSS (containing 50mM glucose, pH6.5) for 30 mins. After that, the cells were treated with HBSS containing NP-CdtB for 2 hours, and then remove the NP-CdtB. Cells were incubated with fresh culture medium for 4 hours, the CLSM was employed to observe the cellular uptake of NP-CdtB in cells. Fluorescent of NP-CdtB was used in the study and the distribution of Cy3-chitosan (red), NP-CdtB (green), and nuclei (blue, stained with DAPI) in the intracellular spaces was observed by a confocal microscope.

(A)



(B)



Figure 5. Detection the efficiency of CDT holotoxin or Nanoparticle -CdtB delivered into cells.

Cells were incubated with 200 nM of (A) NP-CdtB or (B) CDT holotoxin for the indicated periods. The expression of CdtB was determined using western blot analysis against CdtB.

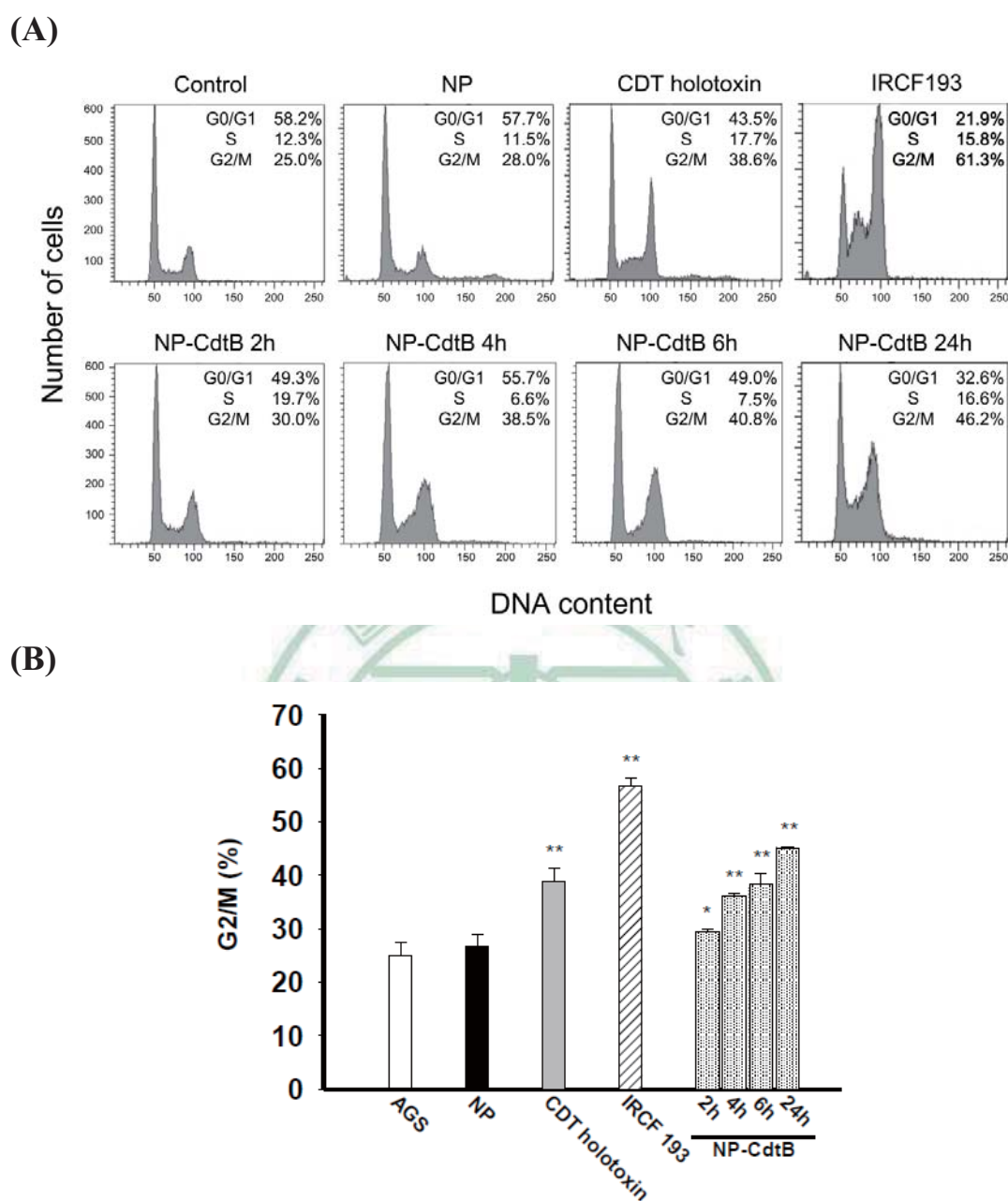


Figure 6. Nanoparticle -CdtB induced cell cycle arrest at G2/M phase in AGS cells.

AGS cells were treated with NP-CdtB (200 nM) for the indicated time (1, 2, 4, 6, 7 and 24 hours). The population of cell cycle arrest was analyzed by flow cytometry. (B) The population of G2/M phase was shown in time-cause dependent manner, the G2/M phase population was increased after AGS cells incubated with NP-CdtB for 2 hours, compare with the negative control (only AGS and nanoparticle). IRCF193 and CDT holotoxin are represented as positive controls. Result are expressed as the Mean±S.E. * $p < 0.05$ compared with control; ** $p < 0.01$ compared with control.

