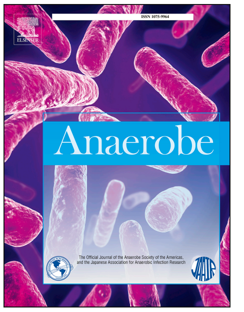

Effects of *Clostridium perfringens* iota toxin in the small intestine of mice

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Abstract

Iota toxin is a binary toxin solely produced by *Clostridium perfringens* type E strains, and is structurally related to CDT from *C. difficile* and CST from *C. spiroforme*. As type E causes hemorrhagic enteritis in cattle, it is usually assumed that associated diseases are mediated by iota toxin, although evidence in this regard has not been provided. In the present report, iota toxin intestinal effects were evaluated *in vivo* using a mouse model. Histological damage was observed in ileal loops treated with purified iota toxin after 4 h of incubation. Luminal iota toxin induced fluid accumulation in the small intestine in a dose dependent manner, as determined by the enteropooling and the intestinal loop assays. None of these changes were observed in the large intestine. These results suggest that *C. perfringens* iota toxin alters intestinal permeability, predominantly by inducing necrosis and degenerative changes in the mucosal epithelium of the small intestine, as well as changes in intestinal motility. The obtained results suggest a central role for iota toxin in the pathogenesis of *C. perfringens* type E hemorrhagic enteritis, and contribute to remark the importance of clostridial binary toxins in digestive diseases.

Keywords

Iota toxin, binary toxins, *Clostridium perfringens*, intestinal permeability, gastrointestinal transit.

1. Introduction

Clostridium perfringens type E infection in domestic animals was first reported in the late 1940s [1]. *C. perfringens* type E has been described to produce hemorrhagic enteritis in calves [2,3], cows [4], sheep [5] and goats [6]. Although these infections have generally been considered a rare occurrence in ruminants, there are numerous reports suggesting that type E isolates may account for approximately 5% of all *C. perfringens* isolates and that could be also associated with 50% of fatal hemorrhagic enteritis in calves [3,7]. In rabbits, like *C. spiroforme* infection [8], *C. perfringens* type E disease has been clinically characterized by general loss of condition, diarrhea and characteristic hemorrhagic lesions of the cecal serosa and mucosa, and eventually the distal ileum and proximal colon with presence of watery mucoid content [8]. As *C. perfringens* type E strains are defined by the production of iota toxin (ITX) [5], it is usually assumed that associated diseases are mediated by ITX, although no definitive evidence in this regard has been provided. ITX belongs to the family of binary actin ADP-ribosylating toxins [9]. Other members of this toxin family are *C. difficile* transferase (CDT), *C. spiroforme* transferase (CST), *C. botulinum* C2 toxin, and *Bacillus cereus/sphaericus* vegetative insecticidal proteins (VIP) [10]. These actin ADP-ribosylating binary toxins are composed of two unlinked proteins, an enzymatic component with ADP-ribosyltransferase activity and a binding component which binds to the cell surface receptor [11,12] and facilitates the enzymatic component entry to the cytosol [9].

Although cellular intoxication by ITX and binary toxins has been extensively studied [9,11–13], information about intestinal alterations induced by ITX are scant and usually limited to descriptions of natural cases of type E diseases [3,4,6]. Therefore, the current work aims to examine the intestinal effects of ITX and to define the role of ITX in *C. perfringens* type E infection, revealing new insights into *C. perfringens* type E enteritis pathogenesis. Also, the results from this study suggest that the mouse is a useful animal model to study type E and ITX pathogenesis *in vivo*.

2. Materials and Methods.

2.1. Animals:

Conventionally reared 20–25 g NHI Swiss outbred male mice were used. Animals were housed in a light cycle, humidity and temperature controlled room. Studies presented here were reviewed and approved by the institutional animal care and use committee (IACUC) from the CICVyA-INTA, protocol 32/2011.

