Praziquantel adversely affects protoscoleces of *Echinococcus granulosus* in vitro

R. C. A. THOMPSON, J. A. REYNOLDS and CICELIA R. RIDDLER

Division of Veterinary Biology and WHO Collaborating Centre for Echinococcosis/Hydatidosis, School of Veterinary Studies, Murdoch University, Western Australia 6150

ABSTRACT

Praziquantel was shown to have adverse effects on protoscoleces of *Echinococcus granulosus* in vitro. Exposure to one dose of praziquantel at a concentration of 10 μg/ml caused protoscoleces to die within 12 to 15 days. Protoscolecidal effects were more marked when cultures were exposed to the drug continuously by the addition of multiple doses. On the basis of these observations, there is a need to re-appraise the metacestocidal potential of praziquantel, particularly with regard to the development of new drug regimes employing multiple dose or sustained release schedules. Possible reasons for the reduced susceptibility of protoscoleces and juvenile worms to praziquantel observed in this study are discussed.

KEY WORDS: *Echinococcus granulosus*, praziquantel, protoscoleces

INTRODUCTION

Although praziquantel is effective in causing the paralysis and eventual breakdown of adult tapeworms *in vivo* (reviewed in Gemmell & Johnstone, 1981; Andrews et al., 1983), praziquantel has variable effects against taenid metacestodes. It is effective against the metacestodes of *Taenia* spp. *in vivo* but has minimal effects, if any, against larvae of *Echinococcus* species (Gemmell & Johnstone, 1981; Andrews et al., 1983). No activity was found against protoscoleces or cysts of *E. granulosus* either *in vitro* or *in vivo* (Heath & Lawrence, 1978; Gemmell & Parmeter, 1983). Similar results were obtained with *E. multilocularis* *in vivo* (Eckert et al., 1977; Thomas & Gonnert, 1978) although protoscoleces from cysts were no longer infective to dogs. However, there is some evidence that long term exposure to praziquantel may increase metacestocidal efficacy. Marshall & Edwards (1982) have shown that sustained release praziquantel is effective in inhibiting the development of secondary cysts of *E. granulosus* in mice. More recently, Li & Jun (1985) reported that in mice infected with secondary cysts of *E. granulosus* and fed a diet containing praziquantel for between six and 24 days, most protoscoleces were killed and there was degeneration of the germinal layer.

In view of the puzzling differences between the effects of praziquantel against larval and adult *Echinococcus* and the report that long term praziquantel treatment did affect the development of secondary hydatid cysts, it was considered important to investigate further the drug effect in our *in vitro* system as a prelude to more detailed studies on larval susceptibility.

MATERIALS AND METHODS

Experimental Design

*In vitro* exposure to praziquantel was at a concentration of 10 μg/ml. However, the length of exposure varied depending on whether drug was added once at the start of the experiment or as multiple replacement doses following each change of media every 36 hours. The present study was divided into three experiments (Table I). In the first, invaginated protoscoleces were either exposed to a single concentration of praziquantel (10 μg/ml) at the start of the experiment or as
TABLE I. Exposure of *Echinococcus granulosus* to praziquantel *in vitro*: Experimental design

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Pre-Incubation</th>
<th>Drug Exposure*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1—Protoscoleces</td>
<td>Nil</td>
<td>Single and Multiple</td>
</tr>
<tr>
<td>2—Group 1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>—Group 2</td>
<td></td>
<td>Multiple</td>
</tr>
<tr>
<td>—Protoscoleces</td>
<td></td>
<td>Multiple</td>
</tr>
<tr>
<td>—Group 3</td>
<td>Pepsin/bile</td>
<td>Multiple</td>
</tr>
<tr>
<td>—Group 4</td>
<td>Bile</td>
<td>Multiple</td>
</tr>
<tr>
<td>3—Group 1—Protoscoleces</td>
<td>Pepsin/Bile**</td>
<td>Single</td>
</tr>
<tr>
<td>—Group 2—Adult worms</td>
<td>Nil</td>
<td>Single</td>
</tr>
</tbody>
</table>

*In vitro* concentration of praziquantel was 10 μg/ml. Length of exposure depended on whether a single initial dose of drug was given or multiple replacement doses following each change of culture media. **Following pre-incubation, *in vitro* development was allowed to continue for a further 7 days before exposure to praziquantel.

multiple concentrations of 10 μg/ml every 36 hours. In the second experiment, the effects of pre-incubating protoscoleces in pepsin and/or bile was examined in order to determine whether these agents increased the susceptibility of the protoscolex to praziquantel directly by affecting the tegument, or indirectly by stimulating evagination. The drug was administered as multiple replacement doses every 36 hours. In the third experiment, protoscoleces were pre-incubated in pepsin/bile evaginating solutions and allowed to develop *in vitro* for seven days before being exposed to a single concentration of 10 μg/ml of praziquantel. In this experiment, cultures containing 37-day-old adult worms removed from experimentally infected dogs were subjected to the same concentration of praziquantel. This experiment was designed to determine whether a short period of development in a strobilar direction rendered the parasite more susceptible to praziquantel. In all experiments, control cultures which were not exposed to praziquantel were maintained.

