**Clostridium perfringens** Epsilon Toxin Increases the Small Intestinal Permeability in Mice and Rats

Jorge Goldstein¹, Winston E. Morris², César Fabián Loidl³, Carla Tironi-Farinatti¹, Bruce A. McClane⁴, Francisco A. Uzal⁵, Mariano E. Fernandez Miyakawa²*

1 Laboratorio de Fisiopatogenia, Departamento de Fisiologia, Facultad de Medicina, Universidad de Buenos Aires, Buenos Aires, Argentina, 2 Instituto de Patobiología, Centro Nacional de Investigaciones Agropecuarias, Instituto Nacional de Tecnología Agropecuaria, Castelar, Buenos Aires, Argentina, 3 Instituto de Biología Celular y Neurociencia “Prof. E. De Robertis”, Facultad de Medicina, Universidad de Buenos Aires, Buenos Aires, Argentina, 4 Department of Molecular Genetics and Biochemistry, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania, United States of America, 5 California Animal Health and Food Safety Laboratory System, San Bernardino Branch, School of Veterinary Medicine, University of California Davis, San Bernardino, California, United States of America

**Abstract**

Epsilon toxin is a potent neurotoxin produced by *Clostridium perfringens* types B and D, an anaerobic bacterium that causes enterotoxaemia in ruminants. In the affected animal, it causes oedema of the lungs and brain by damaging the endothelial cells, inducing physiological and morphological changes. Although it is believed to compromise the intestinal barrier, thus entering the gut vasculature, little is known about the mechanism underlying this process. This study characterizes the effects of epsilon toxin on fluid transport and bioelectrical parameters in the small intestine of mice and rats. The enteropooling and the intestinal loop tests, together with the single-pass perfusion assay and *in vitro* analysis in Ussing’s chamber, were all used in combination with histological and ultrastructural analysis of mice and rat small intestine, challenged with or without *C. perfringens* epsilon toxin. Luminal epsilon toxin induced a time and concentration dependent intestinal fluid accumulation and fall of the transepithelial resistance. Although no evident histological changes were observed, opening of the mucosa tight junction in combination with apoptotic changes in the lamina propria were seen with transmission electron microscopy. This results indicate that *C. perfringens* epsilon toxin alters the intestinal permeability, predominantly by opening the mucosa tight junction, increasing its permeability to macromolecules, and inducing further degenerative changes in the lamina propria of the bowel.

**Introduction**

Epsilon toxin (ETX) produced by *Clostridium perfringens* types B and D is responsible for a highly fatal enterotoxaemia in livestock [1]. This toxin is secreted in the gut lumen as a prototoxin which then becomes fully active by the action of either the host’s intestinal trypsin or a *C. perfringens* metalloprotease [2]. Once fully activated, ETX is absorbed and spreads through the blood-stream, affecting the lungs, kidneys and the brain [3]. Sheep and goats suffering from *C. perfringens* type D enterotoxaemia can experience a disease ranging from a peracute form, with neurological signs and sudden death, to a chronic intestinal disease, including hemorrhagic diarrhea and colitis [1].

The disease associated with *C. perfringens* type D in young lambs is very brief, often less than 2 hours, with many lambs being found dead without premonitory signs, or dying after a few minutes of violent convulsive activity [4]. In lambs and goats inoculated intraduodenally with *C. perfringens* type D culture supernatant containing ETX, nervous signs or death were observed as soon as 30 minutes after inoculation [5]. Similarly, in mice, lethal effects were observed 2 hours after oral administration of ETX [6]. Although this evidence indicates that ETX is promptly absorbed from the gut lumen into the blood circulation, the mechanism involving this process is yet unknown.

Experimentally, necrosis of the colonic epithelium is observed in ETX treated tissues of sheep and goats [7]. This morphological damage can alter the function of the epithelial barrier, allowing toxin absorption through the large intestine. However, evident epithelial damage in the large intestine is rarely seen in natural cases of acute and peracute enterotoxaemia of sheep, thus suggesting that other segments of the gastrointestinal tract are involved in ETX absorption [1]. In fact, Losada-Eaton et al. [8] showed that ETX can be absorbed from both, the large and small intestines of experimentally inoculated mice.

Bullen and Batty [9] reported that filtrates containing ETX, increased immunoglobulin absorption in the intestine of mice and sheep and Fernandez-Miyakawa et al. [10] observed that pefringolysin-O, a 54 kDa thiol-activated hemolysin from *C. perfringens*, was only absorbed from the intestinal tract of mice when ETX was present, but in the absence of histological damage to the intestine. These results suggest that ETX affects the small intestinal permeability by a mechanism independent on histological damage.

