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Research paper

Resistant nematodes in cattle: Pharmaco-therapeutic assessment of the ivermectin- ricobendazole combination

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ABSTRACT

Nematodicidal combinations have been proposed as a valid strategy to achieve effective nematode control in the presence of drug resistance. The goals of this study were: (1) to compare the clinical efficacy (therapeutic response) of ivermectin (IVM) and ricobendazole (RBZ) given subcutaneously either by separate or combined administration to calves naturally infected with gastrointestinal nematodes resistant to IVM, and (2) to evaluate the potential pharmacokinetic (PK) and/or pharmacodynamic (PD) interactions occurring after the co-administration of both anthelmintics. Sixty male calves naturally infected with gastrointestinal nematodes resistant to IVM were randomly allocated into four groups (n = 15). Untreated control: animals not receiving anthelmintic treatment; IVM alone: animals treated with IVM by subcutaneous (SC) injection (0.2 mg/kg); RBZ alone: animals received RBZ by the SC route (3.75 mg/kg); IVM + RBZ: animals treated with IVM and RBZ (0.2 and 3.75 mg/kg, respectively), by SC injection in two separates sites. Eight animals of each treated group were randomly selected to perform the PK study. Plasma samples were taken from those animals up to 28 days post-treatment. IVM and RBZ plasma concentrations were quantified by HPLC. The therapeutic response was determined by faecal egg count reduction test (FECRT). The proportions of third-stage larvae (L3) recovered from coprocultures were used to calculate the efficacy against the main parasite genera. The daily total egg deposition for each experimental group was estimated. Similar pharmacokinetic trends were obtained for both IVM and RBZ allying the single-drug and the combined treatments, which indicates the absence of PK interactions between both anthelmintics. The observed overall clinical drug efficacies were 48% (IVM alone), 94% (RBZ alone) and 98% (IVM + RBZ). *Haemonchus* spp. and *Cooperia* spp. were recovered in the coproculture after IVM treatment, suggesting that resistance to IVM includes both genera. In fact, the efficacy against *Cooperia* spp. was 83% (IVM), 98% (RBZ) and 98% (IVM + RBZ), while the efficacy against *Haemonchus* spp. was 0% (IVM), 97% (RBZ) and 100% (IVM + RBZ). The combination was the only treatment that achieved 100% clinical efficacy against IVM-resistant *Haemonchus* spp. The total egg excretion was reduced to 49.9% (IVM alone group), 6.3% (RBZ alone group) and 1.8% (IVM + RBZ combined group) compared to the untreated control. Although the combined treatment did not significantly increase the overall clinical efficacy in the current natural field conditions, an additive effect was achieved against IVM-resistant nematodes. In fact, the combination obtained significantly higher efficacy against IVM-resistant *Haemonchus* spp. than RBZ alone.

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Additionally, the epidemiological relevance of the reduction in the number of eggs excreted following the combined treatment is not negligible and should be taken into account in future studies. Further work is required to understand the advantages of nematodicidal combinations in different natural anthelmintic resistance scenarios.

1. Introduction

Gastrointestinal (GI) parasitism is a major cause of economic losses in grazing systems of livestock throughout the world. Since chemical control is considered a low cost technology having a high impact on animal production, anthelmintics have been intensively used at short intervals in different grazing systems of cattle production worldwide. Inadequate drug uses, among other factors, have led to the current scenario of anthelmintic resistance, considered a main concern in veterinary medicine today (Kaplan and Vidyashankar, 2012). In Argentina, anthelmintic resistance is widespread and affects the majority of bovine livestock farms (Anziani and Fiel, 2004). It has increased from 60% in 2005 (Caracostantogolo et al., 2005) to 93.5% in 2015 (Fiel et al., 2015). Ivermectin (IVM) and ricobendazole (RBZ) (the sulphoxide derivative of albendazole) are the anthelmintics involved in the cases of parasite resistance (Fiel et al., 2015) with 90% (IVM) and 29% (RBZ) of farms with the presence of resistant parasites to one or both compounds. In an attempt to manage anthelmintic resistance in ruminants, drug combination of two or more anthelmintic compounds has been proposed in some geographic regions as a strategy to delay the development of resistance (Anderson et al., 1988; Geary et al., 2012).