**Parasite Material**

Protoscoleces of *E. granulosus* were collected aseptically from sheep hydatid cysts and washed in four changes of Hanks balanced saline solution (HBSS) containing antibiotics (0.4 μg penicillin ml⁻¹, 100 μg gentamycin ml⁻¹, 2 μg amphotericin ml⁻¹) before being treated as detailed in Table I. Protoscoleces were treated and placed in culture within two hours of removal from the cyst. Only batches of protoscoleces with a mean viability of 80%, as judged by flame cell activity, were used. Adult worms were recovered from experimentally infected dogs and processed for *in vitro* cultivation as previously described (Kumaratilake & Thompson, 1983).

**In vitro Cultivation**

*E. granulosus* were maintained in 30 ml sterile tissue culture flasks (Lux) at 37°C. Cultures were monophasic comprising 10 ml of medium CMRL 1066 (Flow Laboratories) with supplements as described previously (Thompson et al., 1982) but without the addition of dog bile. The culture fluid was changed every three days. Pepsin and bile pre-incubation solutions were prepared according to Smyth & Davies (1974). Protoscoleces were incubated in pepsin for 20 min and in the bile evagination solution for 16 hours. Micronized praziquantel was dissolved in HBSS using a glass hand homogenizer and added to cultures initially and where necessary, following each change of media.
RESULTS

Experiment 1

Within a few minutes of adding the drug, all protoscoleces exhibited shuddering movements and became slightly deformed becoming more square than rounded in profile. After 18 hours, some ballooning and dissociation of the tegument was seen in drug-treated protoscoleces which had also contracted (Fig. 1). They were more shrunken and deformed after 26 hours and, in many cases, were covered in small blebs or blisters (Fig. 2). A few protoscoleces had started to degenerate. At this stage approximately 60% of treated protoscoleces were partly evaginated and active although still retaining a squat appearance (Fig. 2) whereas those still invaginated had become vesicular (Fig. 2). Most of the untreated controls were still invaginated and unaltered morphologically (Fig. 3). After 76 hours little further change was observed in those cultures which had received a single initial dose of praziquantel. However, in cultures which had received a second dose of drug after 36 hours, protoscoleces were darker, less active and both invaginated and evaginated forms were now vesiculating. After 126 hours, those cultures which had been exposed to a single dose of praziquantel had changed little apart from some internal vacuolation (Fig. 4), whereas in cultures treated with three doses, the protoscoleces, although still alive, were inactive, vesicular, possessed bladders and in some cases were losing their hooks (Fig. 5). At 176 hours, single-dose cultures were largely unchanged apart from being more vesiculated, although by this time, untreated protoscoleces had also started to swell and become cystic. Cultures which had by 176 hours received four doses of praziquantel contained large numbers of degenerating and dying protoscoleces (Fig. 6). The remainder were abnormal morphologically and exhibited marked ballooning and dissociation of their tegument (Fig. 7). Protoscoleces in single-dose cultures were less active after 226 hours and darker than untreated controls, whereas multiple-dose cultures contained many dead and dying forms. After 296 hours, a few protoscoleces had died in single dose cultures, whereas 70% of those in multiple-dose cultures were dead. Cultures were terminated after 368 hours at which stage 70% of protoscoleces in single-dose cultures were dead and the remainder mostly large and vesicular (Fig. 8), in contrast to untreated control cultures in which most of the protoscoleces were still alive. Over 95% of protoscoleces in multiple-dose cultures were dead at 368 hours (Fig. 9).