ETX has the ability to induce water accumulation in the small intestine of sheep and goats [7]. This fluid imbalance may explain the diarrhea or watery intestinal contents observed in *C. perfringens* type D enterotoxaemia, particularly if the animals survive longer than a few hours [4,11]. However, the physio-pathological mechanisms of fluid accumulation in small intestines of ETX-challenged mice and rats are currently unknown.
imbalance induced by ETX in the small intestine are unknown [12]. An augmented paracellular permeability of the small intestine could be responsible not only for the toxin absorption; it could be responsible, at least in part, for the fluid accumulation observed in the small intestine of sheep and goats. However, the available data to support this hypothesis is not only scanty but indirect.

In an attempt to address these issues, the aims of the present study were as follows: (i) to assess whether ETX can induce changes in the fluid transport of the mouse small intestine (ii) to investigate the effects of ETX on the electrophysiological parameters of the small intestine in vitro and ex vivo, and (iii) to determine whether ETX-induced intestinal changes alters epithelial permeability.

Results

Effects of ETX on intestinal fluid

Intestinal fluid accumulation in the mouse small intestine was initially determined by the enteropooling assay. Figure 1A shows an experiment carried out to confirm the ability of the enteropooling assay to evaluate enterotoxicity with *C. botulinum* C2 toxin, used as a validated intestinal control model. The results of this experiment show that *C. botulinum* C2 toxin, which had been previously described to have enterotoxic effects in the intestine of mice, produced fluid accumulation dependent of the toxin concentration 6 hours after the toxin was orally administered.
The effects of ETX in the intestine of mice were then evaluated following the same experimental protocol. Figure 1B shows that intestinal fluid values was about 3 times greater than control 6 h after toxin delivery (Fig. 2, P<0.05). This increased fluid accumulation was observed as soon as two hour after toxin ingestion (Fig. 1B, P<0.05) and this effect was dependent of toxin concentration (Table 1). These ETX effects in the fluid transport were confirmed when 1,000 lethal dose fifty (LD50)/ml were incubated in ligated ileal loops during 3 h (Fig. 1C, P<0.05). Also, the water transport was affected in jejunum when the intestinal segments were incubated with 500 LD50/ml and changes were measured by the single-pass perfusion technique (Fig. 1D). Changes became statistically significant after 45 minutes of toxin delivery (P<0.05).

The effects of systemic ETX in the enteropooling assay were further studied. ETX was administered intravenously in mice and enteropooling was evaluated 3 hours later. A reduction in fluid accumulation dependent of the toxin concentration was observed (Table 2). Although the small intestine length was reduced when ETX was administered orally, this effect was not observed when this toxin was injected systemically (Table 1 and 2).

In vitro effects of ETX on the electrical parameters of mouse ileum

Ileal segments of mice mounted in Ussing-type chambers were incubated with ETX. The basal electrical parameters were not altered when concentrations up to 8,000 LD50/mL were incubated in vitro during 60 minutes in the mucosal side of the intestinal tissues (Fig. 2A and 2B). However, when 30 LD50 of ETX were incubated in the serosal side, a statistically significant decrease of the transepithelial resistance (Fig. 2C) was observed after 40 minutes of incubation (P<0.05).

Addition of glucose at the end of the experiments showed equivalent responses in treated and untreated tissues, indicating that glucose-Na+ active cotransport function was unaffected.

Ex vivo effects of ETX on the electrical parameters of mouse ileum

Segments of ileal loops treated during 3 h with different concentrations of ETX and then mounted ex-vivo in a modified Ussing chamber showed alterations of PD, Isc and Rt. A slight increase of Isc was observed when 1,000 LD50 or 2,000 LD50 were incubated in ileal loops; those changes became statistically significant when concentrations of 4,000 LD50 or 8,000 LD50 (Fig. 3A, P<0.05). A drop of Rt was evident when 2,000 LD50 or higher toxin concentrations were used (Fig. 3B, P<0.05). Glucose was added to the mucosal side after the electrical parameters were stabilized. An increase of glucose induced-Isc was observed at higher ETX doses (Fig. 3C). Theophylline stimulated the Isc when it was applied in the serosal side (Fig. 3D).

The same ex-vivo protocol was used with ileal loops incubated with 1,000 LD50 of ETX during different periods of time. An increase of Isc was observed in loops incubated with ETX at 120 or 240 minutes (Fig. 3E, P<0.05). The Rt was significantly reduced after 120 and 240 minutes of toxin incubation but changes were not observed with lower incubation times (Fig. 3F, P<0.05). An increase of glucose induced-Isc was also observed at 240 minutes when compared to control tissues (ΔIsc 30±10 vs 60±10 μA/m², P<0.05). ETX did not produce any significant alteration of the electrical parameter in the ileum 60 minutes after 4 LD50 were injected intravenously.

