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Studies on the Treatment of Fascioliasis X. Anthelmintic Effect of Rafoxanide Against Immature *Fasciola* sp., Especially Worms at Early Stages of Infection, in Rabbits

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A number of drugs have already been used for the treatment of fascioliasis in sheep and cattle. Treatment with their recommended dose levels generally has a good effect against the adult fluke, but little or no effect against the immature fluke. For this reason have been making many investigators efforts to find means and methods of killing the immature fluke of *Fasciola hepatica* in its early stage of development. Kendall and Parfitt (1962) and others obtained good results with hexachlorophene against immature flukes of *F. hepatica* by using a dose larger than the optimal one under experimental conditions. Boray and Happich (1968) and others, however, observed the occurrence of severe intoxication and death after administration with the optimal dose of this compound under field or experimental conditions. Davis *et al.* (1966) and Lucas (1967) obtained significant results from administration with an optimal dose or a dose a little larger than this of nitroxinil against immature liver flukes.

The anthelmintic efficacy of rafoxanide particularly against immature flukes of *F. hepatica* in sheep was reported first by Mrozik *et al.* (1969) and later by Boray (1969).

The purpose of this paper is to record the information collected by an experiment designed to evaluate the efficacy of rafoxanide against immature liver flukes of definite ages in rabbits.

Materials and Methods

1. Drug.

Rafoxanide [3,5-diiodo-3′-chloro-4′ (p-chlorophenoxy) salicylanilide] was used for this experiment. It is off-white crystalline powder readily soluble in organic solvents but insoluble in water. Its melting point ranges from 173 to 177°C.

2. Experimental animals.

Twenty-four rabbits were used. They weighed from 1.9 to 2.4 kg. Of them, 21 were subjected to treatment and 3 served as controls. All of them were confirmed to be free from liver flukes by means of fecal examination prior to experimental infection.

Metacercariae were collected from *Lymnaea ollula*, an intermediate host that had been maintained at this laboratory. Snails of this species had been exposed experimentally to miracidia hatched from ova recovered from the gallbladder of cattle infected with *Fasciola* sp.

Eighteen rabbits of the treated group and all the rabbits of the control group were infected with 20 metacercariae each. The remaining 3 rabbits of the treated group were infected with 20 metacercariae four additional times at 3 or 4 days intervals after the first infection, the total number being 100 metacercariae.

3. Method of medication.

The 18 rabbits were divided into three groups, I, II, and III, which were medicat-

ed orally with rafoxanide in a drench form at a dose rate of 10, 25, and 50 mg/kg of body weight, respectively.

The rabbits of groups I and II were given rafoxanide at a dose rate of 10 and 25 mg/kg of body weight, respectively, at 21 and 28 days of experiment. Each rabbit of group III was given rafoxanide at a dose rate of 50 mg/kg body weight at 7 and 14 days of experiment. The three rabbits forming group IV and infected with 100 metacercariae were administered orally with 50 mg/kg six times at a week's intervals beginning at 7 days after infection. The three rabbits of the control group, or group V, were not treated at all.

4. Evaluation of the effect of the drug.

The anthelmintic efficacy of rafoxanide was evaluated by the numbers of liver flukes surviving and lesions detected in the liver in the postmortem examination. The interval between the date of infection and that of autopsy ranged from 56 to 72 days.

5. Observation of side effects.

Each rabbit was observed for appetite and the appearance of feces.

Results

1. Efficacy of rafoxanide against immature liver flukes.

Six rabbits of group I were administered orally with 10 mg/kg of rafoxanide 21 and 28 days after infection with metacercariae. Of them, five rabbits harbored no liver flukes in the liver, but the remaining one rabbit had only one immature fluke surviving. At the same time, six rabbits of group II were given a dose of 25 mg/kg of rafoxanide 21 and 28 days after infection. As a result, liver flukes were completely removed from them.

