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Berberine in toxin-induced experimental cholera

N. K. DUTTA, P. H. MARKER AND N. R. RAO

Haffkine Institute, Bombay, India

Appendix on berberine in toxin-induced experimental cholera

Summary

1. Oral administration of berberine to infant rabbits 18–24 h before the intra-intestinal administration of choleragenic toxins, arrests diarrhoea or significantly prolongs the survival time.
2. The use of berberine in the treatment of clinical cholera is further justified.
3. Berberine is an antidiarrhoeal drug and the host tissues play a major part in the control of diarrhoeal symptoms.

Introduction

Dutta & Panse (1962), while searching for a chemotherapeutic agent against cholera, reported that in rabbit cholera, berberine, an alkaloid from *Berberis aristata,* was as effective as chloramphenicol or tetracycline. Lahiri & Dutta (1967) tried berberine clinically in epidemics of ' gastro-enteritis ' in Calcutta and recommended it as a drug of choice in clinical cholera and acute non-specific diarrhoea. Kamath (1967), Deshpande (1969) and Sharda (1970) reported that in acute diarrhoea recovery was faster with berberine than with other antimicrobial drugs. Berberine is now being used for treating cases of acute gastro-enteritis.

Berberine is vibriostatic (Nair, Modak & Venkatraman, 1967 ; Amin, Subbiah & Abbasi, 1969), but its action is much weaker than that of tetracycline or chloramphenicol (Dutta, unpublished). While this antibacterial action could be responsible for curing rabbits infected with *Vibrio cholerae,* the question also arises whether berberine has any effect in infant rabbits in which diarrhoea is produced by the administration of cholera toxin. The choleragenic toxin derived from *V. cholerae* is the main cause of diarrhoeal symptoms, as was first reported by Oza & Dutta (1963) who demonstrated intense diarrhoea in baby rabbits when they were fed with cell-free sonicated lysate of the organisms. The present investigation was to see whether berberine is effective against toxin-induced diarrhoeal symptoms.

Methods

Choleraic diarrhoea was induced in randomly selected baby rabbits (70–135 g) by the administration of toxin according to the technique of Oza & Dutta (1963).

Toxin prepared from cell-free culture filtrates of *V. cholerae* 569 Inaba (rabbit passaged strain) according to Finkelstein, Norris & Dutta (1964) (hereinafter referred to as ' Syncase '), and sonicated cell suspension prepared by the method of Oza & Dutta (1963) were used. Toxins were lyophilized and reconstituted with distilled water before use. Berberine sulphate solution (10 mg/ml) in distilled water was given orally. For inducing choleraic symptoms in the treated and control groups, ' Syncase ' toxin was used in treatments A to E and G, ' sonicate ' toxin in treatment F.

The cholera toxin was administered intraintestinally as follows. The rabbit was first anaesthetized with ether, a small midline incision was made in the upper part of the abdomen and a portion of the small intestine was pulled out with fine forceps. The toxin was injected with a 25 gauge hypodermic needle, and the abdominal wall was closed in two stages (muscle layer and the skin) separately with silk thread. The entire operation was carried out aseptically.

Predetermined doses of each batch of toxin were used. Ten, 15 and 20 mg per 100 g body weight of each dose level of a batch were injected into sets of two infant rabbits weighing 120–140 g. The smallest dose which killed both the animals within 18 h was used for experiment.

Treatment A. Treated group (five rabbits): berberine sulphate (20 mg) was given 1 h before toxin and 10 mg doses were repeated at 8 h intervals after the administration of toxin until 72 hours. The control group (three rabbits) was given toxin only.

Treatment B. Treated group (four rabbits): berberine sulphate (20 mg) was given 18 h before toxin and 10 mg 1 h after it. Then doses of 10 mg were continued at 8 h intervals for 48 h thereafter. The control group (three rabbits) was treated as under A.

Treatment C. Treated group (ten rabbits). 30 mg of the drug were given 18 h before toxin administration followed by doses of 15 mg each, the first 1 h after, and the second 24 h after toxin administration. The control group (three rabbits) was treated as under A.

Treatment D. Treated group (six rabbits). 25 mg and 20 mg of the drug were given 24 h and 4 h respectively before toxin administration and 10 mg 1 h after it. The control group (four rabbits) was given water orally in amounts equal to the fluid given in each dose of drug to the treated group.

Treatment E. Treated group (five rabbits). The rabbits received the same treatment as in D. Two groups of controls were kept. Group I (five rabbits) was given distilled water ; group II (four rabbits) saline in amounts specified under D.

Treatment F. Treated group (five rabbits): berberine sulphate (20 mg each) was given 20 h and 1 h before toxin administration and two doses of 15 mg each, 4 h and 22 h after the toxin. The control group (four rabbits) was given saline as in E.

Treatment G. Toxin (double strength) was mixed with an equal volume of a solution of 10 mg/ml of berberine sulphate (in a higher concentration, berberine precipitated out) and incubated for 4 h at 37° C. This incubated solution was administered to five infant rabbits intraintestinally in a dose of 1 ml/100 g body weight. In control animals (five rabbits), toxin solution incubated as above was given intraintestinally. Any infant rabbit which survived for 72 h was considered alive for the purpose of these experiments.