Experiment 2

As in Experiment 1, invaginated protoscoleces in Group 1 (Table 1) exhibited some shuddering movement after the addition of praziquantel to the media and assumed a more squat appearance. However, most of the protoscoleces which had been pre-incubated in pepsin and/or bile had started to evaginate and following addition of the drug immediately contracted and became less active than controls. After 48 hours, invaginated protoscoleces in Group 1 had started to become vesicular, although most were partly evaginated in contrast to untreated controls. Bladders had formed on many protoscoleces and in some there was disruption and loss of hooks from the partly evaginated rostellum (Fig. 10). Evaginated protoscoleces in Groups 2, 3 and 4 all appeared to be equally susceptible to the effects of praziquantel and were inactive, shrunken, with bladders and many were losing their hooks. Those still invaginated were vesicular and similar to Group 1. In contrast, untreated controls which had been pre-incubated were active, vermiform and elongating (Fig. 11). From 96 hours onwards, evaginated protoscoleces in all groups started to degenerate and those still invaginated became increasingly
swollen and vesicular. By 192 hours, the numbers of evaginated protoscolecies degenerating and dying had increased and most invaginated protoscolecies in Group 1 were much darker. Most of the drug-treated protoscolecies were dead by 264 hours.

Experiment 3

After seven days in vitro, protoscolecies had evaginated, elongated and were very active (Fig. 12). Lateral excretory canals were evident but there was no sign of the banding which precedes segmentation, or any loss of calcareous corpuscles. Following addition of praziquantel to the media, the juvenile worms immediately contracted, their motility decreased and they became triangular in shape. External blebs and ballooning of the tegument appeared rapidly (Figs. 13, 14). 37-day old adult worms (Fig. 15) exposed to the same dose of drug also immediately contracted and became inactive (Fig. 16). However, adult worms also became dark and opaque, and within five minutes extensive tegumental disruption occurred leading to disintegration of many adult worms. Adult worms had completely lost their form within 12 min and after 30 min most had lost their hooks (Fig. 17). No such extensive changes were observed with juvenile worms until after 18 hours when many were deformed and exhibited ballooning of the tegument and loss of hooks. After 30 hours, juvenile worms were darker and exhibited little activity whereas most of the adult worms were dead and no longer recognizable morphologically, being devoid of hooks, dark and opaque, grossly deformed and vesicular (Fig. 18). After 80 hours, juvenile worms were largely inactive and some were dead. After 130 hours, juvenile worms had lost their calcareous corpuscles and began to vaculate internally. Juvenile worms continued to deteriorate and, after 372 hours, 70% were dead, and the rest were swollen and vesicular. At this time, untreated juvenile worms were still active and had increased in length. Most worms had lost their calcareous corpuscles and showed evidence of banding prior to formation of the first segment.

DISCUSSION

We have demonstrated that praziquantel does adversely affect protoscolecies of *E. granulosus* in vitro. Exposure to a single concentration of 10 μg/ml caused protoscolecies slowly to vesiculate and darken over a period of several days with most dying between 12 and 15 days after treatment. Continuous exposure, by giving multiple replacement doses of praziquantel over a period of 15 days, caused

FIG. 1. Contracted protoscolecies of *E. granulosus* 18 hours after exposure to 10 μg/ml praziquantel in vitro. Note ballooning of tegument (40×). FIG. 2. Protoscolecies 26 hours after exposure to 10 μg/ml praziquantel. Many are covered in small blebs (arrow) or blisters. Most of the protoscolecies in this photograph are partly evaginated but one at top left is invaginated and cystic (20×). FIG. 3. Untreated controls. Note protoscolecies are invaginated and unaltered morphologically (20×). FIG. 4. Protoscolecies 126 hours after exposure to a single initial dose of praziquantel showing internal vaculation (arrow). (40×). FIG. 5. Protoscolecies 126 hours after continuous (multiple doses) exposure to 10 μg/ml praziquantel. Note bladders and loss of rostellar hooks (arrow). (40×). FIG. 6. Protoscolecies degenerating and dying after 176 hours continuous exposure to praziquantel (40×). FIG. 7. Protoscolex 176 hours after continuous exposure to praziquantel showing marked ballooning and dissociation of tegument (40×). FIG. 8. Large vesicular protoscolex (arrow) 368 hours after exposure to a single initial dose of praziquantel (20×). FIG. 9. Dead protoscolecies 368 hours after continuous exposure to praziquantel (20×). FIG. 10. Partly evaginated protoscolecies 48 hours after continuous exposure to praziquantel showing bladders and loss of hooks (40×).
more marked protoscolecidal effects and earlier death. These results are striking in view of the low solubility of praziquantel in aqueous solution. The long term exposure of protoscoleces *in vitro* to a number of separate doses of praziquantel has not previously been investigated and, in fact, protoscoleces have not been maintained *in vitro* for longer than 24 hours following drug treatment. We

![Images](11-18)