Six rabbits of group III were infected with liver flukes and given a dose of 50 mg/kg of rafoxanide 14 days after infection, but no liver flukes were recovered from the liver in any one of them. Four and seven flukes, however, were detected from two rabbits, respectively, out of three treated with the same dose of the drug 7 days after infection (Table 1).

Three rabbits of group IV were infected intermittently five times with 20 metacercariae each, or with a total of 100 metacercariae, and given a dose of 50 mg/kg of rafoxanide six times. As a result, no liver flukes were observed in any of them (Table 2).

Details in the number of flukes recovered from untreated control rabbits are shown in Table 3. Expressed as a proportion to the number of metacercariae administered, the rate of recovery of flukes amounted to 15.0% in No. 22, 50.0% in No. 23, and 60.0% in No. 24. All the metacercariae recovered showed normal viability evidently.

2. Liver lesion.

Table 4 shows pathological changes revealed 56 to 72 days after infection in rabbits exposed to metacercariae of *Fasciola* sp., the effect of rafoxanide given from 7 to 28 days after infection on these changes, and liver lesions in untreated rabbits.

In the untreated control rabbits, the greater part of the surface of the liver was covered with fairly firmly adherent patches of fibrin. Adhesions were present among the liver stomach, omentum, and diaphragm, and among the individual lobes of the liver. Numerous tract lesions, small hemorrhages, and multiple nodules were scattered on the visible part of the hepatic surface, as well as on the cut surface of it. Invasion of the liver by liver flukes resulted in atrophy of the left lobe accompanied by compensatory hypertrophy of the right lateral lobe. The wall of the extrahepatic bile duct was thickened and its lumen was several times larger than normal. Focal necrosis and cloudy swelling were found in the liver parenchyma. Connective tissue increased around the bile ducts. The intrahepatic bile ducts were distended with their walls thickened. The gallbladder often increased to twice its normal size and was filled with black or brown bile.

In the treated rabbits (cases of no recovery of liver flukes), the surface of the liver pre-

Table 1 Efficacy of rafoxanide against immature flukes of *Fasciola* sp. at 7 to 28 days after infection in rabbits

Group	Rabbit number	No. of metacercariae given	Dose rate mg/kg	Days from infection to admin.	Days from infection to autopsy	No. of worms recovered		Ova in bile	Side effect
						Adult	Immature		
I	1	20	10	21	69	0	0	—	—
	2	20	10	21	69	0	0	—	—
	3	20	10	21	69	0	0	—	—
	4	20	10	28	72	0	0	—	—
	5	20	10	28	72	0	1	—	—
	6	20	10	28	72	0	0	—	—
II	7	20	25	21	71	0	0	—	—
	8	20	25	21	71	0	0	—	—
	9	20	25	21	71	0	0	—	—
	10	20	25	28	72	0	0	—	—
	11	20	25	28	72	0	0	—	—
	12	20	25	28	72	0	0	—	—
III	13	20	50	7	68	0	4	—	—
	14	20	50	7	68	0	0	—	—
	15	20	50	7	68	0	7	—	—
	16	20	50	14	69	0	0	—	—
	17	20	50	14	69	0	0	—	—
	18	20	50	14	69	0	0	—	—

Side effect. — : No apparent toxic effects were observed.

Table 2 Efficacy of rafoxanide in rabbits infected with 100 metacercariae of *Fasciola* sp.

Group	Rabbit number	No. of metacercariae given	Dose rate mg/kg	Days from infection to autopsy	No. of worms recovered		Ova in bile	Side effect
					Adult	Immature		
IV	19	Given with 20 metacercariae 4 additional times at 3 or 4 days intervals after the first infection, total of 100 metacercariae	Administer orally with 50 mg/kg six times at a week's intervals beginning at 7 days after infection	71	0	0	—	—
	20			71	0	0	—	—
	21			71	0	0	—	—

Side effect. — : No apparent toxic effects were observed.

