Retour au menu

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Besnoitia besnoiti : chemotherapeutic trials *in vivo* and *in vitro*

SHKAP (V.), PIPANO (E.), UNGAR-WARON (H.). *Besnoitia besnoiti* : essais chimiothérapeutiques *in vivo* et *in vitro*. *Rev. Elev. Méd. vét. Pays trop.*, 1987, **40** (3) : 259-264.

L'efficacité thérapeutique des sulfonamides, de la pyriméthamine, du triméthoprim, du lactate d'halofuginone, de l'oxytétracycline, de l'acéturate de diminazène et de la pentamidine a été examinée *in vivo* lors d'une infection expérimentale fatale à *Besnoitia besnoiti* chez des mérions (*Meriones tristami shawii*). Une protection complète de l'animal a été obtenue avec une seule dose d'oxytétracycline à longue action injectée par voie intramusculaire à raison de 200 mg/kg, alors qu'un taux de survie de 50 p. 100 a été observé à la dose de 20 mg/kg. Le traitement à l'oxytétracycline soluble à raison de 25 mg/kg pendant 9 jours consécutifs, a donné des résultats allant jusqu'à 76 p. 100 de survie. Les autres médicaments n'ont pas été efficaces. Dans les expériences *in vitro*, l'oxytétracycline a été totalement inactive sur les endozoïtes *B. besnoiti*. Le lactate d'halofuginone seul, chimiquement lié ou mélangé aux immunoglobulines provenant de veaux sains, ou infectés par *Besnoitia*, a été examiné *in vitro* dans des milieux de Vero infectés par *B. besnoiti*. Le pourcentage de cellules parasitées a été nettement réduit à 0,5 p. 100 pour les cultures traitées avec le médicament seul, après une incubation de 96 heures, alors que le pourcentage était de 54 p. 100 chez les cellules témoins non-traitées. La liaison ou le mélange d'halofuginone avec les anticorps réduit l'activité du médicament. Cependant, l'inhibition observée dans la multiplication des endozoïtes ne peut pas être attribuée à une activité spécifique anti-*Besnoitia*, puisque une réaction similaire a été obtenue avec des immunoglobulines provenant de bovins sains ou infectés par *Besnoitia*. *Mots clés* : Mérion - *Meriones tristami* - *Besnoitia besnoiti* - Besnoitiose - Chimiothérapie - Anticorps.

INTRODUCTION

Besnoitia besnoiti is a tissue cyst-forming coccidian protozoon causing besnoitiosis (5, 22). The disease is of considerable economic importance because of its high morbidity rate and resulting damage to the productivity of affected bulls, which often become sterile (2, 3, 16). Hitherto, there has been no noteworthy treatment described and no drugs have been found effectively controlling bovine besnoitiosis. Since experiments with cattle are costly, screening of drugs using small laboratory animals or cell cultures infected with *B. besnoiti* could aid in finding the proper drug and treatment schedules for the control of this disease in cattle. Gerbils (*Meriones tristrami shawii*) and rabbits were found as suitable *in vivo* models for studies on bovine besnoitiosis (13, 16, 20).

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In *in vitro* studies, Vero cultures (green monkey kidney cells) were proved to be a convenient substrate for the multiplication of *B. besnoiti* (4, 20). Preliminary studies on chemotherapy in experimentally induced *B. besnoiti* infection of laboratory animals were performed by SHKAP *et al.* (18, 19). This report presents an extended study exploring the therapeutic potency of various drugs *in vivo* in experimentally *Besnoitia*-infected gerbils and in Vero cell cultures *in vitro*.

MATERIALS AND METHODS

Parasites

B. besnoiti parasites primarily isolated from a naturally infected bull were maintained in Vero cell culture by serial passages as described by SHKAP *et al.* (20).

Animals

Gerbils (*Meriones tristrami shawii*) 2-3 months old of both sexes used in all experiments were bred at the Kimron Veterinary Institute. Each gerbil was weighed before treatment in order to calculate the amount of drug to be administered.