2.2. Iota toxin:

Iota toxin (ITX) was purified from a type E culture as described by Stiles [14]. The purity of ITX was >95% as assessed by densitometry on 12% SDS/PAGE followed by Coomassie Blue staining. Activity and synergistic effect of Ia and Ib were tested on Caco-2 cell monolayers [15]. For *in vivo* assays, ITX concentration was expressed based on the activity on cell monolayers as ITX units (U), which are defined as the reciprocal of the highest dilution inducing cytopathic changes on cell monolayers. According to total protein concentration and the results of cell cytotoxicity tests, it was possible to determine that 200 U/ml of ITX corresponded to a concentration of 1 µg/ml of Ia and 2 µg/ml of Ib.

2.3. Lethality of ITX in mice:

The intragastric (i. g.) or intravenous (i. v.) lethality of ITX was determined using groups of 4 mice each. Animals received two-fold dilutions of ITX in 1% peptone water. Before i. g. challenge, mice were fasted overnight but allowed access to water until 2 h before the start of the experiment. Groups of mice were inoculated by i. g. gavage with 0.5 ml of 1.5% PBS $NaHCO_3$ containing purified ITX (50, 100, or 200 U/ml) or buffer solution without ITX. Another set of mice were i. v. injected with a total volume of 0.5 ml of PBS containing purified ITX (50, 100, or 200 U/ml) or buffer solution without ITX. All mice were observed for up to 72 h to monitor lethality, which was defined as death or development of significant respiratory or neurological signs. Mice showing significant respiratory or neurological signs were immediately euthanized and included in lethal dosed fifty (LD_{50}/ml) calculations [16].

2.4. Effects of ITX on mice intestinal loops.

2.4.1. Mice intestinal loop test: Mice were fasted during 18 h and deprived of water 2 h before the experiments. Anesthesia was then induced by intraperitoneal (i. p.) injection of 100 mg/kg of ketamine and 5 mg/kg of diazepam. The abdomen of each mouse was disinfected with povidone-iodine solution (Pervinox) immediately before surgery. A midline laparotomy was performed, and the ileum or colon was exposed. Only one 2 cm long intestinal loop was prepared in the ileum or the colon of each animal by a double ligation. Care was taken to avoid overdistension of bowel loops and interference with the blood supply, eliminating a possible ischemic component to the toxin-induced damage. During surgery, the serosal surface of the loops was kept wet by frequent soaking with normal saline solution. After injecting the inoculum, the abdominal incision was closed by separate muscle and skin sutures. The surgical procedure lasted approximately 3 min per animal. Mice were kept under anesthesia by periodic administration of ketamine-diazepam mix until the end of the experiments, 4 h after inoculation, and euthanized by cervical dislocation.

2.4.2. Inoculum: In all experiments, a 0.5 ml aliquot of a Ringer's solution containing specified amounts of purified ITX was injected into each intestinal loop. For the dose-response experiments a mixture containing Ringer's solution with 0, 100 or 200 U/ml of purified ITX was injected into each loop. . Additional loops received an injection of Ringer's solution containing 200 U/ml of purified ITX that had been pre-incubated for 30 min at room temperature with neutralizing mice anti-ITX polyclonal antibody or with anti-ITX IgY [17]. The amount of both anti-ITX antibodies (mice and egg yolk) used was the minimum amount that neutralize ITX cytopathic changes on Caco-2 cell monolayers. Control loops were injected with ITX pre-incubated with antibodies obtained from pre-immune sera of mice or laying hens.

2.4.3. Histological analyses: At the end of the experiments, intestinal loops were excised and fixed by immersion in 10 % neutral buffered formalin at pH 7.2 for a minimum of 48 h, after which they were dehydrated through graded alcohols to xylene and embedded in paraffin wax. Samples were cut to obtain 4 μm thick sections. Tissue sections were prepared and stained either with hematoxylin and eosin (H/E) or used for immunohistochemistry (IHC) and examined by optical microscopy.

2.4.4. ITX immunohistochemistry: Deparaffinized tissue sections were treated with 1% hydrogen peroxide in methanol to block endogenous peroxidases, followed by heat-induced antigen retrieval in 0.01 M citrate acid buffer (pH 6). After that, sections of intestines were overlaid sequentially with an egg yolk polyclonal anti-ITX antibody (1/100, vol/vol), and peroxidase labeled rabbit anti-egg yolk (1/100, vol/vol; Sigma Aldrich Co) for 1 h each. Antibodies were diluted in PBS. Control sections were treated using the same buffer but omitting the primary antibody. Finally, preparations were revealed with diaminobenzidine and hydrogen peroxide solution (DAB cod K3468; DAKO) and observed by optical microscopy.

