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Clostridium perfringens Type D Epsilon Prototoxin and Toxin Effects on the Mouse Body Weight

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Background: *Clostridium perfringens*is one of the most important pathogens in human and animals and protection against this bacteria in domestic animals will be only achieved by vaccination, otherwise, high mortality rate and great financial burden will be issued.
Objectives: We aimed to evaluate the effects of *C. perfringens* Type D prototoxin and toxin on the mouse body weight.
Materials and Methods: After preparation of filtrate and freeze dried crude prototoxin, three series of experiments were set up. At the first step, minimum lethal dose per milliliter (MLD/mL) was determined and according to MLD, 50% endpoint (LD_{50}) was determined. Finally, mice with 18 to 20 g body weight were injected with the different concentrations of activated freeze dried prototoxin.
Results: Two days after injection, a decrease in body weight was observed in experimental groups, while no decrease in body weight was observed in the control group. The results indicated that the activated *C. perfringens* culture filtrate temporarily inhibits mouse general metabolism.
Conclusions: As secreted prototoxin is activated in the small intestine of infected animals, vaccination of the domestic animals in the proper time by the appropriate vaccine could prevent these effects.

Keywords: Clostridium perfringens; Minimum Lethal Dose (MLD); Body Weight; Mouse

1. Background

Clostridium perfringens, an important pathogen of humans and livestock, is a gram-positive, rod-shaped, anaerobic, spore-forming, and heat-resistant bacterium of genus *Clostridium*, which produces numerous toxins and is responsible for severe diseases in both humans and animals. It is also a secondary pathogen in various diseases, such as necrotic enteritis (1, 2). Individual strains produce subsets of toxins and four of them, ie, ι (iA), α (cpa), β (cpb), and ε (etx) toxins, are used for classification of *C. perfringens* into five isotypes A, B, C, D, and E (3). In comparison to the other toxigenic Clostridial species including *Clostridium tetani*, *Clostridium botulinum*, and *Clostridium* difficile, *C. perfringens*is the paradigm species for genetic studies because of its oxygen tolerance fast growth rate (generation time of 8-10 minutes under optimal conditions) and ability to be genetically manipulated (4). *Clostridium perfringens* Type D is the causal agent of enterotoxemia of the sheep. This organism produces several major and minor toxins. Epsilon (ε), as one of its major toxins, is lethal for animals (5) and was first described by Wilsdon (6) and in 1933 it was named Epsilon (7). In the small intestine of infected animals, the toxin is produced in the form of prototoxin, which is activated by proteolytic enzymes in the small intestine (8) as well as by other proteolytic enzymes in vitro (9). Prototoxin activation by trypsin is due to cleavage and removal of a small 14-amino acid peptide from the amino terminal (10). There is a tryptophan residue and a histidyl residue in the structure of epsilon toxin, which are respectively important and essential for its lethal activity (11). The effect of *C. perfringens* Type D culture filtrate on the mouse small intestine permeability and also in vitro effects of its epsilon toxin on the mouse, guinea pig, and rabbits cells have been described (12). Electron microscope and I125 labelled epsilon toxin studies (13) have detected epsilon binding sites on the cells and its distribution in various organs of the mouse. (14). *Clostridium perfringens* epsilon toxin induces contraction of the isolated ileum of rat due to an indirect action mediated through the nervous system (15). It also possesses a repressor activity on cardiovascular system of rats (16). Vaccination of the domestic animals in the correct time using the correct vaccine is very important. Clostridial vaccines are manufactured using secreted toxin by virulent *C. perfringens* types B, C, and D. Large scale vaccine production using virulent strains requires extremely high safety conditions and cost benefit detoxification and quality control steps. (17).

2. Objectives

Vaccine against Clostridial diseases are producing in Iran for about five decades and recently, a new fusion construction containing *C. perfringens* type D, epsilon

toxin gene and type B, beta toxin gene was designed, produced, and expressed in *Escherichia coli*. Laboratory mice and rabbits were injected the recombinant fusion protein according to the European Pharmacopoeia and the resulted in acceptable immunization (18, 19). Elucidating of *C. perfringens* pathogenesis is necessary for its prevention; hence, for the further study of its pathogenesis, the present study aimed to evaluate the effects of Type D culture filtrate on the mouse body weight.