Infection of gerbils

Groups of gerbils were inoculated intraperitoneally (i.p.) with 10^7 culture-grown endozoites of *B. besnoiti* suspended in 0,5 ml phosphate buffered saline (PBS), pH 7,2. This inoculum, previously found as LD_{100} for gerbils, was employed throughout all the *in vivo* experiments.

Drugs and treatments

Sulfonamides (sulfamerazine, sulfamezathine, sulfadoxine and sulfadiazine) alone and in combination with trimethoprim or pyrimethamine were given 48 hrs before induced infection either intramuscularly (i.m.), i.p. or *per os* (by stomach tube or in drinking water).

Pyrimethamine and halofuginone lactate tablets of 25 mg and 50 mg respectively were freshly dissolved in O.OIN HCl, before use to a concentration of 2 mg/ml because of poor water-solubility of the drugs, and were subsequently brought to neutral pH.

The toxicity of halofuginone for gerbils was assessed before medication by oral admnistration at 1-30 mg/kg to 54 animals in 9 groups, 6 animals per group. Halofuginone was delivered directly into the stomach by a tube connected through 196 needle to a tuberculin syringe.

Water soluble oxytetracycline (OTC) was injected i.p. or i.m. for 9 successive days, while the long-acting formulation (OTC-LA) was given i.m. as a single dose.

In a separate experiment 30 gerbils were inoculated i.m. at 200 mg/kg with OTC-LA in order to determine serum drug concentrations. Blood samples were collected 0.5; 1; 2; 4; 8; 12; 24; 48; 56 and 72 hrs after treatment and OTC concentrations were determined according to BENNETT *et al.* (1).

Diminazene aceturate or pentamidine were injected concomitantly to *B. besnoiti* inoculation. The dosage and therapeutic regimens for all drugs used are given in tables I and III. For each drug tested, treated but non-infected gerbils (6 animals in each group) served as controls for drug-toxicity (not shown in the tables). In addition, infected non-treated gerbils were included as controls of induced infection. All animals were observed daily and the number of gerbils dying was recorded. Peritoneal washings of dying animals in each group were examined microscopically for presence of *B. besnoiti* endozoites.

In vitro experiments

Vero cells 4×10^5 per ml of culture medium were seeded into Leighton tubes containing cover slips. About an hour later 4×10^5 *B. besnoiti* endozoites suspended in 1 ml medium containing various concentrations of OTC of halofuginone were added. Cover slips were removed from the tubes 24, 48, 72, 96 and 120 hrs after incubation, fixed in methanol and stained with Giemsa. The percentage of infected cells was determined by counting of 200 cells. Each time interval and drug concentration was represented by 3 separate tubes, and the entire experiment was repeated twice. Each value shown on figures 1 and 2 thus represents the average of 6 counts.

The antibesnoitic effect of halofuginone lactate chemically bound or mixed with immunoglobulins (Ig's) from *Besnoitia* infected or from healthy calves was examined in infected Vero cultures as described above.

Halofuginone lactate conjugation to anti-*B. besnoiti* immunoglobulins

Halofuginone was linked to Ig's obtained from a cow naturally infected with *B. besnoiti* and to the F (ab)'pepsin digest of the immunoglobulins. Conjugation of the drug to Ig's and the F (ab)'obtained from the serum of a control cow was concurrently carried out.

Ig's fractions were obtained by precipitation with ammonium sulfate at 40 p. 100 saturation and the F (ab)'fragments by pepsin digest according to NISONOFF *et al.* (14). Conjugation was carried out by means of the water - soluble carbodiimide reagent, 1-ethyl-3-(3 dimethylaminopropyl)-carbodiimide hydrochloride (EDCI) as described by GOODFRIEND *et al.* (8). An average of 10 p. 100 (W/W) binding of drug to protein was obtained for all conjugations.