2.5. Effects of ITX on intraluminal fluid accumulation:

2.5.1. Enteropooling: Mucosal transport of fluid was determined using the enteropooling assay that evaluates the net accumulation of fluid in the lumen of the small intestine [19]. After 18 h of fasting and 2 h of water deprivation, mice were treated as follows. Groups of 6 mice were dosed i. g. with 0.2 ml of 200 U/ml of ITX in 1.5% PBS $NaHCO_3$ or buffer solution without ITX. Mice in both groups were sacrificed 4 or 20 h later. The small intestine of all mice was clamped at the pyloric sphincter and immediately before the ileocaecal junction, and carefully removed from the abdomen. The small intestine length (L) was measured and then weighed (W1), dried of fluid and reweighed (W2). The difference between W1 and W2 divided by the length [(W1-W2)/L] represents the "enteropooling" in milligrams of fluid per centimeter of intestine, which is an indication of intestinal fluid accumulation [18].

2.5.2. Fluid accumulation in intestinal loops: Mice intestinal loops in ileum and colon were prepared as previously described (Section 2.4.1.). Purified ITX (200 and 100U/ml in Ringer's solution) or control (Ringer's solution without ITX) was injected into each loop. After inoculation, the incisions in the peritoneum, abdominal muscles and skin were closed. Mice were kept under anesthesia by periodic administration of ketamine-diazepam mix until the end of the experiments 4 h after inoculation, when they were killed by cervical dislocation. Intestinal loops were excised and the weight and length of each loop was measured. The net increase in the weight of the loop (in milligrams) was calculated as a relation between the weight of the inoculated loop and the length (in centimeters) of the loop [18].

2.6. Effects of ITX on gastrointestinal transit:

The animals were deprived of food for 18 h prior to gastrointestinal transit measurement but allowed water ad libitum until 2 h before the experiments. Groups of 6 mice were dosed i. g. with 0.2 ml of 200 U/ml of ITX in 1.5% PBS NaHCO3 or 0.2 ml of buffer solution without ITX. A charcoal meal (0.2 ml per mouse) containing a solution of 1.5% arabic gum and 5% charcoal as a marker was given i. g. to conscious mice in ITX treated and control groups. Thirty minutes later, the mice were euthanized by cervical dislocation. The abdominal cavity was opened and the gastrointestinal tract was removed. The traveled distance of the marker was measured and expressed as a percentage of the total length of the small intestine from the pylorus to caecum and this percentage was used as a measurement of gastrointestinal transit [19].

2.7. Statistical analysis:

A Bayesian approach was used [20]. For each of the measured variables, we tested the effect of treatment through stepwise model selection, from the simplest model to more complex ones using the information index Deviance Information Criterion (DIC) as an acceptance/reject rule for the proposed models, if a proposed model had lower DIC than the currently accepted model, it was accepted, otherwise it was rejected. The procedure was repeated until the DIC began to increase.

2.7.1. Proposed models for Enteropooling and Motility:

Step 1:

Null model: composed only of mean and error, in which the mean and error were assumed constant for all the individuals used and treatments applied, analogous to the null hypothesis in frequentist statistical approach. Two parameters.

Step 2:

Time Effect: The motility changed with time. Four parameters.

ITX Treatment Effect: The response variable changed with the addition of ITX. Three parameters.

Step 3 (proposed in case of acceptance of Step 2 models):

Time + ITX Effect: Additive effect of both treatments. Five parameters.

Step 4 (proposed in case of acceptance of Step 3 models):

Time + ITX Effect plus interaction term: Additive effect of both treatments plus a multiplicative interaction term. Six parameters.

2.7.2. Proposed models for loops:

Step 1:

Null Model: Similar to the previous one. Two parameters.

Step 2:

Treatment effect: The response variable changed with the addition of the treatment, independently of the concentration. Three parameters.

Treatment effect 2: The response variable changed with the addition of the treatment, and with different concentration. Four parameters.