3. Materials and Methods

3.1. Bacterial Strain

Clostridium perfringens Type D (C.N409) was obtained from Razi vaccine and serum research institute.

3.2. Crude Suspension Preparation

Twelve litters glass chamber fermenter was used for bacterial growth and toxin production. Liquid culture medium consisted of vitamins, trace elements, and dextrin was prepared as described previously (18). After cooling down and at 37 °C, sterilized culture media was inoculated with 5% of *C. perfringens* fresh suspension. Dextrin and trace vitamin solution was added at the time of inoculation. Incubation period was six hours under controlled conditions and fixed temperature (37 °C) at pH of 7.1.

3.3. Filtrate

Immediately after the growth period, 2 L of culture suspension was removed from the fermenter and after microfiltration by Sartocon Mini cassette (Sartorius, Melbourne, Australia) ultrafiltration system through 0.2 μ module, stored for further studies.

3.4. Freeze Dried Crude Toxin

One litter of the filtrate was activated by 1% trypsin, then treated by 70% ammonium sulphate and mixed slowly (20). The mixture was transferred to 4°C refrigerator and was left overnight. At the next day, toxin was extracted as described previously (21), dialyzed against tap water, concentrated with polyethylene glycol (B.D.L. Carbowax M.W. 6000) up to the final volume of 20 mL, and freeze-dried.

3.5. Freeze Dried Prototoxin

The above mentioned procedures, except activation, were performed and inactive culture filtrate was freeze-dried. At the time of injection, 1 mg of trypsin was added to 100 mg of prototoxin and incubated 1 h at 37 °C.

3.6. Animals

White male and female NMRI mice weighing 18 to 20 g (obtained from Razi Vaccine and Serum Research Institute) were used. Animals were kept at normal period of light and fed with standard pellet.

3.7. Minimum Lethal Dose Determination

Minimal lethal dose (MLD) was determined for the filtrate, toxin, and prototoxin. In the case of toxin and prototoxin, 10 mg of freeze dried material was dissolved in 1 mL of normal saline and thereafter dilutions were prepared. Each of the two mice was injected intravenously by 0.5 mL of each dilution (10000 through 40000 with 5000 intervals).

3.8. Lethal Dose 50 Determination

Lethal dose 50 (LD_{50}) was determined as described previously (22).

3.9. Body weight Measurements

Four groups of mice, each consist of six mice weighing 18 to 20 g (mean 19 g) were used. In each group. The animals were treated by injection of a single sub-LD_{50} dose and were observed for eight days. Body weight was measured daily from the second day through the eighth day of injection. A group of six mice, kept as control, each was injected with a 0.5 mL of normal saline.

3.10. Statistical Analysis

Body weight mean was determined for each group and subsequently, each of the first and the second treatment groups respectively was compared to the third and the fourth groups. Student's t test was performed and the p value less than 0.05 was determined as statistically significant.

4. Results

4.1. Minimum Lethal Dose

Minimum lethal dose per milliliter was determined 19000, 12500, and 40000 mg/mL respectively for filtrate, freeze-dried toxin, and freeze-dried prototoxin that was activated at the time of injection.

4.2. Lethal Dose 50

LD_{50} calculated as 0.000215 mg/mL for 18-20 grams mouse or 0.0107 mg/kg of mouse body weight. The data for determination of LD_{50} are shown in Table 1.

4.3. Toxin Effect on Body Weight

Sub-LD_{50} dose of 0.0001 (0.46% of LD_{50}), 0.000125 (58% of LD_{50}), 0.000156 (72.5% of LD_{50}), and 0.000195 mg/mL (90% of LD_{50}) dilutions of the prototoxin, which was activated at the time of injection, was injected to the mice. The data were statistically analyzed by Student's t test. The P value was determined for body weight dif-

ferences in each of the two groups. Tables 2, 3, 4 and 5 these results. Table 6 summarized results of body weight measurements in four groups of mice, which were injected by one sub-LD_{50} dose of one of each dilution. Figure 1 shows Mean body weight changes in four groups of mice that were injected by different sub LD50 dose of C. perfringens epsilon toxin. This result shows that the 4th group which had been received a single dose 0.000195 mg/ml of toxin, undergoes severe body weight.