The two main reasons for the use of combinations are to enable effective control of nematodes in the presence of single or multiple drug resistance, and to slow the development of resistance against the actives included in the combination (Bartram et al., 2012). GI parasitism in cattle always involves different parasite genera. For that reason, it could be expected that treatment with different anthelmintics administered simultaneously will result in effective parasite control, because parasites that survive one active compound included in the combination could be killed by the activity of the other active compound (Geary et al., 2012; Lanusse et al., 2015). This means that fewer resistant parasites survive combined treatment. In addition, the survivors are then diluted by unselected parasites in refugia, and the resistant worms would take longer time to be predominant. Moreover, the results of a recent modelling study indicated that reversion towards susceptibility was more likely to occur (i.e. at lower fitness costs) when drugs were used in combination than in rotation because the combination removed all resistant worm genotypes except those simultaneously carrying multiple sets of resistance genes, which were the least fit (Leathwick, 2013). In fact, Leathwick et al. (2015) found that when anthelmintics containing multiple actives were used against worm populations with varying levels of resistance to two or more of these actives, resistance did not worsen. What is more, evidence was found for significant reversion towards susceptibility (Leathwick et al., 2015). Thus, several pharmaceutical formulations combining either two or three chemical classes have been developed for small ruminants. However, the available information about drug interactions is rather limited in bovine livestock. It is necessary to determine the pharmacokinetic (PK) and pharmacodynamic (PD) behaviours which may be altered when two or more anthelmintic drugs are administered simultaneously under natural field conditions. Therefore, pharmaco-parasitological studies are required before drug combinations are used for anthelmintic control in cattle.

IVM and RBZ are two widely used drugs that may be combined to treat GI nematodes in cattle. IVM, a macrocyclic lactone, is a broad-spectrum antiparasitic drug, extensively used in veterinary medicine. It is highly effective against adults as well as developing and hypobiotic larvae of most GI nematodes, lungworms (Egerton et al., 1981) and many arthropods in cattle (Campbell et al., 1983). RBZ is the active metabolite of albendazole (ABZ), a member of the methylcarbamate benzimidazole family. RBZ is also a broad spectrum anthelmintic compound, which is effective against lungworms and GI nematodes (Campbell, 1990). In some Latin American countries including Argentina, RBZ is formulated as an aqueous solution for subcutaneous injection to cattle, a route of administration that is widely accepted by veterinarians and farmers.

The goals of the current study were to compare the clinical efficacy (therapeutic response) of IVM and RBZ given both separately and co-administered to calves naturally infected with GI nematodes resistant to IVM (representing a real scenario of anthelmintic resistance in Argentina), and to evaluate the potential PK and/or PD interactions occurring after the co-administration of both molecules.

2. Material and methods

2.1. Field trial

This study was conducted in “Santa Elena”, a cattle commercial farm with a grazing system of meat production representative of the Argentina bovine production. “Santa Elena” is a 6200-ha beef-cattle grazing system located in the centre of the Humid Pampean Region, Argentina. A previous study had found that anthelmintic resistance to IVM was present in this farm, possibly linked to the extensive use of macrocyclic lactones for anthelmintic control and the varied provenance of the animals. The fact that some of the animals had come from different farms may have led resistant parasite strains to be imported from other regions of the province or even the country.

2.2. Animals

Sixty male calves naturally infected with GI nematodes resistant to IVM were involved in this trial. The animals were selected based on worm egg per gram (EPG) counts. Only animals with at least 400 EPG on day -1 were included in the study. Experimental animals had an average of 808 ± 343 EPG counts ranging from 440 to 1940. All calves were ear tagged and their individual body weights were recorded.

The flock from which the animals were selected had been grazing on a two-year-old *Agropyrum* pasture for the previous two months, which ensured that their parasite load was native from “Santa Elena”. Previous to their entrance to this pasture, all the animals were treated with levamisole (LEV) and were therefore free of parasites. During the experiment (one month), animals grazed on a similar pasture close to the animal handling facilities. All the animals had free access to water. Animal procedures and management protocols were approved by the Ethics Committee according to the Animal Welfare Policy (act 087/02) of the School of Veteri-

nary Medicine, Universidad Nacional del Centro de la Provincia de Buenos Aires (UNCPBA), Tandil, Argentina.

2.3. Experimental design, treatments and sampling

All parasitized animals (n = 60) were ranked according to EPG counts and then assigned into four groups of 15 animals each for the PK and efficacy trials. The mean EPG were similar across all groups. Each group received one of the following treatments by the subcutaneous route on day 0: IVM *alone*: IVM (Ivomec®, 1% solution, Merial, Argentina) administered at 0.2 mg/kg; RBZ *alone*: RBZ (Bayverm PI®, 15% solution, Bayer, Argentina) administered at 3.75 mg/kg; IVM + RBZ: animals were treated with IVM (Ivomec®) and RBZ (Bayverm PI®) administered at 0.2 and 3.75 mg/kg, respectively. For the efficacy trial, an untreated group was kept as a control.