Linear Treatment effect: The response variable changed as a linear function of the concentration of the treatment. Three parameters.

2.7.3. Fitting:

In the case of Motility the variable measured was a proportion, so the data was logit-transformed in order to use a normal-likelihood function to fit the models. For enteropooling and loops we used directly a normal likelihood function. Normal distribution with mean zero and ten standard deviation was used as an uninformative prior distribution for all the estimated parameters. A posteriori distribution of the parameters and the DIC index were calculated using the PyMC Markov-Chain Monte Carlo (MCMC) toolkit for the Python programming language [21]. Models were run for 200 000 iterations with a 100 000 iteration discarded as a burn-in period and the second 100 000 were used to calculate the posterior distribution of parameters. We evaluated model convergence using Geweke's method.

3. Results.

3.1. ITX induces lethality in mice:

Mice were i. g. or i. v. challenged with 200 U, 100 U or 50 U of ITX (1 µg/ml Ia + 2 µg/ml Ib, 0.5 µg/ml Ia + 1 µg/ml Ib, 0.25 µg/ml Ia + 0.5µg/ml Ib, respectively). The proposed model for ITX effect with lower DIC was the Null Model (DIC = 43.02), whereas the other proposed models in the stepwise procedure had higher DIC values (time effect DIC =

43.22, and ITX Treatment Effect DIC = 45.03). Therefore, ITX i. g. administration did not produce significant changes in survival times or signs of illness in any of the concentrations tested. Intravenous administration of 100 U and 50 U of ITX did not produce significant changes in survival times or signs of illness. When mice were inoculated i. v. with 200 U of ITX, onset of clinical manifestations (respiratory distress and depression) occurred within the first 6 h. In this group, the average time from inoculation to assay end point was 30 ± 10 h. The intravenous LD_{50} of the ITX was determined to be 1 μg/ml of Ia + 2 μg/ml of Ib per mouse.

3.2. Gross pathology and histological analysis:

ITX treated ileal loops showed grossly red mucosa with thick, mucoid and red content, all of which could be observed from the serosal surface. The intestinal wall appeared thin and lost natural tone. A relatively low fluid accumulation was observed and the intestinal fluid became progressively bloodier as the ITX doses increased. Histological examination revealed that a 4 h treatment with ITX caused clear damage to mucosal epithelium and the severity and extent of this ITX-induced damage showed a dose dependency in ileal loops. Treatment of loops with 200 U/ml of ITX resulted in a severe necrosis of the intestinal epithelium with blunting and fusion of intestinal villi, extensive pseudomembrane formation with polymorphonuclear infiltration in the lumen and mucosa (Fig.1). Morphologic changes in loops treated with 100 U/ml of ITX include mild shortening of the villi with degenerative changes in enterocytes on the tip and center of the villi. Mild polymorphonuclear infiltrate and edema was also observed in the lamina propria and submucosa (Fig.1). These changes were not observed in the control ileal loops (Fig.1) or in loops exposed to purified ITX pre-incubated with one of two different neutralizing anti-ITX antibodies, which totally abolished the ability of ITX to cause histological damage (data not shown). These results show that ITX was the active agent producing the intestinal damage described above. In contrast, no histologic damage was observed when the colon was treated with ITX (100 or 200 U/ml). After 4 h of treatment with ITX, the colon appeared similar to the control colon treated only with Ringer's solution. Together, these results indicated that purified ITX causes histologic damage in mouse small intestine but not in the colon, at least under the experimental conditions used in the present study.

3.3. Intestinal ITX binding:

ITX was injected into intestinal loops of mice and toxin binding was analyzed by indirect IHC. In ITX treated ileal loops, it was possible to observe extensive binding of toxin throughout the entire mucosa. Specific binding to mucosal epithelial cells was limited to the brush border; the cytoplasm and nuclei of labeled cells were unstained (Fig.2). Brush border binding was differentiable from any edge effect because individual cells exhibited intense staining while rare cells in the midst of positively stained cells were negative. Binding was observed at the tip, center and base of the villi. Crypt cells were also stained, but binding in the crypts was moderate compared with villi. No staining was detected in any ileum control loop (Fig.2) or colon loops, either treated or control.