Dilution potential

These so called dilution potentials are indicators for the ion selectivity of the epithelium and changes in these transepithelial potential differences reflect changes in ion selectivity of the paracellular pathway if the ion permeability of the cell membranes remains unaltered. In order to have information about the permeability of the paracellular pathway we measured the dilution potential generated when one half of the NaCl in the serosal solution was substituted with an equimolar amount of mannitol. This flux produced an increase of the dilution potential difference in luminal ETX treated ileum (4,000 LD50), indicating that the

Figure 2. In vitro characterization of the effects of C. perfringens epsilon toxin in the electrical parameters of the murine small intestine. Epsilon toxin was incubated in the mucosal side of ileal sheets mounted in modified Ussing chambers. (A) Short circuit current (Isc) and (B) resistance (Rt) parameters were recorded each 10 minutes in tissues from 5 mice. (C) Rt values of ileal sheets incubated in the serosal side with 8,000 LD50/ml of epsilon toxin. Each bar represents results for 4 mice. Results are expressed as means±SEM. doi:10.1371/journal.pone.0007065.g002
The magnitudes of dilution potential were 4.7 and 5.8.

The permeability of the cation being greater than anion permeability. 

ETX during 3 h and then mounted ex-vivo in a modified Ussing-type chambers were unaffected by concentrations of toxin up to 8,000 LD_{50}/mL incubated in the mucosal side for at least 60 minutes. Segments of ileal loops treated with 4,000 LD_{50} of luminal ETX during 3 h and then mounted ex-vivo in a modified Ussing chamber showed alterations of $I_m$ when compared to control loops (43.5±5.3 mV vs 56.2±5.6) and $R_t$ (67.1±2.6 vs 54.5±5.5).

### Histological analysis

Although no major histological changes were observed in either control or epsilon treated loops, in some loops (control and ETX), mild polymorphonuclear infiltrate was observed in the submucosa and lamina propria, together with mild shortening of the villi.

Mild oedema was observed in the lamina propria of some ETX treated loops together with some degenerative cells, exhibiting pycnotic nuclei. These changes were not observed in the control tissues.

### Mucosal binding of ETX in the small intestine

ETX was incubated in ileal loops of mice for 20 minutes and toxin bound was analyzed by indirect immunofluorescence. ETX bound to the mucosal epithelium of small intestinal samples (Fig. 4). No fluorescence was detected in any of the control tissues analyzed. Binding was observed at the tip, center and base of the villi. However, the fluorescence intensity in the ETX small intestine treated samples was higher on the villi than in the crypts.

### Passage of molecules through the wall of the gastrointestinal tract

In the luminal side of the mucosa, HRP could be seen as strong electron dense deposits. In control mice given HRP intraluminally, HRP penetrated from the lumen only as far as the zonula occludens suggesting that the intestinal barrier function was well maintained (Fig. 5A). Considerable amounts of HRP were seen between intestinal epithelial cells only in mice treated with ETX, filtering through the tight junction, from the lumen of the bowel, towards the lamina propria (Fig. 5B).

The extravasation of Evans blue, which binds with plasma proteins upon intravenous injection, was examined to see whether the fluid accumulation and the changes in the intestinal permeability in vivo is accompanied with the leakage of plasma protein into the intestinal lumen. Figure 5C shows the augmented extravasation of Evans blue 3 h after injection of ETX into ligated intestinal loops of mice ($P<0.05$).

### Electron microscopy analysis of small intestinal samples

No significant changes were seen in the segments analyzed of the control loops (Figs. 6A, 7A). In ETX treated loops of rats and mice, the predominant changes observed were oedema in the lamina propria (Figs. 6C-E, 7B-C), shrunken cells (Figs. 6C, 7B, D) and cellular debris (Figs. 7B, 6C). Degenerative fibroblasts (Fig. 6E) and other apoptotic-like cells were frequently seen (Figs. 7D, E), exhibiting condensed fragmented nucleus and condensed cytoplasm, sometimes containing degenerated organelles (Fig. 7E). Endothelial cells surrounding apoptotic vascular cells were frequent (Fig. 6G), and eosinophils (Fig. 7B), plasma cells and mast cells (Figs. 6F, 7F) were also seen. Some endothelial cells exhibited degenerative changes, such as irregular nuclear shape and the blood vessels were often surrounded by oedema and altered erythrocytes (Fig. 7C).

