CHOLERA TOXIN AND VIBRIO IN THE ADULT RAT INTESTINAL LOOP

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Summary: Like rabbit, rat manifests fluid accumulation in its ligated small intestinal loop in response to cholera toxin. The details of this phenomenon are studied. Fasting for 24 to 27 hr is adequate. Rats fasting on ad lib 5% glucose water lose less body weight than those on plain water. Older rats are sensitive like the 40 day old rats. Pentobarbitone is more convenient and permits prolonged anaesthesia than other. Irrigation of intestine is not necessary. 30 cm length of loop is optimum. Jejunal and ileal portions of the small intestine are about equally sensitive. Only one 30 cm long loop of intestine should be prepared per rat. Loop responds to toxin dose range 10 to 30 mg (as 10.6 to 32 Vg pure enterotoxin). Five hr is the optimum period for fluid collection. The fluid volume is not related to sex but to body weight; therefore, rats of narrow weight range should be used. Electrolyte composition of the loop fluid (unlike its volume) remains remarkably constant. Heat-inactivated cholera toxin, cholera vaccine, staphylococcal toxin, Synsage medium, peptone or prostaglandin E1 do not cause fluid accumulation. The rat loop method can be conveniently used for bioassay and other study of cholera toxin, vaccine and anti-cholera drugs.

6.25 x 10⁹ live cholera vibrio (but not 2.5 x 10⁹) injected into 30 cm intestine loop of fasting adult rats also produced fluid accumulation in 18 hr (but less in 12 hr). Thus rat is sensitive to cholera vibrio but less to live vibrio whereas rabbit is sensitive to both.

Key words: cholera toxin, rat’s small intestine, fluid accumulation

INTRODUCTION

Toxins of bacteria, as of animals and helminths, have interesting pharmacological properties. Their study is of much importance in backward tropical countries. For example, cholera toxin (also known as “cholera exotoxin”, “cholera enterotoxin”, cholera exo-enterotoxin”, “choleraagen”, “cholera permeability factor”, “PF”, “fluid accumulating factor”, “FAF”) manifests specific and well-defined action. Indeed, clinical cholera which affects only human species produces most of its patho-physiological effects because this toxin brings about selective and massive outpouring of water and electrolytes into the lumen of the gastrointestinal tract.

Research on cholera remained handicapped for about 70 years till De and colleagues (4,5) showed that rabbit’s ligated small intestine loop is sensitive to cholera vibrio and toxin.

Later Aziz et al. (2) made a preliminary report that 40 day old rat’s small intestine loop is sensitive to cholera toxin. This finding is not confirmed nor its details worked out although the rabbit
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Small intestinal loop in response for 24 to 27 hr is adequate. Jejunal loop is optimum. Jejunal loop of intestine to 30 cm (as 10.6 to 32 U/g pure fluid volume is not related to the used. Electrolyte composition of activated cholera toxin, cholera toxin and electrolytes into the intestinal lumen does not cause fluid accumulation and other study of cholera toxin.

Confirmation of the reported effect (2):

Twentyfour experiments were performed exactly by the procedure of Aziz et al. (2). Forty day old rats were kept on water and soft biscuits for 72 hr and then on only ad lib 5% glucose water for the next 72 hr. After laparotomy, under ether anesthesia, the lumen of the small intestine was irrigated by normal saline. In each rat 2 loops each of 10 cm length were prepared by ligature starting 2 cm above the caecum. The toxin was injected into one loop and distilled water into the other by a thin sharp needle. After 8 hr, rats were killed by chloroform and fluid in each loop was collected.

