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Short communication

Activity of an anti-inflammatory drug against cryptosporidiosis in neonatal lambs

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Abstract

The efficacy of the anti-inflammatory drug Bobel-24 against experimental infection by *Cryptosporidium parvum* was evaluated in neonatal lambs. The animals were treated by oral administration of the drug at 50 or 500 mg/kg of body weight. The prophylactic/therapeutic treatment was started 4 h before inoculation of the lambs with oocysts and was continued for eight consecutive days. The therapeutic treatment was initiated at the onset of diarrhoea, after confirmation of infection, and was continued for six consecutive days. Infection was monitored by daily examination of faecal samples from the first day until 30 days post-inoculation. The criteria considered in evaluating development of the infection and the drug activity were: oocyst shedding, presence of diarrhoea and weight gain at 15 and 30 days post-inoculation. Bobel-24 was effective as a prophylactic/therapeutic treatment at the lowest dose (50 mg/kg of body weight); in the group treated with this dose of drug there was a longer prepatent period, a shorter patent period and a lower intensity of oocyst excretion than in the untreated control group, and the differences were all statistically significant ($P < 0.05$). Moreover, one animal did not excrete oocysts, and two lambs had diarrhoea, for only 1 and 2 days. In the group treated with the higher dose of the drug, the diarrhoea lasted for a significantly shorter period ($P < 0.05$) than in the untreated group.

Keywords: *Cryptosporidium parvum*; Bobel-24; Anti-inflammatory; Lamb-protozoa; Control methods-protozoa

1. Introduction

Cryptosporidiosis produced by *Cryptosporidium parvum* is considered as a serious enteric disease that causes diarrhoea, dehydration, weight loss, and sometimes death in livestock (Fayer and Ungar, 1986; de Graaf et al., 1999). In neonatal lambs, the prepatent period lasts for approximately 5 days, and clinical symptoms are apparent for between 5 days and 2 weeks. The prominent symptom is mild-to-severe diarrhoea, but other clinical signs include depression, anorexia and abdominal pain. Large numbers of oocysts (10^8–10^{10} oocysts/g) are present in the diarrhoeatic stool. The weight losses and mortality (up to 40%) associated with the disease may induce important economic losses for farmers (Naciri et al., 1984; Yvoré et al., 1984).

Sporozoites of *C. parvum* attach to the intestinal epithelium following excystation. Specific molecules on the surface of both epithelial cells and *C. parvum* sporozoites are involved in the infection process. The

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presence of a *C. parvum* sporozoite surface lectin specific to galactose-*N*-acetylgalactosamine (Gal-GalNAc), and which may mediate attachment of sporozoites to host cells, has been demonstrated (Chen and LaRusso, 1999). The many drugs tested to date, such as halofuginone lactate, paromomycin and cyclodextrins (Mancassola et al., 1995; Chartier et al., 1996, 1999; Viu et al., 2000; Castro-Hermida et al., 2001, 2004) have shown only partial effectiveness in the treatment and prophylaxis of cryptosporidiosis in neonatal lambs and goat kids. Moreover, cryptosporidiosis is difficult to control because of the resistance of the oocysts to environmental conditions and to disinfectants, including sodium hypochlorite, ammonia, chlorine, hydrogen peroxide, glutaraldehyde and formaldehyde (Korich et al., 1990; Castro-Hermida et al., 2006).

Infection by *C. parvum* has been demonstrated to increase concentrations of tissue prostaglandins in pigs, calves and humans (Argenzio et al., 1990; Laurent et al., 1998; Cole et al., 2003). Prostaglandins, which are produced as an integral part of the inflammatory response to infection, inhibit sodium absorption, and their blockade with the non-specific cyclooxygenase (COX) inhibitor indomethacin restores sodium absorption to normal levels (Argenzio et al., 1996). Bobel-24 (2,4,6-triiodophenol), a drug obtained by chemical synthesis, has been shown to be potentially active as an anti-inflammatory agent, and has an action similar to or superior than that of other non-steroidal anti-inflammatory drugs (Parreño et al., 2006; Trocóniz et al., 2006). Some interesting properties of phenolic compounds have been described in relation to their structure. It has been found that Bobel-24 is a dual inhibitor of lipooxygenase and cyclooxygenase, which may be important in the treatment of anti-inflammatory diseases (López-Belmonte and Norton, 1997, 1999; García-Capdevila et al., 1998; Parreño et al., 2006). Moreover, it has been shown that Bobel-24 is an inhibitor of L-selectine expression; this molecule is crucial for the attachment of *Cryptosporidium* sporozoites to epithelial cells (Chen and LaRusso, 2000; Rueda et al., 2008). The aim of the present study was to evaluate the antiparasitic activity of Bobel-24 against *C. parvum* infection in neonatal lambs.