### Discussion

This study aimed to clarify the physiopathology of fluid accumulation and toxin absorption in the rodent small intestine exposed to ETX. Previous studies in ruminants (species naturally affected by ETX) contributed partially to explain the

<table>
<thead>
<tr>
<th>Epsilon toxin IV Dose (LD_{50})</th>
<th>Fluid accumulated (mg/cm)</th>
<th>Dried intestine weight (mg)</th>
<th>Intestinal length (cm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>28.4±1.6</td>
<td>272±10</td>
<td>36.13±0.89</td>
</tr>
<tr>
<td>0.5</td>
<td>32.7±0.4</td>
<td>276±5</td>
<td>32.38±1.66</td>
</tr>
<tr>
<td>500</td>
<td>36.5±2.9</td>
<td>279±8</td>
<td>31.11±0.68</td>
</tr>
<tr>
<td>2000</td>
<td>38.1±0.7</td>
<td>266±25</td>
<td>29.21±0.74</td>
</tr>
</tbody>
</table>

Six mice per treatment were injected intravenously with ETX diluted in peptone water. Mice were monitored during 1 hour and euthanized at the end of the experiments. Enteroooling was assessed after reomtion of the small intestine and determination of wet and dried weight. Final values were relativized to the intestinal length and expressed as mg/cm.

doi:10.1371/journal.pone.0007065.t002

---

**Table 1.** Enteropooling of mice inoculated intragastrically with different ETX concentrations.

<table>
<thead>
<tr>
<th>Epsilon toxin Dose (LD_{50})</th>
<th>Fluid accumulated (mg/cm)</th>
<th>Dried intestine weight (mg)</th>
<th>Intestinal length (cm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>28.4±1.6</td>
<td>272±10</td>
<td>36.13±0.89</td>
</tr>
<tr>
<td>125</td>
<td>32.7±0.4</td>
<td>276±5</td>
<td>32.38±1.66</td>
</tr>
<tr>
<td>500</td>
<td>36.5±2.9</td>
<td>279±8</td>
<td>31.11±0.68</td>
</tr>
<tr>
<td>2000</td>
<td>38.1±0.7</td>
<td>266±25</td>
<td>29.21±0.74</td>
</tr>
</tbody>
</table>

Six mice per treatment were inoculated intragastrically with ETX diluted in 1.5% PBS NaHCO$_3$ solution. Mice were monitored during 3 hours and euthanized at the end of the experiments. Enteropooling was assessed after removal of the small intestine and determination of wet and dried weight. Final values were relativized to the intestinal length and expressed as mg/cm.

doi:10.1371/journal.pone.0007065.t001

---

**Table 2.** Effect of ETX injected intravenously in mice in the enteroooling values.

<table>
<thead>
<tr>
<th>Epsilon toxin IV Dose (LD_{50})</th>
<th>Fluid accumulated (mg/cm)</th>
<th>Dried intestine weight (mg)</th>
<th>Intestinal length (cm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>28.4±1.6</td>
<td>272±10</td>
<td>36.13±0.89</td>
</tr>
<tr>
<td>0.5</td>
<td>26.4±2.3</td>
<td>273±60</td>
<td>31.33±1.57</td>
</tr>
<tr>
<td>4</td>
<td>22.2±0.8</td>
<td>268±32</td>
<td>35.58±1.30</td>
</tr>
</tbody>
</table>

Six mice per treatment were injected intravenously with ETX diluted in peptone water. Mice were monitored during 1 hour and euthanized at the end of the experiments. Enteroooling was assessed after reomtion of the small intestine and determination of wet and dried weight. Final values were relativized to the intestinal length and expressed as mg/cm.

doi:10.1371/journal.pone.0007065.t002
changes produced by ETX in the intestinal tract [7,12]. However, since the impracticality to use those species in determined experimental conditions, rodents were considered as potential models since the brain lesions in sheep and mice exposed to ETX are comparable in many respects and the intravenous and intraperitoneal murine models have frequently been used to study the pathogenesis of the disease [13,14,15,16,17,18,19,20]. Although to the best of our knowledge there is no information about natural infection of mice with C. perfringens type D, a recently developed oral challenge

---

**Figure 3.** *Ex vivo* characterization of the effects of *C. perfringens* epsilon toxin in the electrical parameters of the murine small intestine. Intestinal loops were performed in groups of 4–6 anesthetized mice and injected with different concentrations of epsilon toxin in Ringer solution. In a particular set of experiments, intestines were removed after 3 hours of toxin injection and samples were mounted in Ussing chamber to measure changes in (A) short circuit current (Isc) and (B) resistance (Rt). Those tissues were exposed to (C) luminal glucose or (D) serosal theophylline and the Isc changes were recorded. In another set of experiments, ileal loops were injected with 1,000 LD50 of epsilon toxin and incubated during different periods of time. Values of (E) Isc or (F) Rt were recorded for those intestinal samples. Data are expressed as mean±SEM.

doi:10.1371/journal.pone.0007065.g003
mouse model for studying *C. perfringens* type D infection reproduces some of the clinical findings described in sheep [6], suggesting that ETX can cross the intestinal barrier. Indeed, it has been demonstrated that purified ETX can be readily absorbed from the murine small intestine into the systemic circulation [8].