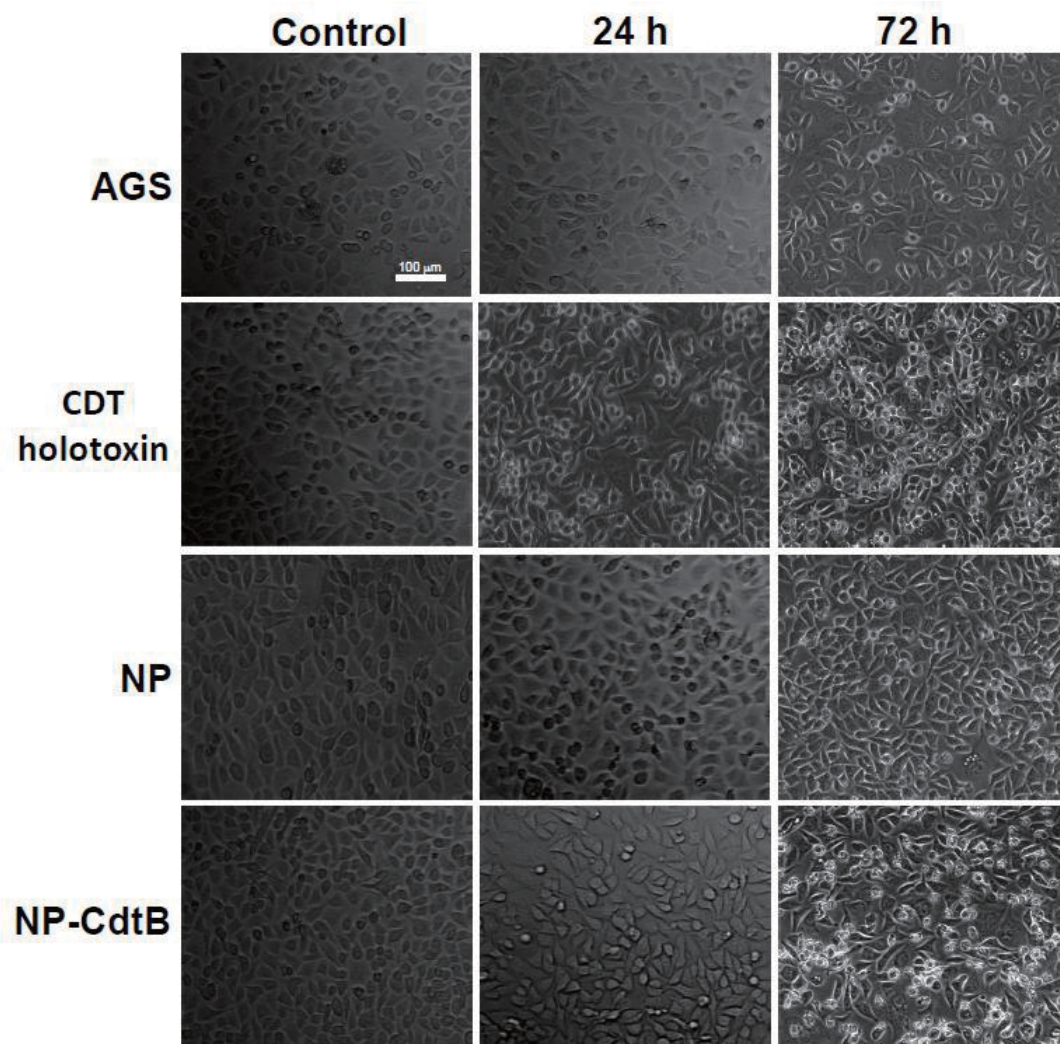


Figure 7. Cells were detachment after Nanoparticle -CdtB treatment for 24 to 72 hours.

The cells were detached after incubation with CDT holotoxin or NP-CdtB for 24 hours. The detached cells was more