TABLE I Effect of sulfonamides, pyrimethamine and trimethoprim on **B. besnoiti** *infected gerbils (6-10 animals in each group).*

Drug	Dose (mg/kg)	Route of administration	No. of treatments	Outcome	
				Percent survived	$\bar{X}$ time to death (days)
SMR + SMT	80 + 80	p.o. (stomach tube)	5-7 (every 48 h)	0	8.7
SMR + SMT	80 + 80	i.m.	4 (every 48 h)	0	6.5
SMR + SMT	80 + 80	i.p.	4 (every 48 h)	0	6.3
SMR + SMT	80 + 80	i.p.	6 (daily)	0	6.3
SDZ	300	p.o. (drinking water)	2 weeks	16.6	11.6
PR	10	p.o. (stomach tube)	5-7 (every 48 h)	0	10.2
SMR + SMT + PR	80 + 80 + 10	p.o. (stomach tube)	4-7 (every 48 h)	0	6.6
SDX + TMP	20 + 4	i.p.	6 (daily)	0	5.6
SDX + TMP	20 + 4	i.m.	6 (daily)	0	6.0
None				0	6.2

SMR – Sulfamerazine, SMT – Sulfamezathine,
SDZ – Sulfadiazine, SDX – Sulfadoxine,
PR – Pyrimethamine, TMP – Trimethoprim.

Statistics

Data were statistically treated using Student's t-test (21).

RESULTS

Experiments *in vivo*

As shown in table I, the various sulfonamides either alone or in combination with pyrimethamine or trimethoprim administered at different concentrations and therapeutic regimens were not effective against *B. besnoiti* in gerbils. All treated animals died, except one in the group receiving sulfadiazine in drinking water. The sulfadiazine and pyrimethamine-treated animals lived longer, the mean survival time in these groups was 11,6 and 10,2 days respectively compared to 6,2 days in the non-treated controls.

TABLE II Toxicity of halofuginone lactate administered **per os** *to gerbils with a stomach tube (6 gerbils in each group).*

Dose mg/kg	Percent survived	Died on day
30	0	2
25	0	1-2
20	0	1-1
15	0	1-3
10	0	2-3
5	0	2-3
3	83.4	1
2	100	—
1	83.4	1

Since no information on the toxicity of halofuginone to gerbils was available, the therapeutic dose was chosen after a preliminary experiment in which gerbils received the drug in doses ranging between 1 and 30 mg/kg. As shown in table II, groups of non-infected gerbils receiving more than 5 mg/kg died within 1-2 days after receiving the drug. On the other hand, at doses of 1 to 3 mg/kg only 2 out of 18 gerbils died (11,1 p. 100). However, all *Besnoitia* infected gerbils treated with 3 mg/kg of halofuginone lactate died, the average survival time was 3,8 days.

All gerbils treated with diminazene aceturate or pentamidine died about the same time as non-treated controls (Table III). Large numbers of *Besnoitia* endozoites were found in the peritoneal washings of dying animals whether or not treated.

OTC injected daily for 9 successive days at 25 mg/kg resulted in the survival of 71.5 and 76 p. 100 of the i.p. or i.m. treated animals respectively. Long-acting OTC administered as a single dose of 20 mg/kg resulted in 50 p. 100 survival, while 100 p. 100 remained alive when treated with a single dose of 200 mg/kg.

Mean serum OTC levels in non-infected gerbils injected i.m. with a single dose of OTC-LA at 200 mg/kg were as follows in (µg/ml) : 12.5 at 0.5 hr ; 10 at 1 hr ; 5 at 2 hrs ; 1 at 4 hrs ; 0.8 at 8 hrs and 0.08 at 12 hrs after treatment. At 24, 30, 48 and 56 hrs post treatment mean serum OTC concentrations ranged between 0.05 and 0.01 µg/ml. The drug was not detected in blood samples collected 72 hours after treatment.

