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SHORT COMMUNICATION

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Echinococcus granulosus: membrane permeability of secondary hydatid cysts to albendazole sulfoxide

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Abstract The objectives of the present study were, first, to establish a methodology for evaluation of the permeability in vitro of hydatid cysts to different drugs and, second, to compare the permeability to albendazole sulfoxide of cysts from untreated animals, cysts from animals treated with 50 mg/kg netobimin for 5 days, and cysts from animals treated with 50 mg/kg netobimin plus 1.1 mg/kg fenbendazole for 5 days. The drug flow follows the Fick law, i.e., the uptake occurs by simple diffusion. We calculated the permeability constant of the cyst membrane by taking into account the disappearance velocity constant, the cyst area, and the incubation solution volume. The permeability value obtained for albendazole sulfoxide was $8.06 \pm 2.30 \times 10^{-6}$ cm s^{-1} in cysts from untreated animals, $5.56 \pm 2.53 \times 10^{-6}$ cm s^{-1} in cysts from animals treated with netobimin, and $7.05 \pm 3.04 \times 10^{-6}$ cm s^{-1} in cysts from animals treated with netobimin + fenbendazole. These permeability values show significant differences ($P < 0.05$).

Introduction

The success of the chemotherapeutic treatment of hydatid disease is based upon the capacity of the drug to operate on the germinal layer and the protoscolices of the hydatid cyst interior at adequate concentrations for sufficient periods. Besides other reasons, the diversity of results obtained by several authors in the treatment of hydatid disease can be explained by the permeability of the cystic membranes to the drug used, as the pharmacokinetic properties of drugs is one factor directly related to their therapeutic effectiveness (Schantz et al. 1982). For the design of an effective chemotherapeutic protocol to treat this zoonosis, permeability studies of the hydatid cysts should be performed.

Even though the high permeability of the cystic membranes to water has been proven (Rotunno et al. 1974; Reisin and Pavisic de Fala 1984), the anthelmintic currently most used in the treatment of human hydatid disease, albendazole (ABZ), and its active metabolite albendazole sulfoxide (ABZSO) are characterized by their low solubility in water and poor absorption. Anthelmintic prodrugs have been developed to overcome these problems (Lanusse and Prichard 1993). Netobimin (NTB) is an inactive prodrug used in veterinary medicine that is converted into active ABZ in the host.

The first aim of this research was to establish a method that would enable us to study in vitro the permeability of the hydatid cyst membrane to new drugs used for therapeutic purposes. The second objective was to compare the permeability of untreated cysts (control cysts) to ABZ's main active metabolite ABZSO, the permeability of cysts derived from infected animals treated with NTB (Schering-Plough, S.A.), and the permeability of cysts derived from infected animals treated with NTB + fenbendazole (FBZ; Hoechst Ibérica, S.A.).

The latter drug is another benzimidazole widely used in veterinary medicine that has proved to be inhibitory of albendazole sulfoxidase activity when incubated in vitro with ABZ (Galtier et al. 1986), decreasing the amount of ABZSO and the production of albendazole sulfone ($ABZSO_2$). Additionally, cystic permeability for $ABZSO_2$ was studied in control cysts. For these studies, hydatid cysts obtained from secondary hydatid disease – produced after intraperitoneal infection of gerbils (*Meriones unguiculatus*) with ovine hydatid material – were used.

Materials and methods

Gerbil infection

The hydatid cysts were obtained at 9–12 months after the infection of female gerbils through intraperitoneal inoculation of 1,200–1,500

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protoscolices of *Echinococcus granulosus* obtained from infected offal of ovines slaughtered at the municipal abattoir in León, Spain.

Drugs and treatments

For study of the membrane permeability to ABZSO of hydatid cysts from treated animals, 50 mg/kg NTB was given by gavage to some of the infected gerbils (three animals) for 5 days, whereas 50 mg/kg NTB plus 1.1 mg/kg (375 μ*M*) FBZ was given for 5 days to another group of infected gerbils (three animals). The cysts were obtained at 72 h after the end of the treatment. The control group (three animals) was infected in the same way but did not receive any drug.