The small intestine of mice incubated with ETX accumulated fluid in a concentration-dependent manner when the enteropooling assay was used. The fluid imbalance was corroborated by ileal loops and intact single-perfusion experiments. The finding suggests that ETX alters the water homeostasis of the murine small intestine, despite the absence of significant morphological change or measurable *in vitro* bioelectrical alterations, similarly as it was reported previously for ruminants [7,12]. All of these data support the use of mice instead sheep or goats to analyse the effects of ETX by *in vivo* incubation and posterior electrophysiological *in vitro* evaluation.

The first important finding of the present study is that ETX increases the small intestinal transepithelial permeability, evidenced as reduction in the electrical resistance, in both mice and rats. Transepithelial electrical resistance can be described as resistance to the passive flow of ions as sodium and chloride. The two major pathways for such flow are through the apical and basolateral membranes in series (transcellular) and through the paracellular space. In epithelia of relatively low resistance, such as the small intestine, most passive ion flow presumably occurs through the paracellular space. This electrical resistance reduction was not due to cell damage since the epithelial cells were morphologically intact, and glucose-Na⁺ active co-transport (a mechanism of transport that occurs only when the tissue is viable) was responsive. Thus, activity of ETX resulting in decreased

---

**Figure 4. Mucosal ETX binding.** Immunofluorescent detection of ETX (A) was negative in control tissues treated only with the vehicle solution. (B) ETX (2,000 LD₅₀/ml) treated small intestinal segments gave a clear signal in the tips of the villi and (C) lower in the crypts.
doi:10.1371/journal.pone.0007065.g004

**Figure 5. Epsilon toxin incubated in the small intestine allowed the passage of macromolecules through the tight junction.** (A) HRP infiltration in the small intestine was not observed beyond the tight junction in control loops of rats and mice. (B and C) in the luminal zone of the mucosa of ETX treated loops, HRP could be seen as strong electron dense deposits, filtering through the tight junction, from the lumen of the bowel, towards the chorion (arrows). (D) Extravasation of Evans blue bound to plasma protein was higher in ETX treated animals than control mice.
doi:10.1371/journal.pone.0007065.g005
transepithelial resistance is likely to do so by decreasing the resistance of the paracellular pathway. As a consequence of alteration of the paracellular pathway, the intestinal mucosa becomes more permeable, and water and electrolytes (under the force of hydrostatic pressure) leak into the lumen [21]. This change in transepithelial permeability could be an important source for fluid imbalance observed in the small intestine of mice, sheep and goats. The hypothesis is that changes in water transport may arise from decreased absorption produced by increased transepithelial permeability. The augmented small intestinal permeability might be a rather slower process for fluid accumulation than intestinal secretion and could explain the fact that diarrhea or watery intestinal contents are observed during enterotoxaemia particularly if the animals survive longer than a few hours [4].

The paracellular pathway can be split into two components that may influence resistance: the tight junction and the remainder of the paracellular space. The intercellular tight junction is the rate-limiting barrier in the paracellular pathway for permeation by ions and larger solutes [22,23]. As the rate-limiting step for paracellular transit, permeability of the tight junction defines the overall barrier function of an intact intestinal epithelium and the tight junctions have a larger conductance for cations [24]. The so-called dilution potentials are indicators for the ion selectivity of the epithelium and changes in these transepithelial potential differences reflect changes in ion selectivity of the paracellular pathway if the ion permeability of the cell membranes remains unaltered. Alteration of the dilution potential by ETX can be interpreted as ETX decreased the cation selectivity of the tight junctions.

Figure 6. Transmission electron microscopy of control and epsilon toxin treated small intestinal loops displaying predominantly edematous changes. In the control loop (A), a blood-vessel exhibiting normal looking endothelial cells and well preserved fibroblast (scale bar = 2.5 μm). (B) In epsilon treated loops, endothelial cell displaying early degenerative changes, such as irregular nuclear shape are seen adjacent to apoptotic cells from the lamina propria (scale bar = 2 μm). (C) Platelet aggregation is seen in one vessel, in an area with evident perivascular edema and irregular-shaped cells (scale bar = 3 μm). (D) a erythrocyte in a vessel is observed surrounded by perivascular edema (scale bar = 7 μm) and (E) degenerated fibroblast with scattered collagen fibers in the edematous lamina propria are also seen (scale bar = 7 μm). (F) Plasma cell in the proximity of an apoptotic-like cell (scale bar = 4 μm) (G) and an endothelial cell surrounding a degenerative a degenerative cell.
doi:10.1371/journal.pone.0007065.g006

