# Toksin B bakterije Clostridium difficile povzroča morfološke spremembe, ki nakazujejo proces avtofagije v človeških črevesnih epitelnih celicah HT–29

# Clostridium difficile toxin B induces morphological changes consistent with autophagy in the human adenocarcinoma cell line (HT–29)

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## Izvleček

**Namen:** *Toksin B (TcdB) bakterije Clostridium difficile sproži celično smrt, ki je rezultat apoptoze ali nekroze, s katerima je povezana avtofagija. Predpostavili smo, da tako $TcdB_{VPI}$ kot tudi $TcdB_{8864}$ s svojim delovanjem vplivata na morfološke spremembe kultiviranih celic, značilnih za avtofagijo.*

**Metode:** *Za in vitro študije interakcij med črevesnimi epitelnimi celicami in toksinoma $TcdB_{VPI}$ in $TcdB_{8864}$ smo uporabili celično linijo HT–29. Celice smo inkubirali od 2 do 24 ur ločeno s $TcdB_{VPI}$ in $TcdB_{8864}$ in jih pregledali s transmisijskim elektronskim mikroskopom.*

**Rezultati:** *V HT–29 celicah smo ultrastrukturne spremembe opazili po 8 urah inkubacije s $TcdB_{VPI}$ in $TcdB_{8864}$. Vidna so spremenjena območja zrnatega endoplazemskega retikuluma, Golgijevega aparata, elektronsko svetle ter elektronsko temne avtofagosomne*

## Abstract

**Purpose:** *Toxin B (TcdB) is a product of the bacteria Clostridium difficile, and can trigger apoptosis or necrosis. In the present manuscript, we proposed that $TcdB_{VPI}$ and $TcdB_{8864}$ would cause morphological changes in cultured cells that are characteristic of autophagy, a constitutive cellular event closely linked with apoptosis or necrosis.*

**Methods:** *We examined an in vitro model of cultured HT–29 cells to determine the occurrence of autophagy. The cells were incubated separately with $TcdB_{VPI}$ and $TcdB_{8864}$ for 2 to 24 hours, and then examined using transmission electron microscopy.*

**Results:** *In HT–29 cells, ultrastructural changes were observed following 8 hours of treatment with $TcdB_{VPI}$ and $TcdB_{8864}$. Upon exposure to both toxins, complex changes occurred that affected the cellular*

*strukture. V citoplazmi so prisotne lipidne kaplje.*

**Zaključek:** *$TcdB_{VPI}$ in $TcdB_{8864}$ povzročita nastanek ultrastrukturnih sprememb v HT–29 celicah. Na osnovi prisotnosti avtofagosomnih struktur v citoplazmi celic sklepamo, da lahko avtofagija pomembno prispeva k prilaganju HT–29 celic na delovanje $TcdB_{VPI}$ in $TcdB_{8864}$ ter s tem na njihovo preživetje.*

*framework, including the dilated regions of the granular endoplasmic reticulum, the Golgi apparatus, as well as electron–lucent and electron–dense autophagosome–like structures. Additionally, lipid droplets were present in the cytoplasm of HT–29 cells.*

**Conclusion:** *Our findings indicate that $TcdB_{VPI}$ and $TcdB_{8864}$ indeed induce ultrastructural changes in HT–29 cells. The presence of these autophagic structures in the cytoplasm of the HT–29 cells suggests that autophagy may be induced during treatment by both toxins. Therefore, this effect could represent an important protective mechanism for cell survival.*

## INTRODUCTION

*Clostridium difficile* is an important pathogen associated with the development and progression of enteric diseases. Toxin B (TcdB) represents one of three known bacterial protein toxins produced by C. *difficile*. Toxin A (TcdA) and TcdB are considered virulence factors because these factors are responsible for colonic damage during C. *difficile* infection (CDI) (1). Four major steps occur during toxin exposure, and include receptor–mediated endocytosis, the translocation of a catalytic glucosyltransferase domain across the membrane (2, 3), the release of the enzymatic moiety by autoproteolytic processing, and glucosyltransferase–dependent inactivation of Rho family proteins (4, 5). Due to the inactivation of the Rho proteins, TcdA and TcdB cause significant morphological and pathophysiological changes in cultured cells. Previous studies indicate that TcdB is capable of inactivating Rho GTPases, thereby, causing apoptosis (6–14) or necrosis (15–17).