2. Material and methods

2.1. Parasite

Oocysts were collected from a naturally infected 15-day-old Friesian calf. Concentration (distilled water/ether), purification (discontinuous Percoll® gradient) and quantification (Neubauer haemocytometer) of oocysts were carried out as described previously by Lorenzo et al. (1993). The isolate was identified as *C. parvum* "cattle" genotype (designated genotype II or C) by use of procedures to amplify and sequence the hsp 70 gene and 18SrRNA gene of *Cryptosporidium* (Xiao et al., 1999; LeChevalier et al., 2003).

2.2. Experimental design

The study was carried out at the *Centro de Investigaciones Agrarias de Mabegondo*. Thirty-seven newborn male and female lambs of the "Gallega" autochthonous breed were divided at random into six groups. The uninfected and untreated control group ($n = 7$) was not infected or treated. Lambs in the other five groups, each consisting of six animals, were experimentally infected within 24 h of birth with 10^6 oocysts of *C. parvum* suspended in 1 ml of sterile distilled water (d0, day of birth and inoculation): the prophylactic/therapeutic treatment 1 and 2 groups were treated 4 h before oocyst inoculation and during eight consecutive days with a daily dose of Bobel-24 of 50 or 500 mg/kg of body weight, respectively. The therapeutic treatment group received the drug for the 6 days following the apparence of *C. parvum* oocysts in the faeces, at a daily dose of 500 mg/kg of body weight. In all cases, the powdered product was suspended in water (37 °C) with sodium carboxymethyl cellulose (0.5%) and Tween 80 (1%), immediately before oral administration to the animals. The excipient control group received only the excipients: sodium carboxymethyl cellulose (0.5%) and Tween 80 (1%), suspended in water at 37 °C. The treatment control group remained untreated.

Each group of lambs was kept with their dams in separate boxes, and strict hygiene was maintained to avoid extraneous infections. The lambs were not vaccinated, and did not receive any vitamins, other nutritional supplements or any type of medication during the experiments. No cases of ovine cryptosporidiosis have been detected in animals maintained at the Mabegondo Research Centre in recent years. All animal handling procedures were completed in accordance with institutional animal care and use guidelines.

The criteria considered for assessing the development of infection and efficacy of the treatment were: oocyst shedding, presence of diarrhoea and weight gains at age 15 and 30 days. The lambs were monitored daily from birth to age 30 days. Faecal samples were collected directly from the rectum of each animal with sterile cotton swabs; each sample was analysed on the day of collection. Faeces were classified, in a blind

fashion, according to their consistency as diarrhoeatic-liquid or soft- and non-diarrhoeatic-solid.

Faecal smears were prepared, stained with carbol fuchsin for visualization of oocysts and examined by microscopy (Heine, 1982). Oocyst shedding was scored semiquantitatively according to the average number of oocysts in 50 randomly selected fields at 1000× magnification: 0 (no oocysts), 1 (≤1 oocyst), 2 (2–5 oocysts), 3 (6–10 oocysts) and 4 (>10 oocysts).

2.3. Statistical analysis

Comparisons of the mean duration of diarrhoea, oocyst shedding, weight at birth and weight gains in the different groups were made by pairwise multiple comparison procedures (Student–Newman–Keuls method) and a one-way analysis of variance (GraphPad InStat® for Windows, version 3.05, GraphPad Software, San Diego, CA, USA). Differences were considered significant at the 0.05 level of confidence.