obvious after treatment with NP-CdtB or CDT holotoxin for 72 hours. Representative image are show. Scale bar, 100 μ m.

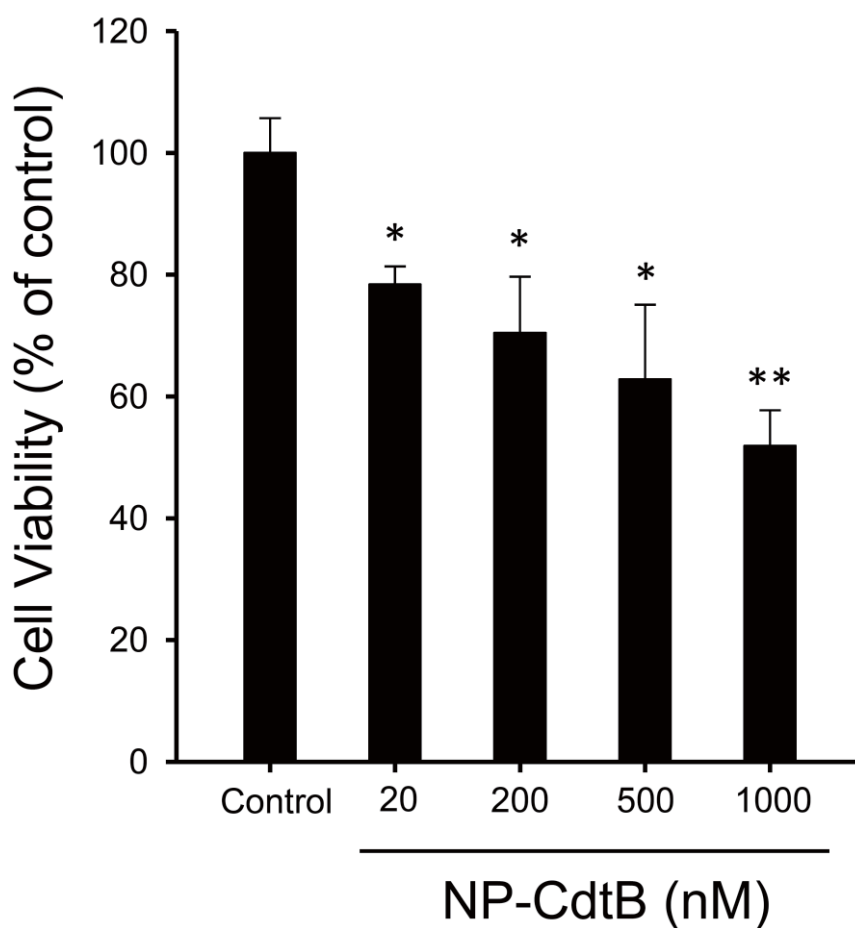


Figure 8. The effects of Nanoparticle -CdtB on cell viability in AGS cells.