Table 1. Lethal Dose 50 Test for the Freeze Dried Prototoxin Activated at the Time of Injection [a]

Toxin Dose, mg/mL	Inoculated/Dead	Mortality Rate, %	Time to Death, h
0.0001	6/0	00	-
0.000125	6/0	00	-
0.000156	6/0	00	-
0.000195	6/1	16.66	24
0.000244	6/3	50	24
0.000305	6/6	100	24
0.000381	6/6	100	24
0.000476	6/6	100	24

[a] lethal dose 50 (LD_{50}) was calculated as 0.000215 mg/mL for 18 to 20 g mouse or 0.0107 mg/kg of mouse body weight

Table 2. Results of the Body Weight Analysis of the First and the Third Groups of Mice Exposed Respectively to 0.0001 mg/mL and 0.000156 mg/mL [a]

Experiments Treatment/mg/mL (0.5 mL)	Weight at The Time of Injection	Weight After 48 Hours (g)	Weigh After 72 Hours (g)	Weight After 96 Hours (g)	Weight After 144 Hours (g)	Weight After 168 Hours (g)	Weight After 192 Hours (g)
1^{st}/0.0001	18.95	16.91 1.65	21.00 1.26	22.50 1.04	22.80 1.18	23.41 1.08	23.90 1.24
3^{rd}/0.000156	19.28	14.50 1.18	18.08 1.07	18.75 0.88	18.91 2.40	19.41 2.50	19.16 2.69
P	-	P < 0.02	P < 0.01	P < 0.001	P < 0.01	P < 0.01	P < 0.01

[a] Data are presented as mean ± standard deviation.

Table 3. Results of the Body Weight Analysis of the Second and the Third Groups of Mice Exposed Respectively to 0.000125 mg/mL and 0.000156 mg/mL [a]

Experiments Treatment/mg/mL (0.5 mL)	Weight at The Time of Injection	Weight After 48 Hours (g)	Weigh After 72 Hours (g)	Weight After 96 Hours (g)	Weight After 144 Hours (g)	Weight After 168 Hours (g)	Weight After 192 Hours (g)
2^{nd}/0.000125	18.75	16.50 2.24	21.00 1.09	21.58 1.39	21.80 2.36	22.00 2.57	22.16 2.36
3^{rd}/0.000156	19.285	14.50 1.18	18.08 1.07	18.75 0.88	18.91 2.40	19.41 2.50	19.16 2.69
P	-	P < 0.05	P < 0.001	P < 0.01	0.05 < P < 0.1	0.01 < P	0.05 < P < 0.1

[a] Data are presented as mean ± standard deviation.

Table 4. Results of the Body Weight Analyses of the First and the Fourth Groups of Mice Exposed Respectively to 0.0001 mg/mL and 0.000195 mg/mL Toxin [a]

Experiments Treatment/ mg/mL (0.5 mL)	Weight at the Time of Injection	Weight After 48 Hours (g)	Weigh After 72 Hours (g)	Weight After 96 Hours (g)	Weight After 144 Hours (g)	Weight After 168 Hours (g)	Weight After 192 Hours (g)
1^{st}/0.0001	18.955	16.91 1.65	21.00 264	22.50 1.07	22.80 1.18	23.41 1.08	23.90 1.24
4^{th}/0.000195	18.93	13.30 1.35	14.60 2.18	18.10 1.47	18.50 1.73	18.75 1.71	19.12 1.70
P	-	P < 0.01	P < 0.001	P < 0.001	P < 0.01	P < 0.01	P < 0.01

[a] Data are presented as mean ± standard deviation.

Table 5. Results of the Body Weight Analysis of the second and the fourth Groups of Mice Exposed Respectively to 0.000125 mg/mL and 0.000195 mg/mL [a]

Experiments Treatment/ mg/ mL (0.5 mL)	Weight at the Time of Injection	Weight After 48 Hours (g)	Weight After 72 Hours (g)	Weight After 96 Hours (g)	Weight After 144 Hours (g)	Weight After 168 Hours (g)	Weight After 192 Hours. (g)
2^{nd}/0.000125	18.75	16.50 2.27	21.00 1.09	21.58 1.39	21.80 2.36	22.00 2.57	22.16 2.36
4^{th}/0.000195	18.93	13.30 1.35	14.60 2.18	18.10 1.47	18.5 1.73	18.75 1.71	19.12 1.70
P			P < 0.001	P < 0.01	P < 0.05	0.05 < P < 01	0.05 < P < 0.1

[a] Data are presented as mean ± standard deviation.