3. Results

C. parvum was not detected in the seven lambs in the uninfected, untreated control group. All six neonatal lambs from the experimentally infected, untreated group (treatment control) began excreting *C. parvum* oocysts at age 5 days. The intensity of oocyst shedding

Table 1
Oocyst shedding and presence of diarrhoea* in control (treatment and excipient) and treated groups of lambs experimentally infected with *C. parvum* "cattle" genotype

Lamb age (days)	Treatment control						Excipient control						Prophylactic/therapeutic treatment 1 (Bobel-24 at 50 mg/kg of body weight)					
	1	2	3	4	5	6	1	2	3	4	5	6	1	2	3	4	5	6
5	1[d]	1[d]	2[d]	2[d]	1[d]	1[d]	2[d]	3[d]	1[d]	2[d]	1[d]	1[d]	0	0	0	0	0	0
6	4[d]	2[d]	4[d]	2[d]	2[d]	3[d]	3[d]	3[d]	2[d]	4[d]	3[d]	2[d]	0	0	0	0	0	1
7	4[d]	4[d]	4[d]	4[d]	3[d]	4[d]	3[d]	3[d]	3[d]	3[d]	4[d]	2[d]	0	0	1[d]	0	0	1
8	4[d]	4[d]	3[d]	3[d]	3[d]	3[d]	3[d]	2[d]	3[d]	2[d]	3[d]	4[d]	0	0	1[d]	1	1	2[d]
9	3[d]	4[d]	2[d]	3[d]	2[d]	3[d]	2[d]	2[d]	2[d]	1[d]	3[d]	4[d]	0	0	2[d]	1	0	1
10	3[d]	3[d]	2[d]	2[d]	1	2[d]	1	2[d]	1[d]	1[d]	3[d]	3[d]	0	1	1	2	0	1
11	2[d]	3[d]	2[d]	2	1	2	1	2[d]	1	1	3[d]	3[d]	0	1	1	2	0	0
12	1[d]	2[d]	1	1	1	1	1	1	1	1	2[d]	2[d]	0	1	1	3	0	0
13	1[d]	2	1	1	1	1	0	1	1	1	2[d]	2[d]	0	1	1	2	0	0
14	1[d]	1	1	1	1	1	0	0	0	2	2[d]	2[d]	0	1	0	1	0	0
15	1	1	0	0	0	0	0	0	0	2	1	1	0	0	0	0	0	0
16	1	1	0	0	0	0	0	0	0	1	1	1	0	0	0	0	0	0
17	1	1	0	0	0	0	0	0	0	1	1	1	0	0	0	0	0	0
18	1	1	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0

Lamb age (days)	Prophylactic/theraeutic treatment 2 (Bobel-24 at 500 mg/kg of body weight)						Therapeutic treatment (Bobel-24 at 500 mg/kg of body weight)					
	1	2	3	4	5	6	1	2	3	4	5	6
5	0	0	2[d]	0	2[d]	2[d]	1	1	1	1	0	1
6	2[d]	0	3[d]	4[d]	3[d]	2[d]	1	1	1	1	2	1
7	2[d]	2	2[d]	3[d]	2	2	1	1	1	1	2	2
8	1	1	2	3	2	3[d]	2[d]	2[d]	2	2	2	2
9	1	1	1	2	1	3[d]	2[d]	2[d]	2	2	1	2
10	1	0	1	2	1	2	3[d]	2	1	1	1	2
11	0	0	1	1	0	1	3[d]	2	1	1	2	2
12	0	0	0	1	0	1	2[d]	1	1	0	2	3
13	0	0	0	0	0	0	2[d]	0	1	0	4	4
14	0	0	0	0	0	0	1	0	1	1	3	2
15	0	0	0	0	0	0	1	0	0	1	2	2
16	0	0	0	0	0	0	0	0	0	0	1	1
17	0	0	0	0	0	0	0	0	0	0	0	0
18	0	0	0	0	0	0[a]	0	0	0	0	0	0

d, Diarrhoea; 0 (no oocyst), 1 (≤1 oocyst), 2 (2–5 oocysts), 3 (6–10 oocysts), 4 (>10 oocysts).
[a] Died.