2.4. PK trial

Eight randomly selected animals from the IVM, RBZ or IVM + RBZ treated groups were used in the PK trial. Jugular blood samples were taken from each group as follows: RBZ group: 0, 1, 2, 4, 6, 8, 10 and 12 h; IVM group: 0, 2, 4, 6, and 8 h and 1, 3, 7, 10, 15, 21 and 28 days post-treatment. The samples from the IVM + RBZ group were taken at the same times and days as those from each individual group. In all the experimental groups, the blood samples were collected using 10 mL heparinised Vacutainers tubes (Becton Dickinson, NJ, USA). Plasma was separated by centrifugation at 2500 rpm for 15 min, placed into plastic tubes and frozen at −20 °C until analysis by High Performance Liquid Chromatography (HPLC).

2.5. Clinical efficacy trial: faecal worm egg counts and coprocultures

Faecal samples were individually collected directly from the rectum of each calf during pre-treatment (day -1) and again on days 15 and 21 post-treatment. A modified McMaster technique with a sensitivity of 10 EPG (Roberts and O'sullivan, 1950) was used to analyse the faecal samples and obtain EPG count. Additionally, 10 g of faecal material (obtained from an individual animal and/or from a pool of each experimental group) was used to prepare coprocultures on each sampling day. The nematode genera and species were identified through the third-stage larvae recovered from these coprocultures (MAFF, 1986). Third stage larvae were collected by the Baermann technique and 100 larvae were differentiated from each sample. Thus, the relative participation of each genus per experimental group was determined.

The anthelmintic clinical efficacy of the different treatments was assessed by the faecal egg count reduction test (FECRT), calculated according to the formula recommended by the WAAVP (Coles et al., 1992):

$$\text{FECRT}(\%) = 100(1 - [T2/C2])$$

where T2 is the arithmetic mean EPG count in the treated group at 15 days post-treatment, and C2 is the arithmetic mean EPG count in the control group at 15 days post-treatment. Additionally, the same formula was used to calculate the clinical efficacy of the different treatments at 21 days post-treatment. The 95% confidence intervals were calculated as reported by Coles et al. (1992). In addition, efficacy against different genera was calculated by dividing the mean faecal egg count of the control group and each treatment group, by the proportion of L3 of each genus in the associated coproculture (McKenna, 1990a). Additionally, the daily total egg deposition observed for each experimental group was estimated considering the weight of each calf, the total amount of faeces of each animal and the EPG count according to Stromberg (1997).

2.6. Analytical procedures

2.6.1. IVM analysis

The extraction of IVM from spiked and experimental plasma samples was carried out following the technique earlier described by Alvinerie et al. (1993) and adapted by Lifschitz et al. (1999). An aliquot of 1 mL plasma sample was combined with abamectin (ABM) (used as internal standard) and then acetonitrile was added to each sample. After mixing and centrifuging, the supernatant was manually transferred into a tube and then injected into a Supelclean LC_{18} cartridge (RP-18, 100 mg, Strata®, Phenomenex, CA, USA), previously conditioned. The compounds were then eluted with methanol and concentrated to dryness under a stream of nitrogen. The resuspension was carried out with a solution of *N*-methylimidazole (Sigma Chemical, St. Louis, MO, USA) in acetonitrile (1:1) (De Montigny et al., 1990). Derivatization was initiated by adding trifluoroacetic anhydride (Sigma Chemical, St Louis, MO, USA) solution in acetonitrile (1:2). Finally, an aliquot of this solution was injected directly into the chromatographic system. IVM concentrations were determined by HPLC using a Shimadzu 10 A-HPLC system with a fluorescence detector (Shimadzu, RF-10 Spectrofluorometric detector, Kyoto, Japan). The internal standard method was used to determine plasma IVM concentrations, using the IVM/ABM peak area ratio to estimate IVM concentration in spiked (validation of the analytical method) and experimental samples. There was no interference of endogenous compounds in the chromatographic determinations. Calibration curves were prepared in the range between 0.2–5 ng/mL and 5–100 ng/mL. The limit of quantification (LOQ) was established at 0.2 ng/mL, which is the lowest concentration measured with a recovery higher than 70% and a CV < 20%. The linear regression lines for IVM analyzed showed correlation coefficients of 0.999. The mean recovery percentages for concentrations ranging between 0.2 and 100 ng/mL (n = 6) was 76% with coefficient of variation (CV) of 9.7%.