Table 3 Infection rate of *Fasciola* sp. in rabbits (Untreated control)

Group	Rabbit number	No. of metacercariae given	Days from infection to autopsy	No. of worms recovered		% recovery* of worms	Ova in bile
				Adult	Immature		
V	22	20	68	3	0	15.0	—
	23	20	56	0	10	50.0	—
	24	20	62	7	5	60.0	—

* Number of worms recovered/number of metacercariae given.

Table 4 Pathological changes of the liver from medicated and unmedicated infected rabbits at 56 to 72 days after infection

Group	Rabbit number	Days from infection to autopsy	Body weight at autopsy (kg)	Liver weight (g)	Number of worms recovered	Liver lesion: Surface: Fibrin on hepatic surface	Surface: Hemorrhage	Surface: Focal necrosis	Surface: Scar on hepatic surface	Surface: Protuberance	Surface: Adhesion	Parencyma: Anemia	Parencyma: Cloudy swelling	Parencyma: Hemorrhage	Parencyma: Focal necrosis	Parencyma: Protuberance	Parencyma: Abscess	Parencyma: Connective tissue increases around the bile ducts	Dilatation of the bile ducts	Dilatation of the ducts choledochus	Atrophy of the left lobe	Hypertrophy of the right lobe	Enlargment of the bilebladder
I	1	69	2.6	60	0	−	−	+	+	−	−	−	−	−	+	−	−	−	−	−	−	−	−
	2	69	2.7	90	0	−	−	+	+	−	−	−	−	−	+	−	−	−	−	−	−	−	−
	3	69	2.5	66	0	−	−	+	+	−	−	−	−	−	+	−	−	−	−	−	−	−	−
	4	72	2.7	69	0	−	−	+	+	+	−	−	−	−	+	−	−	−	−	−	−	−	−
	5	72	2.7	82	1	+	++	++	+	+	−	−	+	++	++	−	−	+	−	−	−	−	−
	6	72	2.6	87	0	−	−	+	+	+	−	−	−	−	+	−	−	−	−	−	−	−	−
II	7	71	2.5	68	0	−	−	+	++	−	−	−	−	−	+	−	−	−	−	−	−	−	−
	8	71	2.5	64	0	−	−	+	+	−	−	−	−	−	+	−	−	−	−	−	−	−	−
	9	71	2.4	67	0	−	−	+	++	+	−	−	−	−	+	−	−	−	−	−	−	−	−
	10	72	2.8	74	0	−	−	+	+	−	−	−	−	−	+	−	−	−	−	−	−	−	−
	11	72	2.5	75	0	−	−	+	+	−	−	−	−	−	+	−	−	−	−	−	−	−	−
	12	72	2.6	71	0	−	−	+	++	−	−	−	−	−	+	−	−	−	−	−	−	−	−
III	13	68	2.5	122	4	+++	++	++	++	++	++	+	+	++	++	+	++	+	−	−	+	+	++
	14	68	2.4	53	0	−	−	+	+	−	−	−	−	−	+	−	−	−	−	−	−	−	−
	15	68	2.4	91	7	+	++	++	++	++	++	+	+	+++	++	+	−	+	−	−	+	+	++
	16	69	2.7	75	0	−	−	+	+	−	−	−	−	−	+	−	−	−	−	−	−	−	−
	17	69	2.7	77	0	−	−	+	+	−	−	−	−	−	+	−	−	−	−	−	−	−	−
	18	69	2.5	71	0	−	−	+	+	+	−	−	−	−	+	−	−	−	−	−	−	−	−
IV	19	71	1.9	53	0	−	−	+	++	−	−	−	−	−	+	−	−	−	−	−	−	−	−
	20	71	2.6	55	0	−	−	+	+	+	−	−	−	−	+	−	−	−	−	−	−	−	−
	21	71	2.6	60	0	−	−	+	++	−	−	−	−	−	+	−	−	−	−	−	−	−	−
V (Untreated control)	22	68	2.5	89	3	+	+	++	++	++	++	+	+	++	++	+	+	+	++	++	+	++	++
	23	56	2.5	109	10	++	++	+++	++	++	+++	++	++	+++	+++	+	−	+	+	−	+	+	+++
	24	62	2.5	129	12	+++	+++	+++	++	+++	+++	++	+++	+++	+++	++	++	+	++	+	+	++	+++

+ : Slight changes. ++ : Middle changes. +++ : Serious changes.

sented small scar and sometimes fibrinous or membranous deposits. Similar lesions were seen on the cut surface of the organ. The bile ducts were normal in size.