Table 2
Mean (±S.D.) weight at birth and weight gain (kg) in control and treated groups of lambs during two periods: from birth to 15 days of age (0–15 days) and from 15 to 30 days of age (15–30 days)

Group	Weight at birth	Weight gain (kg)	
		0–15 days	15–30 days
Uninfected and untreated control	2.9 ± 0.5	3.0 ± 0.8	3.7 ± 1.2
Treatment control	3.4 ± 0.4	2.2 ± 0.3^{b}	2.9 ± 0.6
Excipient control	3.2 ± 0.3	2.5 ± 0.6	2.4 ± 0.6
Prophylactic/therapeutic treatment 1 (Bobel-24 at 50 mg/kg of body weight)	3.3 ± 0.6	3.5 ± 0.8^{a}	3.5 ± 1.2
Prophylactic/therapeutic treatment 2 (Bobel-24 at 500 mg/kg of body weight)	3.3 ± 0.4	2.3 ± 0.7^{b}	3.4 ± 0.9
Therapeutic treatment (Bobel-24 at 500 mg/kg of body weight)	3.3 ± 0.5	1.9 ± 0.6^{b}	3.4 ± 0.9

Means in the same column with different superscript letters are significantly different ($P < 0.05$).

was highest at age 7 days, with a mean score of 3.8 ± 0.4. The patent period ranged between 10 and 14 days (11.3 ± 2.0 days). All animals developed persistent diarrhoea that lasted for 5–10 days (7.0 ± 1.7 days) and the onset of diarrhoea coincided with the start of oocyst shedding (Table 1).

Similar results were obtained in the excipient control group. All six neonatal lambs showed persistent diarrhoea for 5–10 days (7.3 ± 2.1 days). The onset of diarrhoea coincided with the start of oocyst shedding, at age 5 days. The patent period in this group lasted between 8 and 14 days (11.0 ± 2.6 days). Oocyst excretion reached a peak 3 days after it began, with a mean score of 3.0 ± 0.6 (Table 1). There were no significant differences between treatment control and excipient control groups in terms of the criteria studied.

In contrast, in the group that was treated prophylactically/therapeutically with Bobel-24 (50 mg/kg of body weight), three of the lambs did not develop diarrhoea, and two animals had diarrhoea that lasted for only 1 and 3 days, respectively. Moreover, in five of the six lambs that acquired the infection, the mean prepatent period (7.8 ± 1.4 days) lasted significantly longer ($P < 0.05$) than in the other groups. The patent period lasted between 1 and 7 days (5.0 ± 2.4 days) and was significantly shorter ($P < 0.05$) than in control groups (treatment control and excipient control) and in the therapeutic treatment group. The mean score for oocyst shedding was 1.2 ± 0.5, which was significantly lower ($P < 0.05$) than in the two control groups. In the group that received prophylactic/therapeutic treatment with Bobel-24 (500 mg/kg of body weight), diarrhoea lasted for a maximum of 4 days, one lamb did not develop diarrhoea and in the five remaining lambs the diarrhoea stopped before the end of the treatment. The onset of oocyst shedding was confirmed between 5 and 7 days of age (Table 1). The mean score was 1.8 ± 0.8, and the maximum score was recorded at 7 days of age and was significantly lower ($P < 0.05$) than the maximum score for the control groups (treatment control and excipient control). Oocyst shedding lasted between 3 and 8 days (6.0 ± 1.7 days) and was significantly lower ($P < 0.05$) than in control groups (treatment control and excipient control) and in the therapeutic treatment group. During the experiment, one of the lambs died at age 18 days. At this moment, the lamb was not excreting oocysts, did not have diarrhoea, and no other enteropathogens associated with neonatal diarrhoea (rotavirus, coronavirus, *Escherichia coli*, *Clostridium*, or *Salmonella*) were detected in its faeces.