Experiments *in vitro*

The effect of OTC on *Besnoitia*-infected Vero cells was examined after 24, 48, 72 and 120 hrs of incubation with 1, 10 and 100 µg of the drug per ml of culture media. The 200 µg OTC per ml level was apparently

TABLE III Effect of drugs upon **B. besnoiti** *infected gerbils (6-10 gerbils in each group).*

Drug	Dose (mg/kg)	Route of administration	No. of treatments and regimes	Outcome	
				Percent survived	$\bar{X}$ time to death (days)
Diminazene aceturate	5	i.m.	2 (72h interval)	0	7.1
Pentamidine	10	i.m.	3 (every 48 h)	0	6.5
Halofuginone lactate	3	p.o. (stomach tube)	1 (with infection)	0	3.8
Oxytetracycline *	25	i.m.	9 (daily)	76	8.0
Oxytetracycline	25	i.p.	9 (daily)	71.5	6.5
Oxytetracycline - LA **	20	i.m.	1 (with infection)	50	7.6
Oxytetracycline - LA	200	i.m.	1 (with infection)	100	
None				0	6.2

* Water-soluble formulation.
** Long-acting formulation.

toxic since the cells detached from the cover slips in control non-infected cultures. The rate of multiplication of *B. besnoiti* in OTC treated cultures was similar to that of non-treated controls irrespective of drug level and incubation time (Fig. 1). After 120 hrs of incubation about 80 p. 100 of non-treated cells were infected, while addition of the drug at 1 or 100 μg/ml only slightly reduced the percentage of infection to 69 and 79 p. 100 respectively. According to the t-test the differences were not significant.

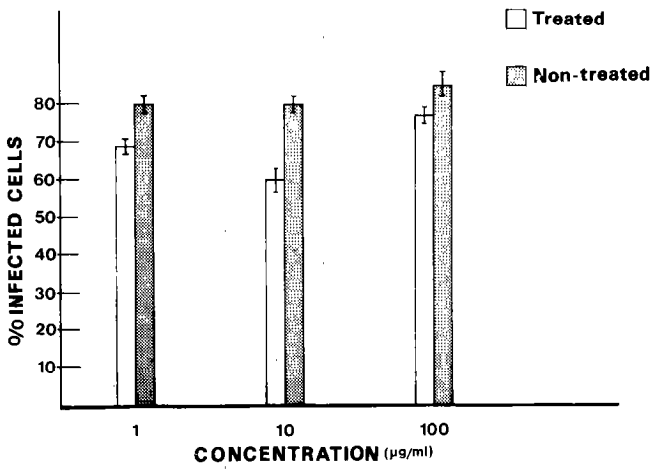

Fig. 1. : Effect of OTC upon B. besnoiti*-infected Vero cells after 120 hrs of incubation.*

The effect of halofuginone lactate in its various concentrations on *B. besnoiti*-infected Vero cells is shown in Fig. 2. After 24 hrs incubation with the drug alone only 8 p. 100 of cells were infected compared to 20 p. 100 in non-treated controls. At 48 hrs of incubation the percentage of infected cells in non-treated cultures increased from 20 to 40 p. 100, while in halofuginone treated cultures only 1 p. 100 of cells contained *Besnoitia* endozoites. As incubation with the drug was prolonged to 96 hrs the percentage of parasitized cells in control cultures increased to 54 p. 100, while only 0.5 p. 100 cells contained endozoites in halofuginone-treated cultures.

Antibodies chemically bound or mixed with the drug inhibited the multiplication rate of *B. besnoiti*. At 96 hrs, the longest incubation period, the percentage of infected cells was in the range of 15-19 p. 100, as compared to 54 p. 100 in the respective non-treated control cultures. As shown in figure 2 similar results were obtained when Ig's from serum of *Besnoitia*-infected or healthy cattle were used.

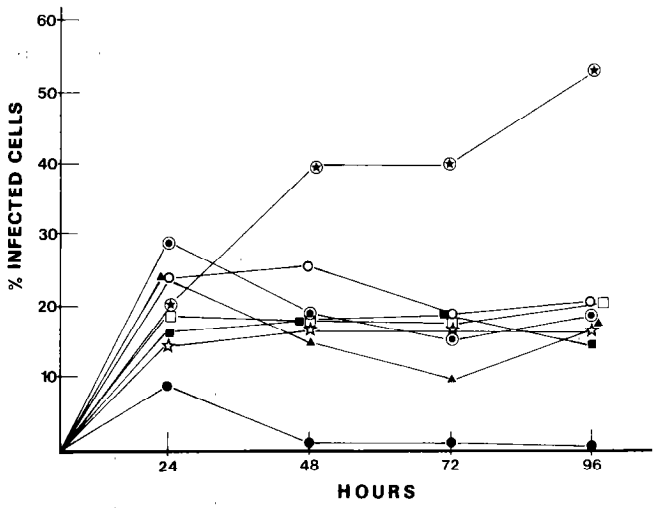

Fig. 2 : Effect of halofuginone lactate alone and in combination with antibodies on Vero cells infected with B. besnoiti *endozoites.*