Although many aspects of TcdB pathogenesis have been investigated (18–23), a more detailed understanding of the toxin–cell interaction could reveal critical information regarding TcdB, especially toxin–induced changes in the biology of the host cell. Therefore, further characterization is necessary to study the cellular control mechanisms as well as the toxin countermeasures in response to toxin exposure. For instance, one such control mechanism could likely be autophagy. Autophagy is defined as a lysosome–dependent mechanism of intracellular degradation that is essential for the turnover of cytoplasm (24). Several forms of autophagy have been described, including macroautophagy, microautophagy and chaperone–mediated autophagy (25, 26).

Macroautophagy is primarily used for the sequestration and degradation of cytoplasm in processes that incorporate specialized cytosolic vesicles or vacuoles that fuse with the lysosome. Unlike macroautophagy, microautophagy involves the direct uptake of cytoplasm at the lysosome surface by invagination of the lysosome membrane. Chaperone–mediated autophagy also takes place at the lysosome membrane, and this process relies on the translocation of unfolded proteins across the membrane (24, 25, 27). Macroautophagy represents the principal process involved in the degradation of cellular compounds (e. g., damaged cytoplasmic organelles by lysosomal enzymes). Therefore, macroautophagy protects cells against the accumulation of damaged cellular components (28).

During macroautophagy (hereafter referred to as autophagy), regional sequestration of cytoplasm within an enveloping double membrane structure creates a vacuole, termed the autophagosome or autophagic vacuole (29). These autophagic structures are classi-

fied according to their morphology. For example, autophagosomes exhibit double membranes and uncompacted cytoplasmic material, and the degradation of the cytoplasmic substrates is initiated when the autophagosome fuses with degradative compartments of the endosomal–lysosomal system (30). The outer membrane of the autophagosome subsequently fuses with a lysosome, exposing the inner single membrane of the autophagosome to lysosomal hydrolases. When the autophagosome fuses with the lysosome, an autophagolysosome is formed. After this fusion, the degradative lysosomal enzymes break down the contents in the vacuole. Autolysosomes are double– or single–membrane structures containing densely compacted amorphous or multilamellar material that is electron–dense (24, 25, 31).

Autophagy is regulated during the response to the availability of nutrients and during the development of multicellular organisms. Autophagy is generally activated under conditions of nutrient starvation, and this process allows cells to degrade nonessential proteins and organelles in order to utilize their components. Consequently, autophagy ensures the survival of cells in extreme conditions (e. g., during starvation), and is an essential process for cellular defense from infectious disease. Autophagy also plays an important role in programmed cell death, and defects in autophagy have been linked to several human diseases, including neurodegenerative diseases and cancer (32). In recent years, research has begun to reveal the molecular machinery that regulates autophagy (33). For instance, many pathogens have been found that interfere with this catabolic process (34). However, the role of autophagy in response to bacterial toxins is still largely unknown.

For this purpose, we analysed structural differences between cells treated with toxins at different periods of time. The human colon cell line HT–29 offers a favourable experimental system for the study of factors involved in the autophagic processes of epithelial cells. In this study, we investigated the influence of two different toxins, $TcdB_{VPI}$ and $TcdB_{8864}$, on the ultrastructure of HT–29 cells. In particular, we focused our study on possible effects of $TcdB_{VPI}$ and $TcdB_{8864}$ on the ultrastructure of HT–29 cells by inducing autophagy. The relationship between autophagy and these two toxins has not previously been studied.

## MATERIALS AND METHODS

### Cell culture

This study was conducted using the human colorectal cancer cell line HT–29 obtained from the American Tissue Culture Collection. The cells were cultured at 37°C in 5% $CO_2$ utilizing Dulbecco's modified Eagle's medium (DMEM) supplemented by 10% heat–inactivated fetal calf serum, 1% nonessential amino acids, 1% sodium pyruvate, penicillin (100 U/mL) and streptomycin (100 µg/mL). *C. difficile* toxins $TcdB_{VPI}$ and $TcdB_{8864}$ that were used in this study were obtained from tgcBIOMICS (Mainz, Germany), and these toxins were produced by the $A^+B^+$ reference strain VPI 10463 and $A^-B^+$ strain 8864. The HT–29 cells were seeded in six well tissue culture plates at a density of $5.0 \times 10^5$ cells per well. After incubation with $TcdB_{VPI}$ and $TcdB_{8864}$ (50 ng/mL) for a particular time (i. e., 2, 8, 12 and 24 hours), both the floating and adherent cells removed by trypsinization were collected.