All of the lambs treated with Bobel-24 (500 mg/kg of body weight) for 6 days following confirmation of infection continued to excrete oocysts for between 8 and 12 days (10.0 ± 1.5 days). Only two lambs developed diarrhoea that lasted between 2 and 6 days, respectively (Table 1). None of the lambs became reinfected throughout the study. Significant differences in weight gain were only observed between age 0 and 15 days (Table 2). During this period, the prophylactically/therapeutically treatment 1 group (Bobel-24 at 50 mg/kg) gained significantly more weight than the prophylactic/therapeutic treatment 2 group, the therapeutic treatment group and the treatment control group. However, it did not gain significantly more weight than the two other control groups (uninfected/untreated and excipient).

4. Discussion

The results of the present study clearly show that in neonatal lambs that received prophylactic/therapeutic treatment with Bobel-24 (50 mg/kg of body weight), the appearance of diarrhoea as well as the duration and intensity of oocyst shedding were considerably reduced. In addition, oocysts were not detected in one of the six lambs. In the remaining groups, treated with Bobel-24 the severity of cryptosporidiosis was reduced.

Attachment of sporozoites to epithelial cells and invasion of the cell membrane are crucial steps in the pathogenesis of cryptosporidiosis. Galactose-*N*-acetyl-galactosamine (Gal/GalNAc) epitopes of glycoproteins on the epithelial apical membrane and Gal/GalNAc-specific sporozoite surface lectins are involved in this mechanism(s). Previous studies have shown that *in vitro* incubation of *C. parvum* sporozoites with Gal/GalNAc and bovine mucin reduces *C. parvum* attachment to biliary and intestinal epithelia by up to 70%. Preincubation of cell monolayers with either lectins specific to Gal/GalNAc, or glycosidases that specifically release Gal/GalNAc oligosaccharides from glycoproteins, decreased attachment by up to 80% (Chen and LaRusso, 2000). This may be why Bobel-24 was able to inhibit up to 99.6% of the infection of HCT-8 cells by *Cryptosporidium* sporozoites (Rueda et al., 2008). In the present study, Bobel-24 was not able to completely prevent infection by *C. parvum* in neonatal lambs. However, oocyst shedding, patent period and diarrhoea were notably reduced after administration of a prophylactic/therapeutic treatment (Bobel-24 at 50 mg/kg). Similar results were obtained by Rueda et al. (2008) with an i*n vivo* model of chronic cryptosporidiosis (SCID mouse). *C. parvum* infection increases the concentrations of tissue prostaglandins (by up to 50% of baseline levels) in both pigs and humans (Argenzio et al., 1990; Laurent et al., 1998) and diarrhoea has been partially attributed to the local production of prostanoid (Argenzio et al., 1993, 1996). Bobel-24 (2,4,6-triiodophenol) is a non-steroidal anti-inflammatory drug that inhibits the synthesis of prostaglandins and thromboxanes. The reduction in the inflammation associated with the parasite infection may account for the antidiarrhoeic effects observed in this study. A global reduction in the level of oocyst excretion was observed, which may be the result of an anti-cryptosporidial effect of the drug, as demonstrated *in vitro* by Rueda et al. (2008). Nevertheless, it is possible that in the present study the decrease in the level of excretion was due to the reduced levels of diarrhoea.

It must be pointed out that no signs of inappetence or toxicity were observed in treated animals. The higher therapeutic activity shown by the lower doses of Bobel-24 in terms of oocyst excretion, patent period, presence of diarrhoea and weight gain during the first 15 days of the animals' lives may be due to the low solubility of the product; the more amount of the product the more difficult preparation of homogeneous and adequate dosages. Problems in both solubility and bioavailability of this compound have been previously suggested by Rueda et al. (2008).

As with other products assayed for the treatment of cryptosporidiosis in small ruminants, such as halofuginone lactate, paromomycin sulphate and α- or β-cyclodextrin (Chartier et al., 1996, 1999; Castro-Hermida et al., 2002, 2004), Bobel-24 was more effective when used prophylactically.

In conclusion, the activity of Bobel-24 demonstrated in the present study suggests that this drug may be suitable for use in the early control of cryptosporidiosis in neonatal lambs. However, further studies are required to improve the drug formulation as well as to establish the optimum dosage and duration of treatment.

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