Because of the problems associated with the statistical analysis of survival times when there are large numbers of survivors, this is dealt with separately in an appendix.

Results

Table 1 shows that in treatment A, the difference in the average survival time of 3·5 h between the treated and control groups was not significant. When berberine

was administered as under treatment B, out of the four animals so treated, three survived. In the corresponding control group which received toxin only, all the animals died. When the dose of berberine was further raised as in treatment C, seven out of the ten animals survived. Here too, berberine significantly prolonged the life of these animals besides preventing death in 70% of the rabbits. Similar results were also obtained with treatments D and E. There was no significant difference in the average survival time of controls given distilled water, and those given saline. An incubated mixture of berberine and toxin produced diarrhoea in the test animals, and average survival time was not significantly greater than that of the corresponding control (treatment G). Administration of berberine was effective in prolonging the survival time and preventing death in three of the five treated animals when challenged with sonicate toxin (treatment F).

In each control group, all the animals died within 11–18 h as against the lowest of the mean survival time, 45·67 h, of the berberine treated groups (treatment D). The statistical analysis of the results is presented in the Appendix using the analysis of variance and ranking test techniques (Rao, 1968).

It will thus be observed that when berberine was administered 1 h before (treatment A) or simultaneously with toxin as in the case of treatment G, there was no improvement in the survival time of the animals. On the other hand treatments B, C, D, E, or F were beneficial to the toxin administered rabbits.

Discussion

Berberine is as effective as chloramphenicol or tetracycline in controlling experimental cholera in infant rabbits infected with cholera vibrios. Our present study relates to the action of berberine on cholera in infant rabbits after the administration of a single fatal dose of choleragenic toxin. The results show that if treatment was begun 24 h before the administration of toxin, then a number of animals could be saved from death or the life of the remaining animals could be prolonged as compared to the control untreated animals.

TABLE 1. *Effect of berberine on toxin*-induced experimental cholera in infant rabbits*

Exp. No.		Class of treatment	No. of animals	Average wt (g)	Survival	Mean survival time (h) ± S.E.	Significance with reference to control‡
1	A	Treated	5	110·4	0/5†	14·80± 1·96	Not significant
		Control	3	108·3	0/3	11·30± 0·71	
2	B	Treated	4	91·7	3/4	66·00± 6·00	Significant
		Control	3	94·3	0/3	11·00± 0·00	
3	C	Treated	10	88·0	7/10	67·00± 3·20	Significant
		Control	3	100·0	0/3	18·00± 0·00	
4	D	Treated	6	98·3	3/6	45·67±11·75	Significant
		Control	4	97·5	0/4	15·00± 0·00	
5	E	Treated	5	119·8	3/5	56·40±10·63	Treated/Control$_{I}$ Significant
		Control (I)	5	112·0	0/5	18·00± 0·00	Treated/Control$_{II}$ Significant
		Control (II)	4	109·7	0/4	17·50± 0·75	Control$_{I}$/Control$_{II}$ Not significant
6	F	Treated	5	98·0	3/5	57·20± 9·30	Significant
		Control	4	100·0	0/4	15·50± 0·40	
7	G	Treated	5	113·0	0/5	14·00± 1·10	Not significant
		Control	5	98·0	0/5	13·20± 0·50	

* Syncase toxin was used in all experiments except experiment No. 6, *viz.* treatment F where sonicate toxin was used. † No. of rabbits surviving for 72 h/total number used. ‡ See appendix.

In vitro studies by many investigators (Nair *et al.*, 1967 ; Amin *et al.*, 1969 ; Mekawi, 1968 ; Dutta, unpublished) have shown that berberine inhibits the growth of several enteric bacteria, namely, *Shigella dysenteriae, Escherichia coli, Staphylococcus aureus,* besides *V. cholerae* though it is not as potent as chloramphenicol or tetracycline.

Experimental cholera induced in infant rabbits by administration of the toxin is a severe disease. Under the conditions of the experiments, non-treated animals succumb to the disease in the course of 18 hours. If the benefit of any drug is to be assessed on such a model, then it has to be administered well before the toxin is given. If a drug could prevent death or prolong the life of the animal, then it would not be unreasonable to assume that it would be useful clinically where toxin formation is progressive and gradual, being dependent on the multiplication of the vibrios. Pretreatment with berberine could save infant rabbits from the adverse effects of choleragenic toxin and could save about 70% of them. Bhide, Chavan & Dutta (1969) showed that the concentration of berberine in the blood of infant rabbits reaches a maximum 8 h after the administration of the drug, and the drug is found in the blood even after 72 hours. For this reason pretreatment was necessary as mentioned before, so that the appropriate concentration of drug was attained in the blood before the toxin reached the site of action. In those animals which lived, diarrhoea was controlled within 40 h and the severity of the symptoms was reduced in those cases where there was prolongation of life.