Detailed study of the reported effect:

Rats of either sex (98-220 g) were denied food for 6, 18, 24, 27 or 36 hr but allowed ad lib plain water or 5% W/V glucose water. Fasting rats were kept comfortably warm in all-metal cage which had wire mesh bottom to drain profuse urine output (there should be no soft bedding at cage bottom which hungry animals tend to eat). They were anesthetized by ip injection of sodium pentobarbitone (35 mg/kg in summer and 25 mg/kg in winter) and, if necessary, ether inhalation was used as a supplement. The abdomen was opened and the intestine was gently taken out but its lumen was not irrigated with saline. A loop was prepared by applying ligatures, the first about 3 cm below the pylorus and the second 30 cm distal. A 30 cm long twine thread proved very convenient for measuring the loop length. Individual ligatures can be strengthened by tying another thread at a short distance. Ligatures should not include larger blood vessels. Cholera toxin solution was injected into the loop and abdominal wall sutured. After 3, 5, 8, 12 or 18 hr, animals were killed by chloroform and volume of the loop fluid was measured.
Effect of the following 4 factors on fluid formation was also studied - (a) body weight of rats, (b) length of loop (20 or 40 cm loops), (c) use of terminal ileal intestine for ligating 30 cm loop and (d) more than one loop per rat.

In some fluid samples, Na+ and K+ were estimated by flame photometer and Cl- by the chemical method (10).

Experiments with live cholera vibrio:

Rats (185-225 g) of either sex were kept on 5% glucose water for 24 hr. A 30 cm loop of the proximal small intestine was tied as described above. Sterile nutrient broth (1 or 2.5 ml) or live culture of cholera vibrio (1 or 2.5 ml of classical Ogawa overnight culture in nutrient broth containing 2.5 x 10^9 bacteria per ml) was injected in some animals. The fluid volume was measured after 5,12 or 18 hr.

RESULTS

Confirmation of the reported effect:

Results in Table I confirm the reported effect. Thus in the 10 cm ligated loops in 40 day old rats, distilled water did not at all provoke fluid formation whereas cholera toxin was found effective. There was no significant difference in the responses by 5 and 10 mg toxin doses; 20 mg toxin produced just significantly (P=0.05) more fluid than 10 mg. According to this method, rats receive only glucose water for 72 hr which results in 18% reduction in body weight.

TABLE I: Fluid accumulation by cholera toxin into 10 cm long loop by the method of Aziz et al. (2) in 40 day old rats.

<table>
<thead>
<tr>
<th>Dose of cholera toxin mg/loop</th>
<th>No. of experiments</th>
<th>Fluid volume in the intestinal loop ml average ± S.E. (p values)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nil (only distilled water)</td>
<td>24</td>
<td>0.0</td>
</tr>
<tr>
<td>5</td>
<td>6</td>
<td>1.7±0.14 (&gt;0.1)</td>
</tr>
<tr>
<td>10</td>
<td>8</td>
<td>1.6±0.09*</td>
</tr>
<tr>
<td>20</td>
<td>10</td>
<td>2.5±0.11 (=0.05)</td>
</tr>
</tbody>
</table>

*Value used as standard for calculating p values by t-test.

Duration of fasting

Rats on 24 hr fast. Prolonging the fast by injecting E. coli leak out of the ileostomy.

Fluid available during fasting

Rats fasting for 24 hr. However, fasting rats hardly reduced fluid formation which reduced somewhat less than on plain water.
Detailed study of the reported effect:

Fluid started gathering in 3 hr and significantly increased to reach the peak after 5 hr. The accumulation showed a statistically significant decline over the next 7 hr. The 18 hr value showed a rise which is difficult to explain (Table II).

<table>
<thead>
<tr>
<th>Interval between toxin injection and fluid collection (hr)</th>
<th>No. of rats</th>
<th>Fluid in the intestinal loop</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Volume ml average±S.E. (p values)</td>
</tr>
<tr>
<td>3</td>
<td>6</td>
<td>1.8±0.29 (0.001)</td>
</tr>
<tr>
<td>5</td>
<td>12</td>
<td>*5.3±0.29 (0.05)</td>
</tr>
<tr>
<td>8</td>
<td>6</td>
<td>4.2±0.86 (&gt;0.05)</td>
</tr>
<tr>
<td>12</td>
<td>5</td>
<td>2.7±0.01 (0.05)</td>
</tr>
<tr>
<td>18</td>
<td>12</td>
<td>6.4±0.56 (&gt;0.05)</td>
</tr>
</tbody>
</table>

*This was used as standard for calculating p values by t-test.