Incubation of cysts

All parasitic material collected from the gerbils was carefully dried and weighed, and the most uniform cysts – those with clear intracystic fluid and a spherical shape that were adherence-free and, among the bigger cysts, those with protoscolices – were selected. After being washed in Krebs-Ringer solution (pH 7.4), cysts were separately incubated at 37 °C in Krebs-Ringer solution (pH 7.4), to which ABZSO or $ABZSO_2$ was added at a final concentration of 20 μg/ml, having previously been dissolved in dimethylsulfoxide (DMSO, 0.1% final concentration). The osmolarity was corrected by a decrease in the final content of NaCl from 7.07 mg/ml (121 m*M*) to 4.58 mg/ml (78.4 m*M*). The samples were kept in a 95% O_2 and 5% CO_2 atmosphere in closed containers (to avoid evaporation) placed in a shaker bath at 50 rpm (Reisin et al. 1977).

The studies were carried out using cysts whose weight was higher or lower than 2 g and incubation medium at volumes similar to or double that of the cyst weight. Under such conditions, samples of 25 μl were sequentially taken from the incubation medium at 0, 15, 30, 45, 60, 90, 120, 150, and 180 min until equilibrium concentrations were reached. The samples were stored at −20 °C for further evaluation.

Drug determination

The concentration of the drug was determined by reverse-phase high-performance liquid chromatography (HPLC) on a nucleosil C-18 column with a mobile phase composed of acetonitrile and deionized water (30/70). Acetic acid (5% v/v) was added to both solvents. The flow rate was 1 ml/min and the wavelength detector was adjusted at 292 nm. Under these chromatography conditions the retention times were 4.5 min for ABZSO and 6 min for $ABZSO_2$. The detection limit of the method was 0.05 μg/ml for both compounds.

Kinetic parameters

According to Reisin et al. (1977), we considered our model to be a two-compartmental system composed of hydatid fluid and incubation medium (the volume of the cyst wall is negligible as compared with the other compartments). The drug flow follows the Fick law of uptake by simple diffusion. Under these conditions the disappearance velocity constant K (per minute) was calculated by linear regression from the equation $Ln\ A = Ln\ A_0 - K\ t$, where A and A_0 are the concentration in the incubation medium at time t and the initial concentration, respectively. The correlation coefficients (r) obtained were > 0.965 in all experiments.

The permeability constant of the cyst membranes (in centimeters per second) was calculated (Reisin et al. 1977) by multiplying the constant K by the ratio of the incubation solution volume (in milliliters) to the hydatid cyst area (in square centimeters). The cystic area was obtained from the cyst weight, w (in grams), according to Rotunno et al. (1974), using the equation $Area = 4\pi\ (3w/4\pi)^{2/3}$. The uptake flow (in nanomolar per square centimeter per hour) was determined by multiplication of the permeability constant by the initial concentration of the drug in the incubation solution.

Statistical analysis

All statistical calculations were based on one-way analysis of variance (ANOVA/MANOVA). Differences were determined using the Least Significant Differences (LSD) test, and $P < 0.05$ was considered to be significant. Results are presented as mean values ± SD.

Results

The studies of permeability for both ABZSO and $ABZSO_2$ were performed in incubations with volumes similar to or double that of the hydatid cyst weight to check the uptake flow under such experimental conditions. When the permeability was calculated the

Table 1 Permeability values recorded for *Echinococcus granulosus* cysts derived from infected gerbils as determined by incubation of cysts with ABZSO and $ABZSO_2$. Data represent mean values ± SD

	Untreated	Netobimin treated	Netobimin + Fenbendazole treated
ABZSO:			
Equal-vol. incubation $K(min^{-1}) \times 10^{-3}$	1.85 ± 0.43 (6)[a]	1.28 ± 0.47 (7)	1.66 ± 0.58 (6)
Double-vol. incubation	0.91 ± 0.20[*a] (7)	0.51 ± 0.13[*a] (5)	0.63 ± 0.17[*a] (6)
Permeability (cm s^{-1}) $\times 10^{-6}$	8.06 ± 2.30	5.56 ± 2.53[*b]	7.05 ± 3.04
Flow (nmol cm^{-2} h^{-1})	2.06 ± 0.59	1.42 ± 0.65[*b]	1.80 ± 0.78
$ABZSO_2$:			
Equal-vol. incubation $K(min^{-1}) \times 10^{-3}$	6.38 ± 0.35 (3)[a]		
Double-vol. incubation	2.85 ± 0.28[*a] (3)		
Permeability (cm s^{-1}) $\times 10^{-6}$	30.7 ± 8.42[*b]		
Flow (nmol cm^{-2} h^{-1})	7.85 ± 2.16[*b]		

[*a] $P < 0.05$ versus equal-volume value; [*b] $P < 0.05$ versus untreated-cyst values for ABZSO incubations

[a] Number of cysts incubated

incubation volumes were considered, and these values did not statistically significantly differ between the use of a double or equal volume for each treatment.