Mekawi (1968) protected mice from death due to cholera infection by injecting them with 0·3 mg of berberine intramuscularly. He also demonstrated that the mice could be protected against cholera toxin. In the latter case, he had attributed this to the detoxifying effect of berberine on cholera toxin.

The study undertaken by Mekawi is not comparable with ours. He had demonstrated the effect of berberine on endotoxin in mice, while we have shown here the activity of berberine on diarrhoeal toxin in infant rabbits. Unlike the work of Modak, Modak & Venkatraman (1970b), the detoxifying effect of berberine on choleragenic toxin could not be elicited though a mixture of toxin and berberine was incubated for 4 h and then administered to the rabbits. The animals died of severe diarrhoeal symptoms and the duration of the disease was not significantly longer than that of the controls.

The mechanism by which berberine controls diarrhoeal symptoms, be it in infected or toxin treated rabbits, still remains speculative. Modak and his co-workers (Modak *et al.*, 1970a) have shown that protein and lipid synthesis were stimulated by berberine 2·5 times more than the control. Since cholera toxins (endo- as well as exo-) contain both protein and lipid components, the formation of toxin by *V. cholerae* in the presence of berberine could be hampered. Analysis of fatty acid composition (lipid components) of *V. cholerae* in the presence of berberine revealed that the alkaloid caused disturbance (presumably biosynthetic) in the relative proportion of various fatty acids. According to Modak *et al.* (1970a), high turnover of proteins also suggests the possible formation of heterogenous proteins. They suggested that berberine might not allow the formation of active and intact toxins.

Pharmacological properties of berberine such as depression of intestinal peristalsis and removal of inflammatory congestion of the mucosal surface of the intestine may promote recovery from diarrhoea.

While the antibacterial effect of berberine on *V. cholerae* could be partly

responsible for its action in clinical cases, the real action of the drug in arresting the severe diarrhoeal symptoms cannot be fully explained. We have shown here that berberine is effective in experimental cholera when toxin was administered and the action of berberine was not due to the interaction between the choleragenic toxin and the drug. Moreover, berberine is not only effective against diarrhoeal symptoms caused by *V. cholerae* and related organisms, but also by other intestinal organisms such as *Shigella, Pseudomonas, Escherichia coli* and *Proteus* (Purohit & Subhadra Rao, 1969), parasites (Deshpande, 1969) and protozoa (Subbiah & Amin, 1967 and Dutta & Iyer, 1968). It appears, therefore, that the host response plays an important part in the recovery from diarrhoea when medicated with berberine.

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Appendix

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There were seven treatments with a control for each treatment group. The analysis of variance with reference to all the experimental treatments is given below:

	D.F.	S.S.	M.S.	F.
Between treatments	6	17006·78	2834·46	9·62
Within treatments	33	9719·22	294·52	
Total	39	26726·00		

The variance ratio 9·62 on 6 and 33 D.F. was significant at the 5% level, indicating real differences in mean values. The latter were written in descending order.

Rank order	1	2	3	4	5	6	7
Treatment	C	B	F	E	D	A	G
Mean	67·00	66·00	57·20	56·40	45·67	14·80	14·00

It appeared that the observed inequality between any two of treatments 1 to 5 was not in doubt, as the values were close. Similarly, treatments 6 and 7 appeared equivalent. The analysis of variance for the apparent equivalent groups 1 to 5 was as follows:

	D.F.	S.S.	M.S.	F.
Between treatments (1–5)	4	1952·38	488·10	1·27
Within treatments	25	9618·42	384·74	
Total	29	11570·80		

The variance ratio 1·27 on 4 and 25 D.F. was not significant at the 5% level, indicating thereby no real differences in mean values with the treatment B, C, D, E or F.

The observed inequality might be due to any treatment of B, C, D, E or F and any treatment of A or G. For this, the analysis of variance between the treatments E, D, A and G is set out below:

	D.F.	S.S.	M.S.	F.
Between treatments (D, E, A and G)	3	7162·58	2387·53	6·21
Within treatments	17	6538·42	384·61	
Total	20	13701·00		

The variance ratio 6·21 on 3 and 17 D.F. was significant at the 5% level. Thus, it is seen that treatment B, C, D, E or F form one group, and the treatment A or G form another group and these two groups appeared unequal. To examine this, we computed the 5% critical difference between the mean values of the treatments D and A, the lowest and the highest in the respective groups.

The critical difference was given by the formula

$$\left[\left(\frac{1}{n_1}+\frac{1}{n_2}\right)\hat{\sigma}^2\right]^{\frac{1}{2}}_{t_\alpha}$$

Where n_1 and n_2 were sample sizes and $\hat{\sigma}^2$ was the estimate of σ^2 which was 384·61 on 17 D.F. and t_α was the α level significant value of $|t|$ on 17 D.F. The critical difference was worked out at 25·05.

The observed difference in mean values (45·67–14·80=30·87) was larger than the critical difference, indicating that the two treatments D and A gave significantly different mean values. This showed that the treatment B, C, D, E and F gave higher survival time than treatment A or G.