### Conventional transmission electron microscopy of HT–29 cells

The morphology of these cells and the changes occurring in their ultrastructure were analysed by conventional transmission electron microscopy (TEM).

In each time period (i. e., 2, 8, 12 and 24 hours), we investigated 40–50 cells in detail, which showed no significant differences in their ultrastructure. In this contribution, representative cells and their structures are shown (Figs. 1–4).

The cells were fixed in 2.45% glutaraldehyde and 2.45% paraformaldehyde in a 0.1 M sodium cacodylate buffer (pH 7.4) at room temperature for 3 hours and at 4°C overnight. The cells were then washed in a 0.1 M sodium cacodylate buffer at room temperature for 3 hours and postfixed in 2% $OsO_4$ at room temperature for 2 hours. The samples were washed in a 0.1 M sodium cacodylate buffer (pH 7.4) at room temperature for 3 hours, and then dehydrated in a graded ethanol series and embedded in TAAB epoxy resin (Agar Scientific Ltd.). For transmission electron mi-

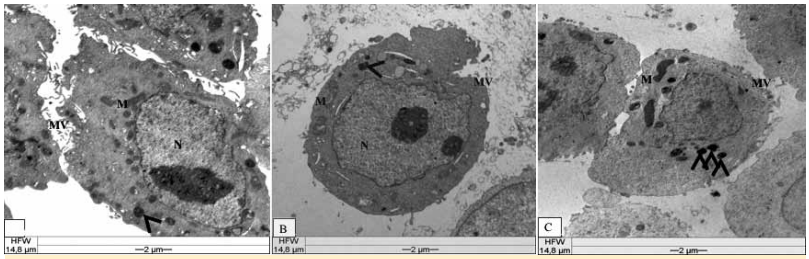


***Figure 1: Ultrastructure of HT-29 cells 2 hours after treatment with toxins.***

*A) $TcdB_{VPI}$; B) $TcdB_{8864}$; C) control micrograph. Toxin-treated cells (Figs. A, B) and non-treated cells (control; Fig. C) showed the same general morphology. Representative micrographs are shown here. The plasma membrane of all cells is differentiated into microvilli (MV). Cells show round or oval nuclei (N). Numerous well-conserved mitochondria (M) are seen. Occasionally, individual autophagic structures (arrows) are present in the cytoplasm of the cells.*

croscopy, ultrathin sections (70 nm) were obtained, and were transferred to copper grids. The ultrathin sections were stained with uranyl acetate and lead citrate, and were observed by a Zeiss EM 902 transmission electron microscope.

## RESULTS

In this study, we documented the evolution of morphological changes in HT–29 human colonic adenocarcinoma cells associated with induction by the toxins $TcdB_{VPI}$ and $TcdB_{8864}$ as a function of time. While representative control cells showed normal morphology without any ultrastructural changes, we found that toxins $TcdB_{VPI}$ and $TcdB_{8864}$ induce morphological changes with ultrastructural features of autophagy in HT–29 cells, using transmission electron microscopy.

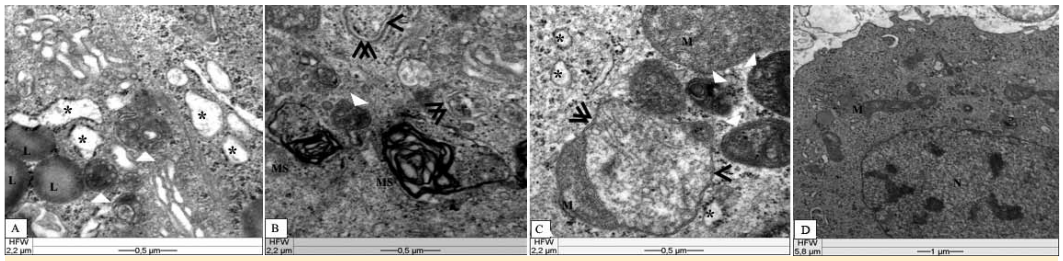


***Figure 2: Ultrastructure of HT-29 cells 8 hours after treatment with toxins.*** *Ultrastructural analysis of HT-29 cells after 8 hours of toxin-treatment indicates the presence of autophagic structures, altered mitochondrial morphology and dilated regions of granular endoplasmic reticulum (gER). Representative micrographs are shown here.*