AGS cells were incubated with various concentrations (20, 200, 500, 1000, 2000 nM) for 24 hours. The percentage of cell viability was analysis by MTT assay (n=3). Result are expressed as the Mean±S.E. *p <0.05 compared with control; **p < 0.01 compared with control.

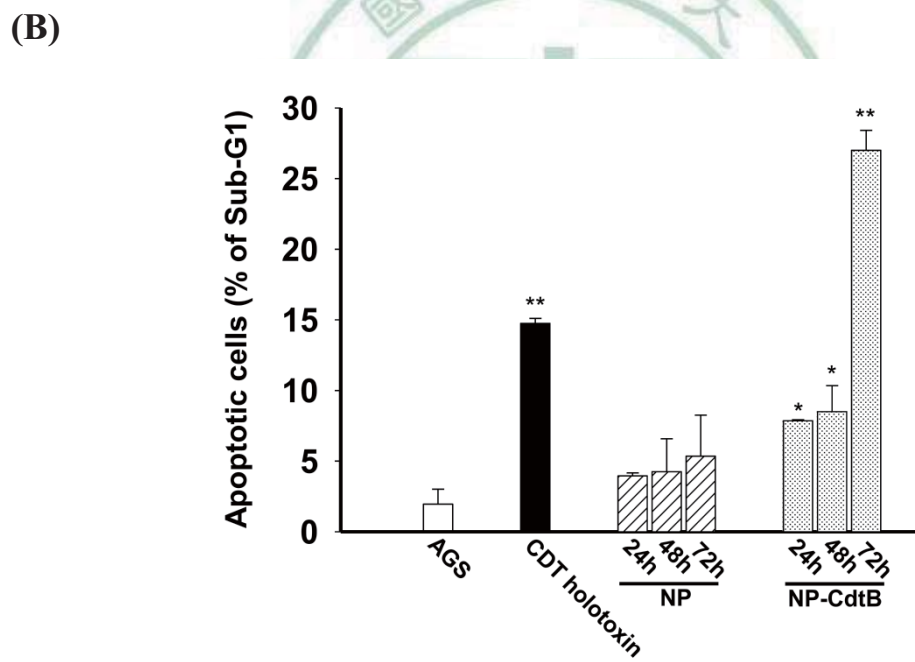
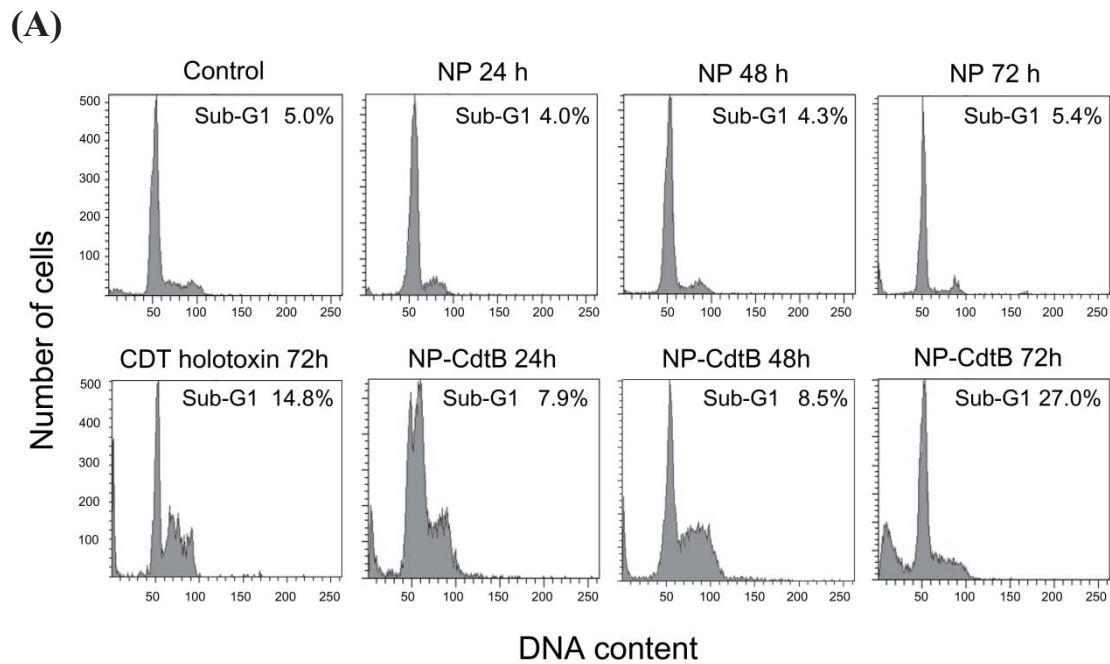