Usually, the intestinal barrier is impermeable to microorganisms, particulate antigens and macromolecules as ETX [25]. However, oral administration of ETX produced an increased passage of immunoglobulins from the intestinal lumen to blood in mice and sheep [9] and increased lethal absorption of perfringolysin-O (protein monomer 52 kDa) orally administered to mice [10]. Also, in the present report, the instillation of ETX into ileal loops resulted in a significant increase in EB recovery when compared with vehicle-challenged loops, suggesting that the permeability of the epithelium was affected. Concordant with the electrophysiology results, it is likely that augmented EB
recovery was, at least in part, the result of an increased intestinal epithelial permeability produced by ETX.

The ultrastructural analysis of epsilon treated loops demonstrated two areas of the ileum which were mostly affected: the lamina propria and the mucosa. In the lamina propria, oedema and apoptotic cell-death were the most relevant changes observed. In the mucosa, on the other hand, subtle opening of the tight junction, evidenced by the intrusion of the HRP through the junction, and oedema, evidenced as electronlucent gaps in the basal area, were the only changes noticed. It is likely that epsilon toxin is capable of opening the tight junction, increasing the mucosa permeability to macromolecules. This could enable the toxin to filter through the mucosa into the lamina propria, inducing other degenerative and inflammatory changes there. The toxin appears to alter the vascular permeability as well, thus enabling the toxin to reach the blood stream, causing other systemic alterations.

This study also showed that the basal short-circuit current of toxin-incubated ileal mucosa ex vivo was increased significantly when compared with control tissues. Although the source of this ETX-induced increase in baseline $I_{sc}$ is not clear, it might contribute to watery intestinal contents in animals suffering the disease. Also, the addition of luminal glucose or serosal theophylline revealed significantly higher $I_{sc}$ stimulations in epithelia from animals pretreated with ETX in comparison with the control situation. At present it can only be speculated whether the differentiation caused by ETX could account for the findings i.e. changes in the cytosolic cAMP and/or cGMP concentrations; a higher number of cells that express enhanced absorptive/secretory function; an increased density of transporters located in the apical membrane or by a faster turnover rate of each transporter; decreased receptor density or enterocyte membrane fluidity modification.

Immunofluorescence showed a marked binding of ETX to enterocytes, but it remains unknown if in vivo intestinal activity of ETX is a direct or an indirect effect of the toxin on epithelial cells. A few active molecules of ETX overcoming the epithelial barrier might stimulate mucosal or submucosal components which, in turn, modulate normal enterocyte function. Correspondingly, serosal application of ETX in the small intestine produced a drop of transepithelial resistance. This in vitro decline of electrical resistance was polar, occurring when the toxin was applied in the basolateral rather than the apical surface of the small intestine. This polarity dependency of ETX induced biological effects may be due to either, easier access of this toxin to target molecule(s) localized on the basolateral side of the small intestine, and/or interaction with a subsequent epithelial factor. Release of mediators from these cells may conceivably affect the paracrine

Figure 7. Transmission electron microscopy of control and epsilon toxin treated small intestinal loops displaying predominantly apoptotic-like changes. In control loops (A), enterocytes with normal looking nucleus and organelles are seen (scale bar = 15 μm). In epsilon treated loops (B) fibroblast with fragmented nucleus and edema with a polymorphonuclear cell (eosinophils) is seen; note as well, the electron lucent gaps between the epithelial cells (scale bar = 10 μm). (C) Abnormal looking red-blood cells together with degenerating cells, some displaying organelle and nuclear fragmentation (scale bar = 4 μm, or (D) cytoplasmatic (see in the inlet an apoptotic cell surrounded by another cells in process of nuclear fragmentation, probably at an early apoptosis stage) and (E) nuclear condensation (scale bar = 10 and 4 μm). (F) Lymphocyte in contact with a mast-cell (upper-left corner) together with some degenerating fibroblasts (scale bar = 4 μm).

doi:10.1371/journal.pone.0007065.g007
Materials and Methods

Animals

Conventionally reared, 20–25 g BALB-c mice and 150–200 g Wistar rats of either sex were used. The study was approved by the Animal Care and Use Committee of the University of Buenos Aires.