*A) and B) $TcdB_{VPI}$; C) $TcdB_{8864}$; D) control micrograph.*

*Non-treated cells (Fig. D) showed the same general morphology as non-treated cells at 2 hours (Figs. 1). A) When we examined the toxin-treated HT-29 cells, we frequently noted an endoplasmic reticulum with dilated regions (asteriks) and some lipid droplets (L) in the cytoplasm. Additionally, electron-dense cytoplasmic autophagolysome-like inclusions (white arrowhead) can be observed. Fig. B shows one double-membraned (two arrows) autophagosome-like structure (arrow) and two multilayered, electron-dense structures (MS). Between these two structures, an autophagolysosome-like structure (white arrowhead) is present. A portion of cytoplasm containing heterogenous, electron-dense and electron-lucent compounds is enclosed by an autophagic membrane (two arrows) to form an autophagosome. C) We can see a double-membrane (two arrows) structure (arrow), containing a mitochondrium (M) and electron-dense cytoplasmatic autophagolysosome-like structures (white arrowheads). The granular endoplasmic reticulum shows dilated regions (asterisks). Mitochondria (M) with normal morphology can be observed only in the control micrograph (Fig. D), whereas changed mitochondria are seen in the cells treated with toxins (e. g. Fig. C). N, nucleus.*

After 2 hours of $TcdB_{VPI}$ and $TcdB_{8864}$ treatment, the plasma membrane of HT–29 cells was differentiated into many microvilli (Figs. 1A–B). These cells showed a round, oval or slightly indented nucleus, normally structured mitochondria as well as a granular endoplasmic reticulum (gER) and Golgi apparatus. In the cells treated for 8,

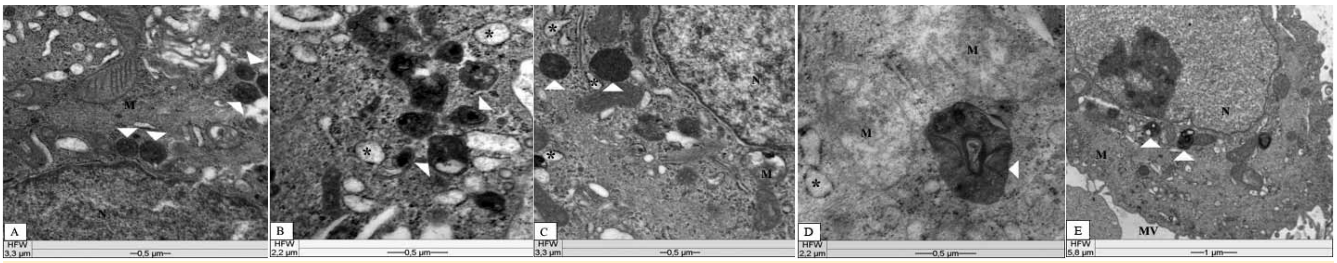


***Figure 3: Ultrastructure of HT-29 cells 12 hours after treatment with toxins.*** *Ultrastructural analysis of HT-29 cells after 12 hours of toxin-treatment reveals an accumulation of autophagic structures, mitochondria and gER with changed ultrastructure. A) and B) $TcdB_{VPI}$; C) and D) $TcdB_{8864}$; E) control micrograph. Representative micrographs are shown here.*

*Non-treated cells (Fig. E) showed the same general morphology as non-treated cells at 2 hours (Figs. 1). In the cells treated with toxins, numerous electron-dense autophagolysosome-like structures (white arrowheads) were seen (e. g. Figs. A, B, C, D). Cell shows dilated regions of gER (asterisks). In cells exposed to $TcdB_{VPI}$ and $TcdB_{8864}$ the mitochondria (M) appear enlarged (e. g. Fig. A), or they have an electron-lucent appearance with only a few visable cristae (e. g. Fig. D). MV, microvilli; N, nucleus.*