Figure 9. Nanoparticle -CdtB induced apoptosis of human gastric adenocarcinoma cells.

(A) AGS cells were treated with CDT holotoxin, NP-CdtB, and nanoparticle for the indicated times (24, 48, 72 hours). The population of apoptotic cell was analyzed by flow cytometry of PI-stained cells (n=3). (B) The data represented percentage of sub-G1 cells was increase with a time-dependent manner compare with control groups (NP and control). Results are expressed as the Mean±S.E. *p <0.05 compared with control; **p <0.01 compared with control.

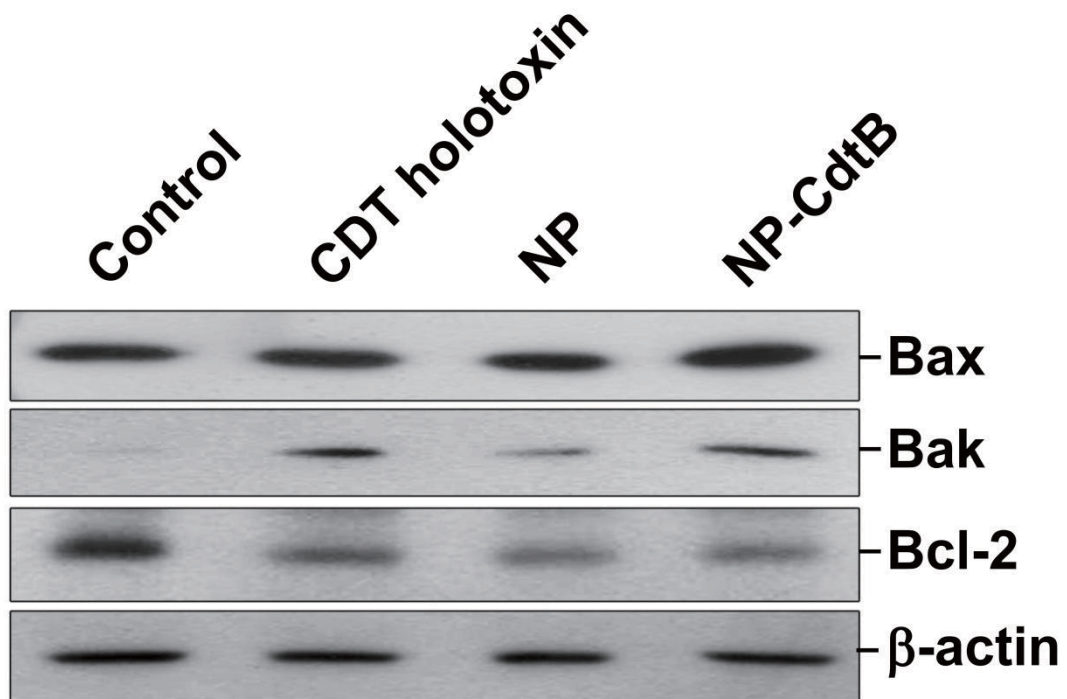