Table 6. Body Weight Means of Four Groups of Mice at Six Different Post Injection Times (x)

Treatment/ mg/ml	Weight at the Time of Injection	Weight After 48 Hours (g)	Weigh After 72 Hours (g)	Weight After 96 Hours (g)	Weight After 144 Hours (g)	Weight After 168 Hours (g)	Weight After 192 Hours (g)
1^{st}/0.0001	18.95	16.91	21.00	22.50	22.80	23.40	23.90
2^{nd}/0.000125	18.75	16.50	21.00	21.58	21.83	22.00	22.16
3^{rd}/0.000156	19.28	14.50	18.08	18.75	18.91	19.41	19.16
4^{th}/0.000195	18.93	13.30	14.60	18.10	18.50	18.75	19.12

5. Discussion

Genus *Clostridium* contains varieties of pathogenic and nonpathogenic species which are found in soil and water; some species are the members of the normal flora in humans as well as animals (23). *Clostridium perfringens*is the causative agent of enterotoxemia in sheep, goats, and cattle; although vaccination is the key action in preventing this disease, it can be prevented with better understanding of its risk factors and pathogenesis that would result in high mortality (24). In the present study, we attempted develop a better understanding of *C. perfringens* epsilon toxin effects on the general metabolism of the laboratory animals. According to the European Pharmacopoeia potency testing of anaerobic clostridial vaccine could be replaced by a proper test such as MLD (EP 2010). In the present study, the determined MLD values indicated that the highest value (40000) belonged to the freeze-dried prototoxin activated immediately prior to the injection. Conversely, the lower value (12500) was seen in active freeze-dried toxin. Therefore, one may infer that activation, concentration, and freeze-drying of prototoxin had led to reduction of its activity. This loss of activity could be interpreted in different ways. It is possible that the trypsin had continued further degradation of the toxin. Another interpretation is that the toxin had been denatured after activation, which is before and during freeze-drying process, the latter interpretation is more probable because epsilon toxin as a protein has a definite half-life and naturally undergoes degradation. Whatever the reason, it was obvious that the prototoxin activated shortly before injection had a much higher activity and, for this reason, this method was chosen for preparation of the toxin. There is a threshold for teratogenic and carcinogenic materials (25). When this threshold is passed, the pathological effects have direct association with the size of the dose. Therefore, amounts of toxin injected were selected on a decreasing scale of a sub-LD_{50} dose. The data of our study indicated that a single dose injection of toxin could cause a body weight loss proportionate to the size of the applied dose, which was followed by the resumption of body weight gain within the next few days. The differences between the first and the third treatments on all occasions were significant, while the differences between the second and the third treatments was significant only up to 96 hours after injection (Table 3). Comparative analysis of the data showed that at 48th and 96th hours after injection, the differences between the first and the fourth treatments were significant. In addition, up to 144 hours after injection, the differences were significant between the second and the fourth groups but thereafter, significant difference was not seen; the standard error of differences of each mean was high and the overlapping of parameters was obvious. According to our results, the fourth group, which had received only one injection of 0.000195 mg/mL toxin (nine-tenths of LD_{50}), developed a severe loss in body weight while this was less remarkable in other groups. Forty-eight hours after injections, the maximum body loss was compared with the mean body weight (19 g); the decrease percentage for the first (0.0001 mg/mL), the second (0.000125 mg/mL), the third (0.000156 mg/mL), and the fourth treatment (0.000195 mg/mL) were 11%, 13.5%, 23.68%, and 30%, respectively. This showed the direct effect of the dose on Wight loss. Furthermore, 72 hours after injections body weight means were 21 g in