3. Side effects.

None of the treated rabbits manifested any clinical sign of intoxication following administration with 10 to 50 mg/kg of rafoxanide.

Discussion

In the treatment of fascioliasis with an anthelmintic, it is desirable that the drug will show efficacy aginst immature and mature flukes. In the past few years many chemical compounds have been synthesized, studied, and recommended for the treatment of fascioliasis of cattle in Japan. Some of these compounds have been introduced into practice as active principles of fasciolicidial drugs, such as bithionol and salicylanilide derivatives. When used at their recommended dose levels, these drugs generally have a good action against the adult fluke, but little or no effect against the immature fluke. Consequently, there remains a need for a safe and effective drug for the prevention of liver flukes from invading the bile duct in the liver.

The present experiment was performed on 24 rabbits to examine rafoxanide for therapeutic effect against infection with immature liver flukes.

It is evident from Table 1 that doses of 10 and 25 mg/kg of the drug are equal in efficacy against immature flukes 3 or 4 weeks of age. A single dose of 50 mg/kg of the drug was fairly effective against immature flukes 2 weeks of age. It had, however, no effect against flukes 1 week of age. It can be concluded from these results that an optimum dose of the drug for a rabbit lies between 10 and 25 mg/kg to remove immature flukes from 2 to 4 weeks after infection. This dose is highly effective against immature flukes which have already entered the bile ducts, dispensing with the necessity of a second treatment against mature flukes.

Mrozik *et al.* (1969) reported that rafoxanide was highly effective against adult flukes and 4- to 6-week-old ones in the parenchymal stage of *F. hepatica* in sheep. Campbell *et al.* (1970) mentioned that rafoxanide was highly effective against 6-week-old flukes at a dose of 7.5 mg/kg or more and against 4- week-old flukes at a dose of 10 mg/kg or more. The drug exhibited variable efficiency against 2-week-old flukes even when administered at a dose larger than 15 mg/kg.

Ross (1970) clarified that rafoxanide had an efficacy against immature flukes in sheep. The present experiments showed that the drug was 45 and 67% efficient against flukes 4 weeks of age when administered at a dose of 7.7 and 10 mg/kg, respectively. A dose of 7.5 and 10 mg/kg was 87 and 97% efficient against flukes 6 weeks of age, respectively. According to Armour and Corba (1970), rafoxanide showed a remarkable effect against immature flukes (2, 4, and 6 weeks of age) in sheep when administered at a single dose of 7.5 or 15.0 mg/kg, destroying 36 to 99 % of 2-, 4-, or 6-week-old flukes harbored.

Recently, Kimura (1973) conducted an experiment on the effect of rafoxanide in which 21 rabbits were first infected with 20 metacercariae of *Fasciola* sp. and then given doses ranging from 50 to 100 mg/kg of rafoxanide. Each of them was administered orally with the drug by the aid of a pipette. Autopsy was performed during a period from 24 to 77 days after infection. A single dose of 50 mg/kg of the drug was highly effective against immature flukes 3, 4, and 5 weeks of age. A single dose of 75 or 100 mg/kg was fairly effective against immature flukes 3 and 4 weeks of age.

On the other hand, rafoxanide revealed an efficacy greater than 90% against mature flukes when evaluated in sheep and cattle at a dose of 2.5 to 10.0 mg/kg (Boray, 1969; Ross, 1970; Campbell *et al.*, 1970; Campbell and Hotson, 1971; Knapp and Presidente, 1971).