Figure 10. Bax, Bak, Bcl-2 activation are involved in Nanoparticle -CdtB mediated cell apoptosis in human gastric adenocarcinoma cells.

AGS cells were incubated with CDT holotoxin, NP-CdtB and nanoparticle for 24 hours. Expression of Bax, Bak, Bcl-2 were examined by western blot analysis. The expression of Bax and Bak were increased after treatment of cells with CDT holotoxin and NP-CdtB.

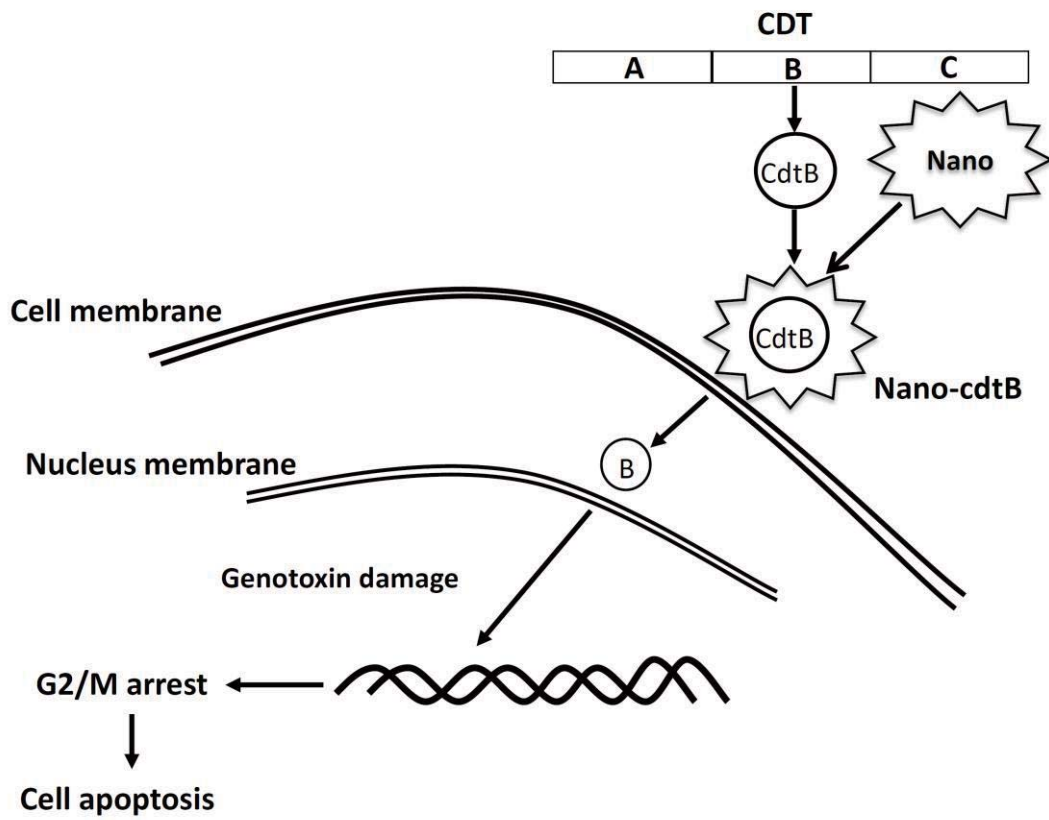


Figure 11. Depiction of the mechanism of Nanoparticle-CdtB delivers into host cell nucleus and induces cell apoptosis.