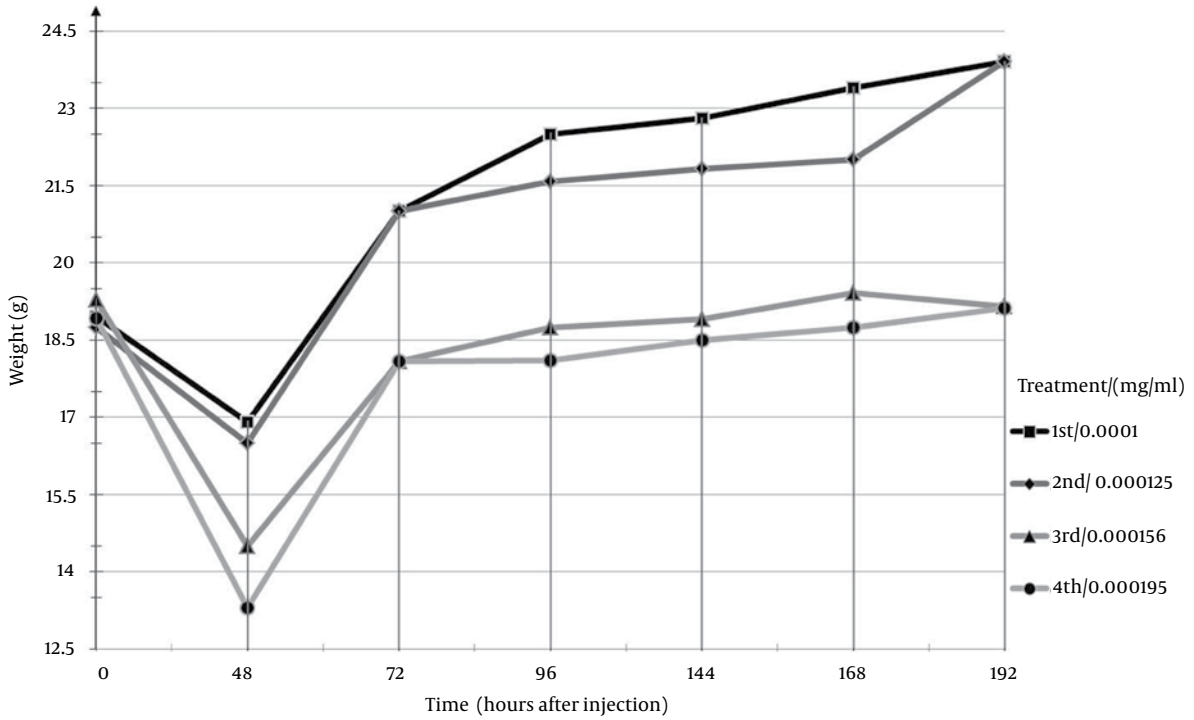

Figure 1. Shows Mean body weight changes in four groups of mice that were injected by different sub-LD_{50} dose of *Clostridium perfringens*epsilon toxin.

the first and the second groups while these were 18.7 5 and 18.1 g in the third and the fourth group, respectively; after that, all animals showed weight gains, which were much slower in the third and the fourth groups in comparison to the two other groups. Data analyzing of this study indicated that sub-LD_{50} doses of freeze-dried prototoxin, which was activated at the time of injection, cannot kill the mice but although temporarily, it can severely affect and disrupt the natural metabolism. If the applied dose is closer to LD_{50}, the more severe and longer effects would be expected. Epsilon toxin increases cAMP concentration in mouse plasma. Moreover, *Escherichia coli* heat resistant enterotoxin, *Vibrio cholera* enterotoxin, and *Staphylococcus aureus* deltatoxin increase the cAMP concentration in vitro in the ilial mucosal cells of guinea pig. Thus, *C. perfringens*epsilon toxin is an enterotoxin, which can bind to specific cell surface receptors of certain cells in blood vessels, kidney, and liver and finally can cause wide damages mediated by adenyl cyclase cAMP system. Toxin may bind to mouse brain blood vessels, endothelium luminal surface of thalamus, cortex, cerebrum white nucleus, pons, and meninges (12, 14). According to the present work and data discussed here, we suggest that wide damages mediated by adenyl cyclase-cAMP system can cause severe and temporary decrease in the body weight. The mechanisms of these effects remain to be elucidating in future investigations.

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