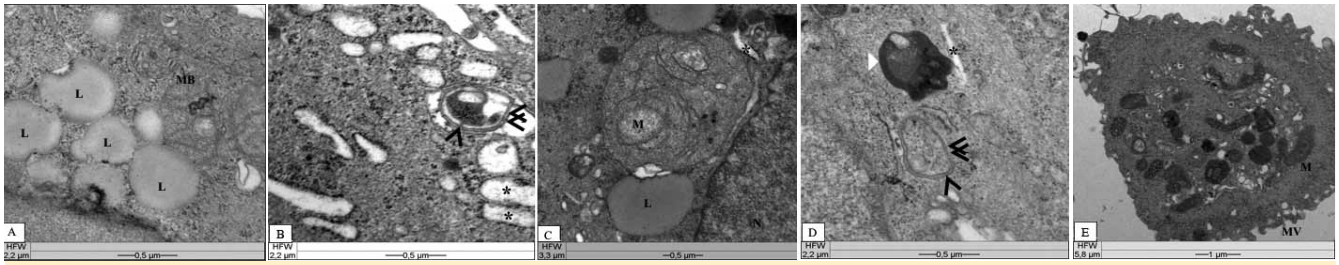


***Figure 4: Ultrastructure of HT-29 cells 24 hours after treatment with toxins.*** *A) and B) $TcdB_{VPI}$; C) and D) $TcdB_{8864}$; E) control micrograph. Representative micrographs are shown here.*

*A) The cytoplasm of HT-29 cells treated for 24 hours with toxin B contains multivesicular bodies (MB), and lipid droplets (L). In Fig. B, a double membrane, autophagosome-like structure (arrow) containing electron-dense material can be seen. The double membrane is indicated by two arrows. The cell shows mainly dilated gER (asterisks). C) Lipid droplets (L), and a mitochondrium showing signs of changed inner organization (M) are present in the cytoplasm. D) A double-membrane (two arrows), autophagosome-like structure (arrow) containig electron-lucent material and an electron-dense autophagolysosome-like structure (white arrowhead) are present in the cytoplasm of the cell. Non-treated cells (Fig. E) showed the same general morphology as non-treated cells at 2 hours. The plasma membrane is characterized by microvilli (MV); in the cytoplasm numerous mitochondria (M) were present. N, nucleus.*

12 and 24 hours with $TcdB_{VPI}$ and $TcdB_{8864}$, the loss of microvilli was observed. However, the cells contained autophagic structures, mitochondria with changed inner structure, and granular endoplasmic reticulum (gER) with dilated regions after 8 hours of toxin treatment (Figs. 2A–C).

The autophagic structures found in the cytoplasm of HT–29 cells were recognized according to their morphological characteristics. Autophagosomes were characterized by their double membranes and uncompacted cytoplasmic material (Figs. 2B, C). Autophagolysosomes are double– or single–membrane structures, containing electron–dense material, which can be densely compacted and amorphous (Figs. 2A, C), or multilamellar in appearance (Fig. 2B). In some cells, a portion of cytoplasm containing heterogenous electron–dense and electron–lucent material is enclosed by an autophagic double membrane to form

an autophagosome (Fig. 2B). Additionally, some double–membrane structures, presumably autophagosomes, contain mitochondria or their remains (Fig. 2C). In control cells incubated with only a sterile medium, cell compartments show normal morphology. For example, the plasma membrane of HT–29 cells is differentiated into microvilli; the nucleus of the cell is round or oval, and the mitochondria are well conserved (Fig. 2D).

When autophagy was stimulated by incubating HT–29 cells with $TcdB_{VPI}$ and $TcdB_{8864}$ for 12 hours, the mitochondria, gER and Golgi apparatus show changes in the ultrastructure. In the cells treated with toxins, numerous electron–lucent, autophagosome–like structures and electron–dense autophagolysosome–like structures (Figs. 3A–D) were observed. Non–treated cells (Fig. 3E) showed the same general morphology as non–treated cells at 2 hours (Fig. 1). Autophagic structures were rarely observed.