3.4. Effects of ITX on intestinal fluid accumulation:

The effects of ITX in the fluid homeostasis of the intestine were initially determined by the enteropooling assay in mice. After i. g. challenge with 200 U/ml of purified ITX, no significant differences were observed when compared to the control group at 6 h (6.14 vs. 6.92 mg/cm) and 20 h (9.78 vs. 9.72 mg/cm). The effects of ITX on intestinal fluid accumulation were also evaluated in ligated ileal loops with 200 and 100 U/ml of purified ITX. Of the proposed models for loops, the lower DIC value was corresponding to Linear treatment effect (DIC = 52.73), whereas the other proposed models had higher DIC (Null Model, DIC = 53.54, Treatment Effect DIC = 54.12, Treatment Effect 2 DIC = 54.62). Therefore we found that increased fluid accumulation was observed in ITX treated loops, and it was linearly dependent on toxin concentration (Fig.3), with a slope of 0.016 +/- 0.01, and the intercept 0.77 +/- 0.516. Intestinal fluid accumulation was increased by 3 and 5-fold respect to control intestines after 4 h of treatment with 100 U and 200 U ITX, respectively.

3.5. Effect of ITX on gastrointestinal transit:

The charcoal meal method was used to test if i. g. delivered ITX inhibits intestinal motility. Administration of 200 U/ml of ITX had no effect on the luminal movement of the marker (charcoal), expressed as a percentage of the total length of the small intestine from the pylorus to caecum, when compared with the control group (data not shown).

4. Discussion

Iota toxin and infection by *C. perfringens* type E strains are associated with sudden death and hemorrhagic enteritis in different animal species [2–5]. Although ITX production is limited to type E strains, structurally related binary toxins are widely spread among other species of enterotoxic clostridia and *Bacillus* [22]. Based on sequence homology, immunological cross-reactivity and biologically active chimeras formation, clostridial binary toxins are classified in two families, the iota family and the C2 family [23]. Besides

ITX, the iota-family also includes *C. difficile* CDT and *C. spiroforme* CST [23]. These toxins not only share structure and sequence homology but might also share biological functions such as the observed strain specific increase of intestinal attachment of *C. perfringens* bacterial cells [17] in other clostridial species including *C. difficile* [24].

Although cellular intoxication by ITX and binary toxins has been extensively studied [9,11–13], there is scant information about intestinal alterations induced by binary toxins and their role in digestive diseases, usually limited to descriptions of natural cases [2–4,25]. In the present report, mice were used as animal model to study the effects of ITX in the gastrointestinal tract. According to previous studies, mice are sensitive to ITX [26] and provide several advantages over other species as model given its small size and wide availability [27–29]. The LD_{50} for i. v. ITX was in agreement with previous reports [14,30], which is a relatively high LD_{50} (above 1 µg/mouse) compared to other toxins that produce enterotoxemia (i.e. epsilon toxin, 3 ng/mouse), raising questions about the role of ITX in clinical signs and systemic changes described in natural cases of type E disease, usually defined as type E or iota enterotoxemia [3].

Several reports of disease associated with *C. perfringens* type E in cattle show that the dominant necropsy finding is hemorrhagic enteritis affecting jejunum and ileum [3,4], and severe necrosis of the mucosal epithelium as the main microscopic lesions of the small intestine. The histopathological findings of the present study are consistent with those described in natural cases of cattle type E enteritis [3,4]. Intraintestinal inoculation of purified ITX in ileal ligated loops of mice reproduced microscopic changes in a dose-dependent manner, which could be prevented using anti-ITX neutralizing antibodies.

The development of progressive microscopic lesions with increasing ITX concentrations provides new insights into the pathogenesis of type E diseases. First, treatment of ligated ileal loop with a low toxin concentration causes necrosis of enterocytes at the tip of the villi and degenerative changes in the enterocytes of the middle region of the villi. These conditions may represent those present at the onset of the disease, where the first cells to be affected seem to be enterocytes of the tip of the villus. With a higher concentration of toxin, it is possible to observe mucosa areas with completely detached epithelium, wich can also be considered as a progression of the action of lower concentrations of toxin. Also, coincident with reports of type E disease in ruminants, mice challenged with ITX did not develop alterations in the large intestine after intragastric and colonic loops challenge.