The results show a significant increase in K values in incubations performed using a volume similar to the cyst weight as compared with the double-volume experiment (Table 1). The permeability values increased slightly with cyst weight, but without significant differences, as has also been reported by Rotunno et al. (1974).

Table 1 shows the permeability and uptake flow values obtained for ABZSO and $ABZSO_2$ in hydatid cysts from control animals, in hydatid cysts from animals treated with NTB, and in hydatid cysts from animals treated with NTB + FBZ. The results show that the permeability values recorded for ABZSO and $ABZSO_2$ ($8.06 \pm 2.3 \times 10^{-6}$ and $30.7 \pm 8.42 \times 10^{-6}$ cm s^{-1} respectively) were below the diffusional water permeability (2.2×10^{-4} cm s^{-1}) reported by Reisin and Pavisic de Fala (1984). A significantly higher permeability and uptake flow was found in incubated cysts from untreated animals relative to cysts from animals treated with NTB alone. In cysts from gerbils treated with NTB + FBZ, no significant difference was found relative to cysts from untreated animals. Significant differences were observed between the values of permeability and uptake flow noted for ABZSO and $ABZSO_2$.

Discussion

The study confirms that ABZSO penetrates the cyst membrane, as it can be detected in the hydatid fluid. This observation is in agreement with that reported by Morris et al. (1987), who estimated the concentration at the cyst level as being 13–22% of the serum concentration. This is a remarkable finding because it is of critical importance that ABZSO, the therapeutically active metabolite of ABZ, reach the parasite structures, protoscolices, and germinal layer of the cyst interior, as this is one of the factors to be considered in evaluation of the effectiveness of any chemotherapy for hydatid disease (Chinnery and Morris 1986).

The permeability differences observed between control cysts and cysts from animals treated with NTB alone indicate a loss of cyst permeability due to the treatment. When the gerbils were treated with NTB plus FBZ, an inhibitor of albendazole sulfoxidase activity, they did not show significant differences as compared with control cysts (Table 1). Xiao et al. (1992) observed that the glucose content decreased significantly in the hydatid fluid of cysts from mice treated with mebendazole after intravenous glucose administration at the end of the treatment, suggesting that mebendazole had an inhibitory effect on the transport of exogenous glucose to the cyst wall. According to this finding, the chemotherapeutic treatment could decrease the cyst's permeability to ABZSO, which means that prolonged treatments would be required to achieve therapeutic effectiveness.

Saimot et al. (1984) reported the importance of cystic and intracystic ABZSO concentrations but did not find a significant correlation between the drug concentrations in cysts and the therapeutic efficacy. Taylor et al. (1989) stressed the importance of the concentration and action time whereas Gil Grande et al. (1993) emphasized the sole dependence of chemotherapeutic success on the period of exposure to parasitic material, as they did not find any significant relationship between ABZSO concentrations in the cyst and the therapeutic effectiveness of the drug.

When FBZ is used as an inhibitor of the abovementioned enzymatic system the main effect on ABZ metabolism is a decrease in the conversion of ABZ into inactive metabolites and an improvement in the pharmacokinetic parameters of the pharmacologically active metabolite ABZSO (Lanusse and Prichard 1991). Under these conditions the permeability of the cyst to this metabolite did not decrease significantly. Both of these events could improve the effectiveness of the treatment.

The higher degree of permeability observed for $ABZSO_2$ does not imply a stronger effectiveness, as it is well known that this metabolite has no therapeutic effect and does not reach high serum levels (Chinnery and Morris 1986).

The need to identify new candidate chemotherapeutic drugs against hydatid disease and to promote this type of research has been stated by the World Health Organization (report of the WHO group meeting, Besacon, France, October 1992). In that sense, in vitro studies might be very useful for investigation of the permeability of the cystic membrane to the drugs under study, thus reducing the amount of animal experimentation, which involves elevated costs and long periods to obtain infected animals (Chinnery and Morris 1986). Once such permeability has been proven, further in vitro studies could be performed to establish the minimal effective concentrations that, along with pharmacokinetics studies in vivo, could be used to establish an effective chemotherapy.

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