In the HT–29 cells treated for 24 hours with $TcdB_{VPI}$ and $TcdB_{8864}$, many double membrane, autophagosome–like structures (Fig. 4B, D) and electron–dense, autophagolysosome–like structures (Fig. 4D) were observed. The cytoplasm of HT–29 cells contained multivesicular bodies (Fig. 4A) and numerous lipid droplets (Figs. 4A, C). The granular ER showed many dilated regions (Fig. 4B). Almost all mitochondria seen in the cytoplasm of these cells displayed a changed inner organization (e. g., a loss of cristae or electron–lucent parts inside the inner part of the mitochondrium) (Fig. 4C). The ultrastructure of non–treated cells (Fig. 4E) appeared similar to non–treated cells at 2 hours. For example, the plasma membrane was characterized by microvilli, and numerous mitochondria were present in the cytoplasm.

Our findings suggest that the vacuoles induced by $TcdB_{VPI}$ and $TcdB_{8864}$ are likely related to the autophagic pathway despite the fact that toxin B is cytotoxic. No morphological heterogeneity was observed in control cells.

## DISCUSSION

The primary goal of this study was to gain insight into the potential impact of TcdB on the ultrastructure of HT–29 cells. Using transmission electron microscopy, we found that TcdB is capable of inducing cell rounding in less than 2 hours; however, cell death does not occur until almost 24 hours following treatment, as assayed by a variety of methods (15). Our study results indicated that cell rounding and cell death are temporally distinct events (11).

In the present work, we used purified $TcdB_{VPI}$ and $TcdB_{8864}$ from the bacteria *C. difficile*. The experimental evidence described here indicates that the autophagic process activated by both toxins proceeds to completion. For example, the toxins $TcdB_{VPI}$ and $TcdB_{8864}$ generated vacuoles that display features consistent with a degradative compartment. The present findings also revealed ultrastructural changes in the cell compartments (e. g., rough endoplasmic reticulum, mitochondria, and loss of microvilli). Additionally, the presence of autophagosome–like structures and autophagolysosome–like structures was observed, which is a characteristic process associated with autophagy. In contrast to apoptosis, other features were observed including the condensation of the cytoplasm and the preservation of the organelles, essentially without autophagic degradation (35).

Autophagy is thought to be an evolutionarily conserved and self–limiting survival strategy (36). While this pathway preserves cell viability in several cellular systems, this process has also been linked to cellular destruction (37). The results presented here provide insight supporting the role of autophagy as a cell survival mechanism for $TcdB_{VPI}$ and $TcdB_{8864}$. Despite lacking a causative relationship between these two events, increased autophagy activity has been associated with cell death. On the other hand, cell death can be accelerated when autophagy is suppressed, thereby, suggesting a pro–survival role for autophagy (38). The discovery of a relationship between autophagy and cell damage induced by $TcdB_{VPI}$ and $TcdB_{8864}$ can provide an important tool for investigating the role of autophagy as a cell survival response. Likewise, our findings suggest that autophagy could also represent a mechanism by which toxin B hinders apoptosis or necrosis, possibly by removing damaged organelles that could trigger cell death.

In this study, we found that $TcdB_{VPI}$ and $TcdB_{8864}$ appear to modulate autophagic activity after 8 hours, a

process that is required for the survival of the infected host cell. We also demonstrate that this autophagic response potentially overrides the effects of these toxins on HT–29 cells in order to prevent cell death. Therefore, these findings support the concept that different pathways are involved in the execution of programmed death in cells. It is worthwhile to note that the duration of exposure and toxin concentration of TcdB can elicit different responses depending on their magnitude. From our findings, we propose that $TcdB_{VPI}$ and $TcdB_{8864}$ function by at least two mechanisms to regulate the cell death of the host cell, including (1) the induction of apoptosis and (2) the inhibition of anti–apoptotic autophagy. These hypotheses are largely based on the autophagy–like structures we observed in cultured cells using transmission electron microscopy. Once the occurrence of autophagy has been detected by this method, these autophagic structures can be confirmed using additional tools (e. g., immunocytochemistry and immunoblotting). Future studies are necessary to further elucidate the mechanism(s) that underlie the effects of $TcdB_{VPI}$ and $TcdB_{8864}$ on HT–29 cells.

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