Although *C. perfringens* type E infection is generally associated with sudden death, some reports describe the occurrence of diarrhea in cattle [3,6]. Previous *in-vitro* and *in-vivo* studies suggest that ITX can have a key role in alterations of intestinal fluid homeostasis. *In-vitro* studies show that ITX can induce permeability changes in Caco-2 cells monolayers [15]. *In-vivo* studies with other clostridial binary toxins, like CDT from *C. difficile* [31,32] and BEC from *C. perfringens* [33,34], also described fluid accumulation in the intestinal lumen. In the present report, fluid imbalance was determined by two different approaches, enteropooling and intestinal loops. Although the enteropooling technique estimates the net movement of fluid through the intestinal wall [18], the differences influid accumulated in the small intestine after treatment with ITX were not statistically significant. . It was possible to observe a statistically significant increase in liquid accumulation in a dose dependent manner when ligated ileal loops were used. These results suggest an enterotoxic action of ITX and reinforce the concept that binary toxins could share biological effects. For example, as while *C. difficile* infection in most species is considered a large bowel disease, strains producing only CDT induce fluid accumulation in ileum as it was observed using different animal models like rabbit [32] or golden hamster [31].

Histopathology analysis of treated loops showed that ITX causes degenerative changes and necrosis of small intestinal enterocytes, suggesting that fluid accumulation could be associated with loss of intestinal epithelial barrier integrity. Apical and lateral region of the villi were the most affected areas, and a functional decrease of these enterocytes, which are primarily responsible for the absorption of water, would lead to a net increase in the amount of liquid present in the intestinal lumen. Higher concentrations of ITX induce epithelium detachment with the concomitant fluid passage from blood and lymph to intestinal lumen.

Since gastrointestinal motility disturbances is animportant mechanism involved in digestive disorders, ITX effect upon intestinal motility was measured by intestinal dye retention, a method widely used in different studies [19]. No differences were observed between ITX treated animals and controls, suggesting that ITX has no effect on gastrointestinal motility. However, further studies with different approaches would be necessary to confirm this result.

The present study shows that ITX produces intestinal damage consistent with lesions observed in natural cases of type E enteritis. Therefore, it is possible to propose a central role for ITX in *C. perfringens* type E pathogenesis and probably for other binary toxins in determined diseases like CDT in *C. difficile* enteritis and, eventually, colitis. According to our findings, changes in mucosal epithelium could be one of the main mechanisms involved in type E pathogenesis. The current study serves as a starting point to propose potential mechanisms involved in initial stages of *C. perfringens* type E pathogenesis.

Competing Interests:

The authors have declared that no competing interests exist.

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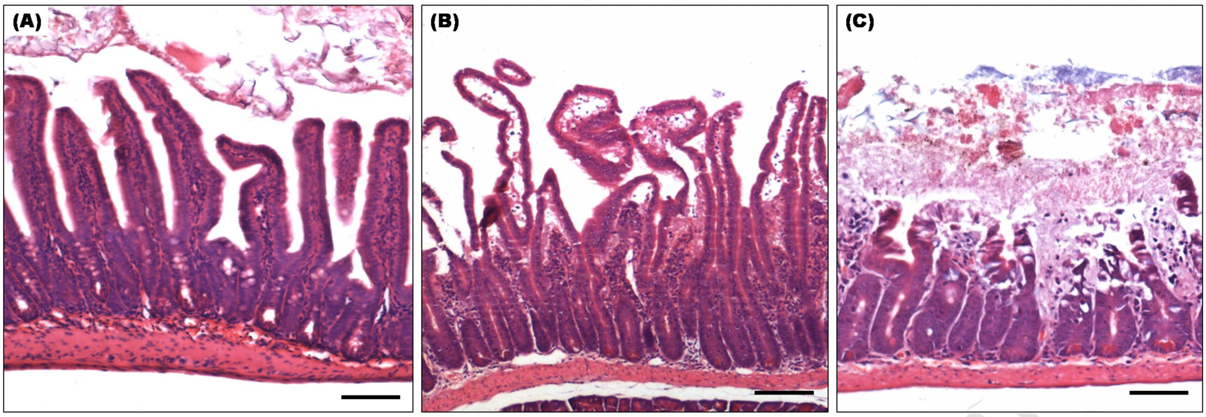
Figure legends