2.6.2. RBZ/metabolites analysis

RBZ and its metabolite, albendazole sulphone ($ABZSO_2$), were extracted from plasma as previously described (Alvarez et al., 2008). A 1.1 mL-aliquot of plasma sample placed in an eppendorf was centrifuged at 16200*g* for 15 min. Then, 900 μL was transferred into a tube and spiked with oxibendazole (OBZ) as internal standard. Spiked samples were placed into a preconditioned C_{18} column (Strata®, RP-18 100 mg, Phenomenex, CA, USA). Then the compounds were eluted with methanol and concentrated to dryness under a stream of nitrogen. Finally, samples were reconstituted with 100 μL of acetonitrile and 200 μL of water. Fifty μL of this solution was injected directly into the chromatographic system. RBZ and $ABZSO_2$ plasma concentrations were determined by HPLC (Shimadzu 10 A-HPLC System, Kyoto, Japan) with a UV detector following a method previously developed (Alvarez et al., 1999). Identification of RBZ and $ABZSO_2$ were undertaken by comparison with the retention time of pure reference standards. Retention times for RBZ, $ABZSO_2$, and OBZ were 4.31, 6.66 and 9.24 min, respectively. There was no interference of endogenous compounds in the chromatographic determinations. Calibration curves in the range between 0.05–1 μg/mL were prepared for each compound. Plasma calibration curves had a correlation coefficient ≥ 0.998. Mean absolute recovery percentages for concentrations ranging between 0.05 and 1 μg/mL (n = 6) were 75.1 (RBZ) and 87.9% ($ABZSO_2$) with CV of 6.9 and 7.2%, respectively. The LOQ was calculated as described for IVM. The LOQ was established at 0.05 μg/mL for RBZ and $ABZSO_2$. In all cases, concentration values below the

Table 1
Plasma pharmacokinetic parameters (mean ± SD) for ivermectin (IVM) obtained after its subcutaneous administration (0.2 mg/kg) both alone and co-administered with ricobendazole (RBZ) (3.75 mg/kg) to naturally parasitized calves.

IVERMECTIN		
Pharmacokinetic parameters	**IVM Alone**	**IVM + RBZ**
T_{max} (d)	1.57 ± 0.98	1.75 ± 1.04
C_{max} (ng/mL)	46.3 ± 18.7	47.9 ± 19.6
$AUC_{0\text{-}LOQ}$ (ng.d/mL)	348 ± 80.8	390 ± 93.2
$AUC_{0\text{-}\infty}$ (ng.d/mL)	357 ± 81.7	398 ± 94.7
MRT (d)	7.58 ± 2.81	6.95 ± 1.28
$T_{½abs}$ (d)	4.80 ± 1.36	4.23 ± 0.96
$T_{½el}$ (d)	1.24 ± 1.20	0.63 ± 0.51

T_{max}: time to peak plasma concentration; C_{max}: peak plasma concentration; $AUC_{0\text{-}LOQ}$: area under the plasma concentration vs. time curve from 0 to the quantification time; $AUC_{0\text{-}\infty}$: area under the concentration-time curve extrapolated to infinity; MRT: mean residence time; $T_{½el}$: elimination half-life; $T_{½abs}$: absorption half-life.

Table 2
Plasma pharmacokinetic parameters (mean ± SD) for ricobendazole (RBZ) obtained after its subcutaneous administration (3.8 mg/kg) both alone and co-administered with ivermectin (IVM) (0.2 mg/kg) to naturally parasitized calves.

RICOBENDAZOLE		
Pharmacokinetic parameters	**RBZ Alone**	**RBZ + IVM**
T_{max} (h)	7.75 ± 1.67	9.00 ± 2.39
C_{max} (μg/mL)	0.85 ± 0.31	0.77 ± 0.31
$AUC_{0\text{-}LOQ}$ (μg.h/mL)	10.8 ± 3.44	10.9 ± 4.70
AUC_{∞} (μg.h/mL)	11.2 ± 3.41	11.4 ± 4.87
MRT (h)	10.1 ± 1.05	10.5 ± 1.01
$T_{½abs}$ (h)	4.15 ± 0.90	4.47 ± 1.14
$T_{½el}$ (h)	1.58 ± 0.32	1.98 ± 0.46

T_{max}: time to peak plasma concentration; C_{max}: peak plasma concentration; $AUC_{0\text{-}LOQ}$: area under the plasma concentration vs. time curve from 0 to the quantification time; $AUC_{0\text{-}\infty}$: area under the concentration-time curve extrapolated to infinity; MRT: mean residence time; $T_{½el}$: elimination half-life; $T_{½abs}$: absorption half-life.

LOQ were not considered for the kinetic analysis of experimental data.