Injecting Evans blue with cholera toxin into the loop showed that the fluid did not leak out of the loop over 18 hr.

Duration of fasting (Table III):

Rats on 24-36 hr fast gave significantly better results than those on 6 or 18 hr fast. Prolonging the fasting period to 72 hr (2) did not improve response to cholera toxin.

Fluid available during fasting (Table III):

Rats fasting on plain water respond to cholera toxin as well as those on glucose water. However, 2 advantages were noted in offering glucose water - (a) Though fasting rats hardly drank plain water, they profusely drank glucose water (and get polyuria) which reduced solid residue in their small intestine, (b) As ascertained from the weights of the entire group of 40 rats, 27 hr fasting on glucose water caused less weight loss (0.4%) than on plain water (3.8%).
Body weight and sex:

In medium weight rats (98-220 g; 10 animals) which are used in most of the work here, average response to 30 mg cholera toxin in 5 hr was 5.6 ± 0.16 ml fluid per loop. In smaller rats (48-70 g; 6 animals), it was significantly less (2.7 ± 0.37 ml; P <0.001); in heavy rats (250-330 g; 6 animals), the value, though higher, does not differ significantly (6.6 ± 0.84 ml; P >0.1). Sex of the rats did not influence the fluid accumulating response to cholera toxin. Thus, in one experiment with 30 mg of Miller’s first batch toxin, 9 males showed 5.1 ml ± 0.20 fluid and 7 females, 5.1 ml ± 0.26. In another experiment, with 100 mg of Miller’s second batch toxin, 8 males showed 3.6 ml ± 0.38 fluid and 6 females, 4.0 ml ± 0.48. The difference between the values of two sexes were not significant (P>0.1).

Loop length:

100 mg dose of second batch of Miller’s toxin produced, in 5 hr, significantly (P <0.001) more fluid accumulation in 30 cm long intestinal loop (5.1 ± 0.33 ml; 11 rats) than in 20 cm loop (3.5 ± 0.40; 9 rats). 40 cm loop did not show better response (4.9 ± 0.47; 9 rats, P >0.05) than 30 cm loop.

Sensitivity of proximal and distal intestine:

Distal ileum of rat small intestine was as sensitive to cholera toxin as the proximal jejunal portion. Thus, with 75 mg cholera toxin (2nd batch) acting for 5 hr, 2.5 ± 0.16 ml (5 rats) fluid gathered in the 30 cm proximal jejunal portion and in other 5 rats, 2.6 ± 0.20 ml gathered in the distal ileum.

Number of loops per rat:

In 8 experiments, 2 loops each of 30 cm length were prepared per rat and in both loops 100 mg cholera toxin (2nd batch) was injected. After 5 hr, 2.8 ± 0.66 ml fluid accumulated in the 8 proximal loops and 1.7 ±0.63 ml in the 8 distal ones; these 2 values do not differ significantly (P >0.1). However, in experiments where only one loop was tied per rat, the average response per loop was significantly greater and more uniform (5.1 ± 0.47 ml; 17 rats; P <0.001).

Toxin dose:

The higher doses of any cholera toxin batch produced more fluid accumulation than the smaller ones (Table IV).