**FIG. 11.** Untreated control culture containing protoscoleces 48 hours after incubation in pepsin and bile evagination solutions (20x). **FIG. 12.** Untreated culture containing evaginated protoscoleces/juvenile adults after 7 days *in vitro* (20x). **FIGS. 13 & 14.** Contracted, seven-day-old juvenile worms 3 min after exposure to a concentration of 10 µg/ml praziquantel, showing formation of blebs (Fig. 13) and ballooning of the tegument (Fig. 14) (40x). **FIG. 15.** Untreated 37-day-old adult worms *in vitro* (8x). **FIG. 16.** Contracted 37-day-old adult worms, 3 min after exposure to a concentration of 10 µg/ml praziquantel (8x). **FIG. 17.** Degenerating 37-day-old adult worms, 30 min after exposure to praziquantel. Note loss of hooks and tegumental disruption (20x). **FIG. 18.** Dead adult worm 30 hours after exposure to praziquantel (8x).
therefore feel that a reappraisal of the metacestocidal potential of praziquantel is warranted and that contrary to the opinions of other workers (Andrews et al., 1983; Gemmell & Parmeter, 1983), further studies of the action of praziquantel against metacestodes of Echinococcus are justified.

Our observations complement those of Marshall & Edwards (1982) who found that sustained exposure in vivo significantly inhibited secondary cyst development of E. granulosus in mice. Further, the results of Li & Jun (1985) indicate that continued treatment with praziquantel over several days can cause degeneration of the germinal layer in secondary cysts. Emphasis should now be given to the assessment of drug regimes employing multiple dose treatment schedules or sustained release formulations so that the therapeutic effects of praziquantel against hydatidosis can be reassessed in established model systems (Anon., 1981). Since drug interactions with praziquantel have not been reported, consideration could also be given to achieving greater metacestocidal efficacy by combining the use of praziquantel and mebendazole, a drug known to penetrate, and possess efficacy against, metacestodes of Echinococcus (Reisin et al., 1977; Eckert et al., 1978; Eckert, 1986).

Praziquantel may also be of value by rendering cysts in domestic animals non-infective to the definitive host. Not only does praziquantel cause the eventual death of protoscoleces, but the morphological and degenerative changes which precede death would affect their ability to develop in a strobilatate direction. Further, most drug-treated protoscoleces evaginate and would not survive passage through the stomach of the definitive host. These assumptions are supported by in vivo studies in which protoscoleces from treated cysts were no longer infective to dogs (Thomas & Gonnert, 1978). Consequently, in certain endemic areas where domestic cycles predominate, transmission of E. granulosus may be more effectively broken by treating domestic animals as well as dogs.

Compared to adult Echinococcus, protoscoleces and juvenile worms are much less susceptible to the effects of praziquantel. Invaginated protoscoleces are the least affected, probably because the scolex is protected. Marchonho & Andersen (1983) have shown that the basal region of the protoscolex is covered in a layer of mucopolysaccharide. Since the apical region of the protoscolex (suckers, rostellum and hooks) is not covered by this layer but is invaginated within the basal region, the mucopolysaccharide coating may protect the protoscolex from the detrimental effects of praziquantel. However, invaginated protoscoleces and juvenile worms also exhibit reduced susceptibility suggesting additional survival factors. In this respect, the calcareous corpuscles may play a role. The large numbers present in the protoscolex and juvenile worm disappear just before segmentation, at around 11 days after infection. It is possible that the calcareous corpuscles act by sequestering the drug. Alternatively, variation in susceptibility could be related to permeability or metabolic differences between protoscoleces and adult worms.

The strong cestocidal activity of praziquantel was demonstrated by the in vitro exposure of 35-day-old adult worms, which rapidly degenerated and died. In contrast, seven-day-old juvenile worms took several days longer to die. However, the fact that juvenile worms rapidly contracted and lost motility would be sufficient to cause their dislodgement from between the villi in vivo and subsequent expulsion from the body. In relation to this, trials in vivo suggest that praziquantel is effective in removing adult Echinococcus as young as three days old from dogs (Andrews et al., 1983).

Future research will hopefully elucidate the factors responsible for differential susceptibility to praziquantel between metacestode and adult stages of Echinococ-
cous, and as a consequence, lead to the development of new regimes for metacestocidal therapy.

ACKNOWLEDGEMENTS
This work was supported by a grant to Dr. Thompson from the Australian Research Grants Scheme. We thank Bayer for the micronized praziquantel.

REFERENCES

Accepted 2nd July, 1986.