Toxin

Purified epsilon prototoxin was prepared from an overnight culture of *C. perfringens* type D (strain NCTC 8346) in Trypticase-yeast-glucose medium, under anaerobic conditions at 37°C. The overnight cultures were centrifuged at 10,000 rpm for 30 min at 4°C, and the supernatant containing ETX was saved for toxin purification. The toxin was then precipitated by ammonium sulfate dialyzed against phosphate buffer solution pH 7.4 (PBS). It is referred as crude fraction. Two columns were prepared with DEA E and CM Sepharose (Pharmacia, Sweden), respectively, equilibrated in 10 mM Tris, pH 7.5. The toxin was applied to the DEA E column, and the effluent was monitored at 220 nm. The initially eluted peak was saved and applied to the CM column. Again the eluent was monitored at 220 nm, and the first peak was collected, dialyzed against PBS, and freeze-dried. Epsilon prototoxin purity was checked by SDS-PAGE. Prior to its use in these experiments, the prototoxin was reconstituted and activated by incubation at 37°C during 30 min with 0.1% trypsin (Sigma). Semi-purified *Clostridium botulinum* C2 toxin was a gift of Dr. Patricia Geoghegan.

Enteropooling or intraluminal accumulation of fluid

Mucosal transport of fluid was determined using the enteropooling assay [30] that evaluates the net accumulation of fluid in the lumen of the small intestine [31]. After 18 h of fasting and 2 h of water deprivation, mice were treated as follow. Two groups of 6 mice were were dosed orally with 1,000 LD₅₀ of ETX, and sacrificed 2 and 6 h later. Other 4 groups of 6 mice were were dosed orally with 0, 125, 500 or 2,000 LD₅₀ of ETX in 0.5 ml of 1.5% PBS NaHCO₃. Further groups of 6 mice were were injected intravenously with 4, 0.5 or 0 LD₅₀ of ETX in 0.5 ml of 1% peptone water and analyzed 3 hours later. All of these animals were were sacrificed with CO₂.

As positive control of the enteropooling assay, groups of 4 mice were were injected intragastrically with 0, 12, 50 and 200 LD₅₀ of C2 botulinum toxin in 0.5 ml of 1.5% PBS NaHCO₃ and evaluated 6 hours later. C2 is a recognized enterotoxic clostridial toxin which induces fluid accumulation in the small intestine of mice [32]. Mice in negative control group were were inoculated with 0.5 ml of 1.5% PBS NaHCO₃.

The small intestine of all the mice was were clamped at the pyloric sphincter and immediately before of the ileo-caecal 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] shows the “enteropooling” in milligrams of fluid per centimeter of intestine and it is an indication of fluid accumulation.

Intestinal loop test

Mice were fasted during 18 hs and deprived of water 2 h before the experiments. The mice were anesthetized by intraperitoneal injection of 0.5–0.6 g/kg of Tribromoethanol (Avertin). The abdomen was opened, and starting approximately 2 cm proximal to the ileo-cecal valve, one loop of 5-cm was isolated with cotton ligatures on the small intestine. After inoculation of 0.3 ml of PBS containing 1,000 LD₅₀/ml of ETX and PBS solution as control the abdomen was closed by applying cyanoacrylate adhesive. The mice were kept under anesthesia by periodic administration of avertine until the end of the experiments, 3 h after the inoculation, when they were killed by cervical dislocation. The intestinal loops were excised, and the weight and length of each loop were measured. The net increase in the weight of the loop (in milligrams) was calculated as a relation between the weight of the loop inoculated and the length (in centimeters) of the loop.

Perfusion Fluid and Protocol

An in vivo perfusion method for measuring the absorption of water by the mouse small intestine previously described was followed with slight modifications [33]. Briefly, ~10 cm small intestinal loops were constructed as described above. One group to test ETX and one control group (4 mice each) were used. A
polyethylene perfusion catheter was inserted into the gut just below the proximal ligature and secured in place by a silk ligature. A collecting cannula was inserted into the lumen of the jejunum 10 cm further down to collect the perfusion fluid. The abdominal wall was closed with three sewing points of 4-0 silk. The isolated segment of small intestine was quickly rinsed with perfusion fluid, the inlet catheter was attached to a perfusion pump (Minipuls II, Gilson Instruments, speed 80, Technico, producing a flow of ~2 ml/15 min), and 15-min samples were collected from the output catheter. The perfusion fluid was a Ringer solution containing (in mM) 115 NaCl, 4 K2HPO4, 0.4 KH2PO4, 25 NaHCO3, 1.2 MgCl2, and 1.2 CaCl2, pH 7.4 and glucose was added. 10 mM mannitol was substituted for glucose in the mucosal bath solution. Glucose (10 mM) was included in the serosal solution and perfusion began with an equilibration period of 45 min; these samples were discarded. Crude fraction of mucosal and serosal fluids at the end of the perfusion, and the segment of jejunum was removed and its length measured.