Bioassay of two other cholera toxin samples:

The results in Table IV indicate that Miller’s second batch and Gaitonde’s sample are less potent than Miller’s first batch.
which are used in most of the work is 6.6 ± 0.16 ml fluid per loop, less (2.7 ± 0.37 ml; P <0.001); however, does not differ significantly since the fluid accumulating response mg of Miller's first batch toxin, ± 0.26. In another experiment, used 3.6 ml ± 0.38 fluid and 6 of two sexes were not significant produced, in 5 hr, significantly fluid loop (5.1 ± 0.33 ml; 11 rats) showed less fluid accumulation than 10% of the proximal ch) acting for 5 hr, 2.5 ± 0.16 ml, did not show better response (4.9 ml) of choleratoxin as the proximal intestinal loop (5.1 ± 0.33 ml; 11 rats) allowed and in other 5 rats, 2.6 ± 0.20 ml fluid was prepared per rat and in both groups (5 & 6), 2.8 ± 0.66 ml fluid per loop was given at 10 min intervals where only one loop was tied in other 8 distal ones; these 2 values were not significantly different and more uniform greater and more uniform in Miller's first batch and Gaitonde's sample.

<table>
<thead>
<tr>
<th>Duration of fasting (hr)</th>
<th>Fluid allowed during fasting</th>
<th>No. of rats</th>
<th>Fluid in the intestinal loop</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Volume ml average ± S.E. (p values)</td>
</tr>
<tr>
<td></td>
<td>5% glucose water</td>
<td>10</td>
<td><strong>3.8 ± 0.28</strong> (&lt;0.001)</td>
</tr>
<tr>
<td></td>
<td>Water</td>
<td>10</td>
<td><strong>4.1 ± 0.26</strong> (&lt;0.001)</td>
</tr>
<tr>
<td></td>
<td>5% glucose water</td>
<td>8</td>
<td>4.2 ± 0.24 (&lt;0.001)</td>
</tr>
<tr>
<td></td>
<td>5% glucose water</td>
<td>6</td>
<td>5.1 ± 0.68 (&gt;0.1)</td>
</tr>
<tr>
<td></td>
<td>5% glucose water</td>
<td>10</td>
<td>*5.6 ± 0.16 (&gt;0.1)</td>
</tr>
<tr>
<td></td>
<td>Water</td>
<td>7</td>
<td>5.0 ± 0.43 (&gt;0.1)</td>
</tr>
<tr>
<td></td>
<td>5% glucose water</td>
<td>12</td>
<td>5.3 ± 0.29 (&gt;0.1)</td>
</tr>
</tbody>
</table>

*Value used as standard for calculating p value by t - test.
**Difference between these values not significant (p>0.1 by t - test).

**TABLE IV: Bioassay of 3 cholera toxin samples. Rats fasted for 27 - 36 hr on glucose water; one 30 cm long loop tied in proximal small intestine.**

<table>
<thead>
<tr>
<th>Source of cholera toxin</th>
<th>Dose of cholera toxin mg/loop</th>
<th>No. of rats</th>
<th>Volume of fluid in the loop ml average ± S.E.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>3</td>
<td>2.9 ± 0.80</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>3</td>
<td>2.8 ± 0.66</td>
</tr>
<tr>
<td>Miller's toxin</td>
<td>30</td>
<td>12</td>
<td><em>5.3 ± 0.29</em></td>
</tr>
<tr>
<td>1st Batch</td>
<td>100</td>
<td>6</td>
<td><em>6.4 ± 0.35</em></td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>3</td>
<td>0.8 ± 0.44</td>
</tr>
<tr>
<td></td>
<td>75</td>
<td>5</td>
<td>2.8 ± 0.16</td>
</tr>
<tr>
<td>Miller's toxin</td>
<td>100</td>
<td>17</td>
<td>5.1 ± 0.47</td>
</tr>
<tr>
<td>2nd Batch</td>
<td>30</td>
<td>2</td>
<td>0.0</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>2</td>
<td>0.0</td>
</tr>
<tr>
<td></td>
<td>150</td>
<td>4</td>
<td>5.8 ± 0.35</td>
</tr>
<tr>
<td></td>
<td>200</td>
<td>6</td>
<td>5.1 ± 0.38</td>
</tr>
<tr>
<td></td>
<td>300</td>
<td>2</td>
<td>6.0</td>
</tr>
</tbody>
</table>

*Difference between these two values is significant (p<0.02 by t - test).
**Electrolytes:**

Na⁺, K⁺, and Cl⁻ values of the loop fluid were fairly constant and were not influenced by fluid volume or toxin dose or duration (Tables II-III).