⊛ Besnoitia-*infected cells, non-treated,*

● *halofuginone lactate,*

◉ *Ig's from a healthy cow,*

o *Ig's from* Besnoitia-*infected cow,*

■ *halofuginone chemically linked to anti-*Besnoitia *Ig's,*

▲ *halofuginone chemically linked to Ig's from a healthy cow,*

□ *halofuginone mixed with anti-*Besnoitia *Ig's,*

☆ *halofuginone mixed with Ig's from a healthy cow.*

DISCUSSION

The results of the *in vivo* study show that gerbils could be cured from acute *Besnoitia* infection by a single i.m. injection of OTC-LA at 200 mg/kg. Lower doses of either OTC-LA or the water-soluble OTC formulation were effective, but treatment was 76 p. 100 successful when OTC was administered for 9 successive days. The rapid elimination of the drug from the blood of gerbils, (only minute amounts of OTC were found in sera as early as 24 hrs after drug administration) may probably explain the differences in results obtained when different OTC-formulations were used. In a preliminary report SHKAP *et al.* (18, 19) found OTC-LA effective in eliminating *B. besnoiti* infection in gerbils and preventing clinical besnoitiosis in rabbits. The use of a rodent model, however, does not provide information on the potential efficacy of the drug in cattle that have reached stages of disease accompanied by cysts

formation. Therefore, at present, it remains to be documented whether OTC-LA might be of practical use in the chemotherapy of bovine besnoitiosis. GARIN *et al.* (6) and GILBRIDE (7) reported that tetracycline compounds are effective against the taxonomically related parasite *T. gondii.*

The sulfonamides alone and in combination with trimethoprim or pyrimethamine had no effect upon *B. besnoiti* infection in gerbils. Although the time to death in gerbils treated with sulfadiazine or pyrimethamine was significantly longer than in the non-treated controls, all animals eventually died. These results complement previous reports of POLS (16) and BIGALKE (3) on the unsuccessful treatment of *Besnoitia*-infected cattle and rabbits with these drugs.

The anticoccidial halofuginone (12), which was also reported to be efficient against the blood protozoon *Theileria annulata* infection *in vivo* and *in vitro* (11, 17), failed to protect gerbils against *B. besnoiti* (Table III). Moreover, the very short mean time to death (3,8 days) as compared to the infected non-treated group (6,2 days) points out the high toxicity of halofuginone for gerbils at 3 mg/kg. Although large numbers of *Besnoitia* endozoites were found in dying animals, it is not completely clear whether the gerbils died because of infection or drug toxicity. In addition, it seems that halofuginone delivered directly into the stomach was not effective against *B. besnoiti* endozoites multiplying in the peritoneal cells.

Diminazene aceturate and pentamidine were ineffective in treating *Besnoitia* infection. These drugs are successfully used for chemotherapy of bovine babesiosis (10, 15), and are known as trypanocidal compounds (9).

Results of the *in vitro* studies were inconsistent with those obtained with the *in vivo* model. Thus, although OTC displayed a high protective effect *in vivo*, the drug was completely inactive *in vitro*. The biochemical events involved in the drug-parasite-host interactions have to be elucidated in order to better understand the results obtained.

Remarkable inhibition of intracellular *B. besnoiti* multiplication was obtained with halofuginone in the *in vitro* model. It is obvious that binding or mixing of the drug with antibodies reduced halofuginone's activity. Moreover, since addition of Ig's from *Besnoitia*-infected or healthy cattle exhibited a similar effect (Fig. 2), it seems that the inhibition of endozoites multiplication observed cannot be attributed to a specific anti-*Besnoitia* antibodies activity.