Fig 1: Histology of ITX treated small intestine loops. After 4 h of treatment, loop tissues were formalin fixed and embedded in paraffin. H/E was used to stain 4 um-thick sections of intestinal tissue. Control loops (A). Loops treated with 100 U/ml (B) and 200 U/ml (C) of purified ITX. Scale bar, 100 μm.

Fig 2: Immunohistochemistry of ITX treated small intestine. (A) Sections of intestinal tissue treated with control buffer. (B) Sections of intestinal tissue treated with 100 U/ml of purified ITX. In ITX treated loops the brush border is variably labeled with dense staining of individual cells adjacent to cells not stained (arrows). Scale bar, 20 μm.

Fig 3: ITX alters fluid homeostasis in the small intestine. Ligated ileal segments (loops) were excised 4 hours after injection of ITX and intestinal water was determined. Data shown are mean values obtained by using 4 mice (1 ileal loop/mouse). Error bars represent the SEM. Results are expressed as means ±SEM based on data from 4 loops for each ITX dose.


(A)
(B)
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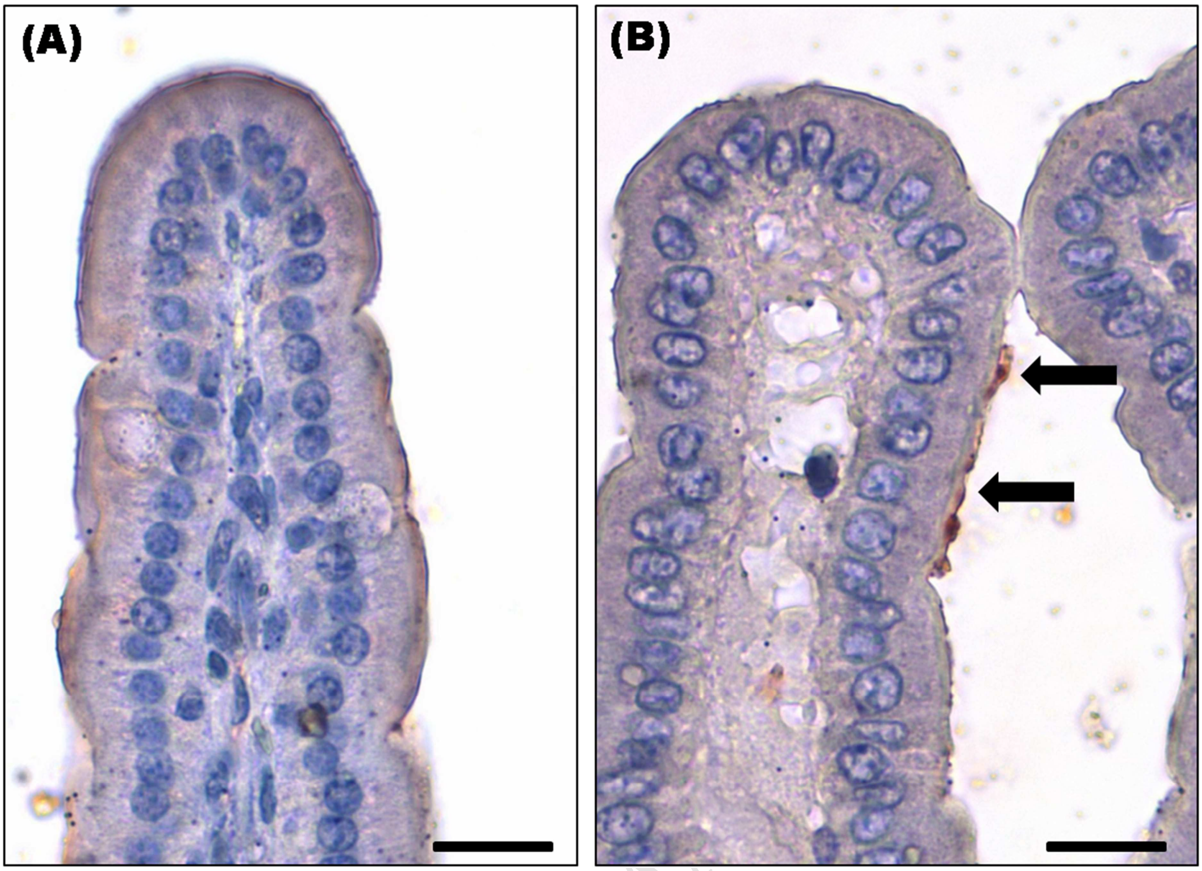
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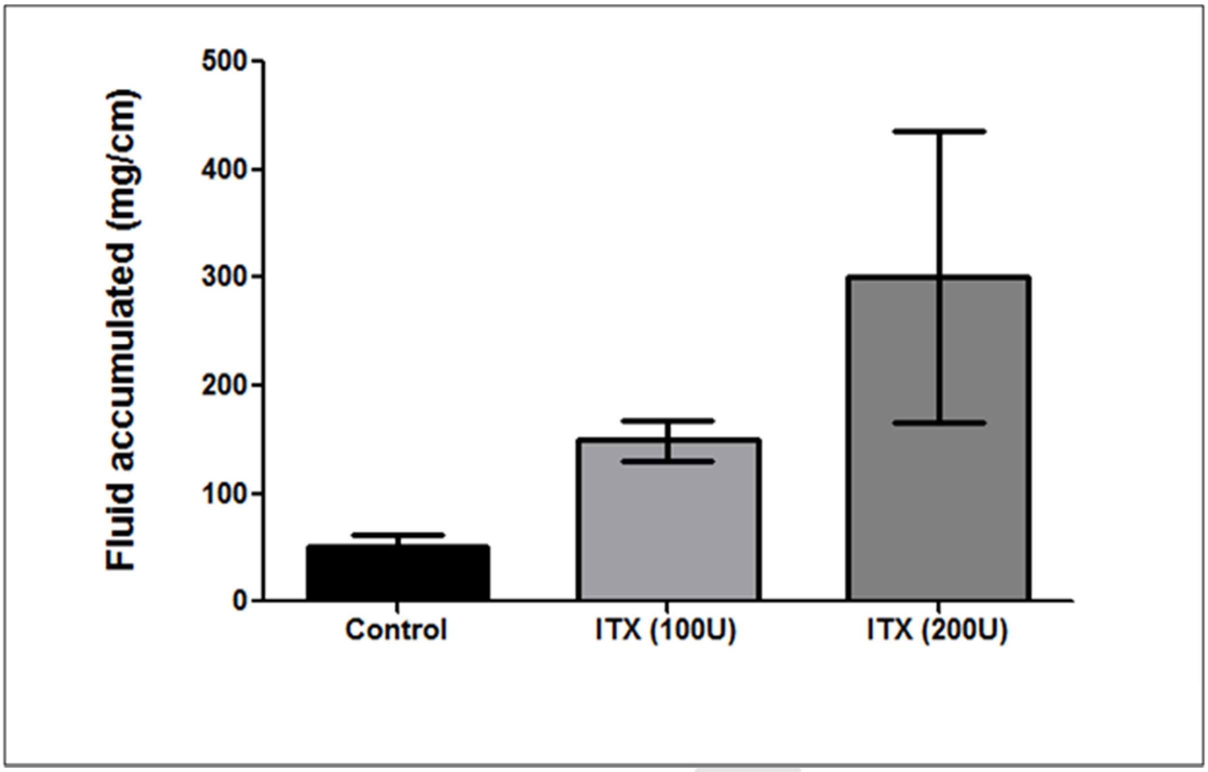
Fluid accumulated (mg/cm)
500
400
300
200
100
0
Control
ITX (100U)
ITX (200U)

Highlights:

1. Iota toxin intestinal effects were evaluated in a mouse model.
2. Iota toxin causes histological damage in mouse ileal loops.
3. Luminal iota toxin induced fluid accumulation in the small intestine.
4. Mice are sensitive to intravenously administered iota toxin.