**Bioelectric Measurements**

The effects of ETX in the mouse intestinal electric parameters were analyzed in *in vitro* and *ex vivo*. For *in vitro* experiments, segments of ileum were obtained from normal mice which were fasted for a minimum of 2 h and only water was provided. For *ex vivo* experiments, ileal loops were performed as described above, except that loop length was 1.5 cm and the volume injected was 0.1 ml. The mice were killed by a brief exposure to a 100% CO2 gas atmosphere (to induce narcosis) and a midline abdominal incision was used to excise the ileum. The excised segment was flushed with any intestinal content with cold saline solution and opened along the mesenteric border in ice cold-oxygenated Ringer solution. Each intestinal sheet (1 cm in length) without any visible Peyer’s patch with a support of nylon gauge were mounted in modified Ussing chambers (Warner instruments, CA) that compensates for electrode offset and the use of an automatic voltage clamp device (EC-800; Warner Instruments, CA) that contains a pair of Silver electrodes connected to the serosal and mucosal leads. The intestinal segments used with mice except that loop length was 2.5 cm and the volume injected was 0.3 ml at a concentration of 8,000 LD50. Intestines were incubated during 4 hours.

**Extravasation of plasma protein into the intestinal lumen**

*In vivo* small intestine mucosal permeability was evaluated following a modified version of Lange’s method [34]. Studies were conducted in anesthetized mice and the experimental protocol was essentially similar to the method described above for the intestinal loop test. After 3 h of incubation with 1,000 LD50 of ETX or PBS alone, a 0.1 ml of the azo dye Evans blue (EB) in PBS (15 mg/ml) was administered intravenously. The mice were euthanized 10 minutes later by cervical dislocation. The loops were dissected, opened, and rinsed with acetylcysteine in order to remove the adherent mucus layer. Each loop was weighed and incubated with formamide for 24 hr to elute the amount of EB absorbed, which was quantitated spectrophotometrically at 560 nm.

**Histopathological analysis**

Representative samples of intestinal tissues were taken immediately after the end of each experiment. Samples were immersed for 48 h in 10% buffered formalin pH 7.2, after which they were dehydrated through graded alcohols to xylene and embedded in paraffin wax. Sections were cut at 4 µm and stained with hematoxylin and eosin and examined by light microscopy.

**Immunofluorescence detection of ETX binding to mouse intestine**

ETX (2,000 LD50/ml in Ringer’s solution) or control (Ringer’s solution without ETX) was incubated during 20 minutes in intestinal loops (ileum or colon) as described above. After incubation, intestinal loops were excised and the intestinal content was flushed with Ringer’s solution and tissues were immediately frozen in Tissue-Tek OCT compound (EM Science). Air-dried frozen sections of intestines were overlaid sequentially with goat polyclonal antibody anti-ETX at 1/1,000, vol/vol, and fluorescein-conjugated rabbit anti-goat (1/100, vol/vol; Sigma Aldrich Co) for 1 h each. The toxin and antibodies were diluted in PBS.
Control sections were applied by using the same buffer but omitting the primary antibody.

Electron microscopy

Control and treated ileum segments of mice and rats were removed immediately, flushed, cut into 0.5-cm rings, and fixed in 4% formaldehyde buffered 0.1 mol/L phosphate buffer saline, pH 7.25, for 24 h at 4°C. Sections were then rinsed in Tris(hydroxymethyl) aminomethane buffer and postfixed in 1% osmium tetroxide for 60 min. After dehydration with ethanol, the sections were treated with propylene oxide and embedded in Epon polybed 812 (Polycysiences, Warrington, PA). The samples were first assessed by light microscopy with blue toluidine to select the areas for TEM studies. Ultrathin sections were cut from selected areas [35]. Ultrathin sections were contrasted with 1% OsO4 and 1% uranyl acetate, dehydrated and flatembedded in Durcupan. The sections were contrasted with lead citrate, examined and photographed on a Zeiss 109 electron microscope.

Statistical analysis

Statistical analysis was carried out using Statistix software (version 2.0). One-way analysis of variance (ANOVA) and t-test was used and assumed significance for a P value of <0.05. Throughout, descriptive statistics are reported as the mean± the standard error of the mean (SEM).

Acknowledgments

We are grateful to A. Eaton, C. Ibarra and Ana E. Elizondo for their help. J. Goldstein, C.F. Loidl, C. Tirion-Farinatti and M.E. Fernandez-Miyakawa are members of the National Council of Scientific and Technological Research (CONICET) of Argentina.

Author Contributions

Conceived and designed the experiments: JG WEM BAM FAU MEFM. Performed the experiments: JG WEM CFL CATF MEFM. Analyzed the data: JG WEM CFL MEFM. Contributed reagents/materials/analysis tools: JG CFL MEFM. Wrote the paper: JG WEM MEFM.

References