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SHKAP (V.), PIPANO (E.), UNGAR-WARON (H.). *Besnoitia besnoiti* : chemotherapeutic trials *in vivo* and *in vitro*. *Rev. Elev. Méd. vét. Pays trop.*, 1987, **40** (3) : 259-264.

Therapeutic potency of sulfonamides, pyrimethamine, trimethoprim, halofuginone lactate, oxytetracycline, diminazene aceturate and pentamidine was examined *in vivo* during fatal experimental *Besnoitia besnoiti* infection in gerbils (*Meriones tristami shawii*). Complete animal protection was achieved with a single dose of long-acting oxytetracycline given intramuscularly at 200 mg/kg, while 50 p. 100 survival was observed after a 20 mg/kg dose. Treatment with a water-soluble formulation of oxytetracycline at 25 mg/kg for 9 successive days resulted in up to 76 p. 100 survival. The other drugs were not effective against besnoitiosis in gerbils. In the *in vitro* experiments, oxytetracycline was completely inactive on *B. besnoiti* endozoites. Halofuginone lactate alone, chemically bound or mixed with immunoglobulins from *Besnoitia*-infected or from healthy calves was examined *in vitro* in Vero cultures infected with *B. besnoiti*. Percentage of parasitized cells was markedly reduced to 0.5 p. 100 in cultures treated with the drug alone at 96 hours of incubation compared to 54 p. 100 in control non-treated cells. Binding or mixing of halofuginone with antibodies reduced the drug activity. However the observed inhibition of endozoites multiplication cannot be attributed to a specific anti-*Besnoitia* activity, since a similar effect was obtained with immunoglobulins from *Besnoitia*-infected or healthy cattle. *Key words* : Gerbil - *Meriones tristami* - *Besnoitia besnoiti* - Besnoitiosis - Chemotherapy - Antibody.

SHKAP (V.), PIPANO (E.), UNGAR-WARON (H.). *Besnoitia besnoiti* : ensayos quimioterapéuticos *in vivo* e *in vitro*. *Rev. Elev. Méd. vét. Pays trop.*, 1987, **40** (3) : 259-264.

Se examinó *in vivo* la eficacia terapeútica de las sulfonamidas, de la pirimetanina, del trimetoprim, del lactato de halofuginona, de la oxitetraciclina, del aceturato de diminazeno y de la pentamidina durante una infección experimental fatal a *Besnoitia besnoiti* en *Meriones tristami shawii*. Se obtuvó una protección entera del animal con una sola dosis de 200 mg/kg de oxitetraciclina con larga acción inyectada por via intramuscular, mientras que se observó un porcentaje de 50 p. 100 de supervivencia con una dosis de 20 mg/kg. La administración de 25 mg/kg de oxitetraciclina soluble durante 9 días consecutivos dió resultado hasta 76 p. 100 de supervivencia. No fueron eficaces demás medicamentos. Durante los ensayos *in vitro*, la oxitetraciclina fué totalmente inactiva en los endozoitos *B. besnoiti*. Se examinó *in vitro* en medios de Vero, infectados por *B. besnoiti* el lactato de halofuginona solo, quimicamente ligado o mezclado con inmunoglobulina proviniendo de terneros sanos o infectados por *Besnoitia*. El porcentaje de células parasitadas fué mucho reducido a 0,5 p. 100 para los cultivos tratados con el medicamento solo, después de una incubación de 96 horas, mientras que era de 54 p. 100 el porcentaje en las células testigas no tratadas. La relación o la mezcla de halofuginona con los anticuerpos reduce la actividad del medicamento. Sin embargo, no se puede atribuir a una actividad específica anti-*Besnoitia* la inhibición observada en la multiplicación de los endozoitos ya que se obtuvó una reacción similar con inmunoglobulinas proviniendo de los bovinos sanos o infectados por *Besnoitia*. *Palabras claves* : *Meriones tristami* - *Besnoitia besnoiti* - Besnoitiosis - Quimioterapia - Anticuerpo.

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