**Control experiments:**

Heat-inactivated cholera toxin (100 mg/loop; 9 rats), Syncase medium which is used for vibrio culture to obtain cholera toxin (100 mg/loop; 6 rats), highly purified staphylococcal toxin (0.1 - 5 mg/loop; 5 rats) and anti-cholera vaccine (Central Research Institute, Kasauli; containing 1200 crores vibrios per ml; 0.25 ml per loop; 8 rats) which has very little exotoxin were found entirely ineffective. Also, berberine (5 mg; 6 rats), TAB vaccine (0.25 ml; 3 rats), milk (2 ml; 3 rats), peptone (200 mg; 3 rats), prostaglandin E₁ (0.3 - 1.0 mg; 9 rats) and distilled water (2 ml; 3 rats) were entirely ineffective. These results indicate specificity of the action of cholera toxin.

**Experiments with live cholera vibrio:**

When 2.5 ml of live vibrio culture was injected into the loop, 5.15 ± 0.24 ml (10 rats) fluid accumulated in 18 hr. In 6 of these rats, 18 hr loop fluid samples were subcultured and all of them showed growth of vibrios. The response was significantly less when 12 hr were allowed (2.0 ± 0.19 ml; 8 rats; P < 0.001) and almost nil when 5 hr were allowed (0.2 ml; 10 rats).

When 1 ml of live vibrio culture was injected, the response was poor even after 18 hr (0.6 ml; 3 rats). In control study, injecting 2.5 ml sterile nutrient broth did not produce any fluid accumulation even after 18 hr (0.0 ml; 11 rats).

**DISCUSSION**

The present work confirms the sensitivity of rat intestine loop to cholera toxin. It also suggests that young rats, short loops and prolonged fasting by the method of Aziz et al. (2) reduce fluid formation and thus increase the possibility of error of measurement. Better alternatives are worked out and presented in this work.

In rat loop, cholera toxin induces maximal fluid accumulation in 5 hr whereas vibrios require 18 hr; this could be because the live vibrios in the loop slowly produce toxin.

Since 1953 rabbit is widely used in ligated intestinal loop method of experimental cholera (4,5,6). Therefore, a relevant question that arises here is, how sensitive rat is to cholera toxin and vibrio as compared to rabbit. In this connection, it is interesting to note that in 4 rabbits, Miller's 2nd batch toxin produced, in 18 hr, 8.3 ml fluid with 12 mg dose and 10.2 ml with 75 mg dose (Table IV). Table IV shows that with 75 mg dose, and durations done, these results broach sensitivity to the cholera toxin.

On the other hand, rabbit loop is known in rats, in the present study, as much as 6.25 ml.

In rat only 5 ml per rabbit. However, it is cheaper to purchase 5 hr instead of 18 hr.

Altogether, the results of this study.

Both in rabbit and rat induced loop fluid can significantly produce fluid (9). There is a sensitive parameter.

The rabbit is more sensitive than the cutaneous inflamed rat.

Sincere thanks are due to the members of Scientific Unit, Department of Scientific and Industrial Research, Kasauli for their cooperation.
The electrolyte composition of the toxin-induced loop fluid was remarkably constant and close to that of serum. Also, berberine can significantly reduce the volume but not the electrolytes in the toxin-induced loop fluid (9). Therefore, in the ligated intestinal loop method, electrolyte composition is not a sensitive parameter.

The rabbit intestinal loop which is extensively used in experimental cholera, is more sensitive than the rat intestinal loop but is much less sensitive than the rat neck subcutaneous inflammation (1).

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