The relationship between development of fluke and efficacy of drug is very important. Broome and Jones (1966) reported that anthelmintics had a comparative efficacy against adult and immature flukes. The present experiments showed that both adult (12-week-old) and immature (3-week-old) flukes were equally susceptible to the action of 2, 2′-dihydroxy-3, 3′, 5, 5′, 6-pentachlorobenzanilide when investigated under identical conditions *in vitro*, suggesting that immature flukes might have received some sort of protection from their particular habital *in vivo*. Such protection may be provided in two ways. First, the drug may be discharged into the bile duct as an active metabolite (glucuronide), which may concentrated preferentially around adult flukes inhabiting the bile duct. Secondly, the activity of the drug may considerably be reduced in the presence of blood and serum, presumably by protein binding. This reduction may reduce the effectiveness of the drug against immature flukes in the liver tissue.

Boray *et al.* (1967) studied a difference in anthelmintic efficacy against adult and immature flukes in sheep. It was found that there was hardly any difference in size or frequency distribution between flukes 4 and 6 weeks of age. Six-week-old flukes however, were appreciably more susceptible to anthelmintics than 4-week-old flukes. This suggests that in these stages of age the size of the fluke may not be so important as its age or physiological development.

In the test for eradication of immature flukes, an essential point is in what stage of worm after infection a drug should be administered to animals. The word "immature fluke" mentioned in every previous report of experiment, the stage of immature flukes at the time of treatment is not the same in all the experiments. As stated before, the immature fluke of the last infection-stage doses extremely heavy damage to host. If no immature flukes are eliminated in this stage, it will be really difficult to reduce severe liver lesions. It is required, therefore, to eliminate immature flukes in the early stage of infection.

Summary

Rafoxanide was effective against immature flukes of *Fasciola* sp. in rabbits when given in a diet over a period from 7 to 28 days after infection.

(1) Nineteen rabbits were infected experimentally with 20 metacercariae. A dose of 10, 25, or 50 mg/kg of the drug was highly effective against immature flukes 2, 3, and 4 weeks of age. A single dose of 50 mg/kg was slightly effective against immature flukes 1 week of age.

(2) Three rabbits infected intermittently five times with 20 metacercariae, or with a total of 100 metacercariae, were administered six times with a dose of 50 mg/kg of rafoxanide each. None of them were found to harbor any liver fluke in the liver.

(3) The mean percentage of recovery of *Fasciola* sp. was 41.2%, as compared with untreated controls.

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肝蛭症の治療に関する研究
(10) Rafoxanide による家兎感染幼肝蛭の駆除試験，特に感染初期虫体に対する効果

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家兎肝蛭の感染初期に Rafoxanide を投与し，幼肝蛭にたいする駆除効果について検討した．

家兎6頭に本剤 10 mg/kg を肝蛭感染後21日および28日に投与した．そのうち5頭には肝蛭はみとめられなかつたが，1頭には幼肝蛭1匹が棲息していた．また，25 mg/kg を感染後21日および28日に投与したものでは，幼肝蛭を完全に駆除することができた．さらに肝蛭感染後14日に 50 mg/kg を投与した家兎においても，幼肝蛭を駆除することができたが，同薬量を感染後7日に投与した家兎3頭において，そのうち2頭に肝蛭4，7匹を検出した．

肝蛭メタセルカリア20コを断続的に5回，計 100 コを感染させた家兎に，本剤 50 mg/kg を反復投与したところ，肝蛭はいずれの肝にも全くみとめられなかつた．

以上のとおり，家兎感染幼肝蛭にたいする Rafoxanide の駆除効果は，感染 14 日頃以降の感染初期虫体を殺滅し，またこれら家兎の肝病変もきわめて軽微であつた．このことから，本剤は肝蛭幼虫の駆除剤として有効な製剤であるといえる．