2.7. Pharmacokinetic analysis of the data

The concentration vs. time curves for IVM and RBZ/$ABZSO_2$ in plasma for each individual animal after the different treatments was fitted with the PK Solution 2.0 software (Summit Research Service, CO, USA). The peak concentration (C_{max}) and time to peak concentration (T_{max}) were recorded directly from the measured concentration data. The elimination half-life ($T_{½el}$) and absorption half-life ($T_{½abs}$) were calculated as $\ln 2/\lambda_{el}$ and $\ln 2/k_{abs}$, respectively, where λ_{el} is the elimination rate constant and k_{abs} represents the first order absorption rate constant. The rates were calculated by performing regression analysis using data points belonging to the terminal or absorption phase concentration-time plot. The area under the plasma concentration-time curve from zero up to the quantification time ($AUC_{0\text{-}LOQ}$) was calculated by means of the trapezoidal rule (Gibaldi and Perrier, 1982) and further extrapolated to infinity ($AUC_{0\text{-}\infty}$) by dividing the last experimental concentration by the terminal elimination rate constant (λ_{el}). Statistical moment theory was applied to calculate the mean residence time (MRT) according to Perrier and Mayersohn (1982).

2.8. Statistical analysis of the data

The PK parameters and concentration data are reported as arithmetic mean ± Standard Deviation (SD). Mean PK parameters for IVM and RBZ obtained after its administration both alone and co-administered were statistically compared using Student *t*-test. Faecal egg counts (reported as arithmetic mean ± SD) were compared by non-parametric Kruskal–Wallis test. A value of $P < 0.05$ was considered statistically significant. The statistical analysis was performed using the Instat 3.0 software (Graph Pad Software, CA, USA).

3. Results

Fig. 1 presents the mean (±SD) plasma concentration profiles for IVM after the SC administration both alone and co-administered with RBZ to parasitized calves. IVM plasma levels were measured up to 28 days post-treatment. Table 1 summarizes the plasma PK parameters for IVM obtained after those treatments. The presence of RBZ/$ABZSO_2$ did not affect the plasma disposition kinetics of IVM after the SC administration. No statistical differences between both treatments were observed for all PK parameters ($P > 0.05$).

RBZ and $ABZSO_2$ were the main analytes recovered in plasma after SC administration of RBZ. Fig. 2a shows the mean (±SD) plasma concentrations profiles of RBZ after its SC administration both alone and co-administered with IVM. This compound was detected in plasma between 2 h and 24 h post-treatment. No statistical differences between both treatments were observed ($P > 0.05$). Therefore, the plasma disposition kinetics for RBZ did not show differences between the single-drug and the combined-based treatment. Table 2 summarizes the plasma PK parameters for RBZ both alone (RBZ group) and co-administered with IVM (RBZ + IVM group).

The mean (±SD) plasma concentrations of $ABZSO_2$ after the SC administration of RBZ both alone and co-administered with IVM are shown in Fig. 2b. The sulphone metabolite reached a Cmax of 0.79 ± 0.18 (RBZ group) and 0.85 ± 0.28 (RBZ + IVM group) μg/mL at 12 and 11.5 h post-treatment, respectively. The $ABZSO_2$ $AUC_{0\text{-}LOQ}$ was similar in both treatments with values of 9.70 ± 1.73 and 10.2 ± 3.11 μg h/Ml for RBZ group and RBZ + IVM group, respectively. No statistical differences between both treatments were observed for Tmax, Cmax and AUC parameters. Therefore, the presence of IVM did not affect the plasma disposition kinetics of $ABZSO_2$ after the SC administration of RBZ alone or in combination.

Table 3 shows the overall faecal egg counts (arithmetic mean) obtained for all experimental groups on days 15 and 21 after treatment, including the results of the FECRT and upper and lower confidence limits (95%). The results of the FECRT indicate the presence of GI nematodes resistant to ivermectin (48% and 40% of egg reduction on days 15 and 21, respectively). Significant ($P < 0.05$) differences were observed between IVM group and the other treated groups. In fact, the EPG counts on days 15 and 21 of IVM group did not differ ($P > 0.05$) with the control group. The faecal egg count reduction (FECR) of both, RBZ administered alone and as a combined treatment were over 90%. Although the highest anthelmintic efficacy (98%) was observed for the IVM + RBZ group, no significant differences in post-treatment EPG counts were found between this group and RBZ alone ($P > 0.05$).

Table 4 shows the clinical efficacies against *Cooperia* spp., *Haemonchus* spp., *Ostertagia* spp. and *Oesophagostomum* spp. for the different treatments, including the results of the FECR and upper and lower confidence limits (95%). IVM alone failed to control *Haemonchus* spp. and *Cooperia* spp., showing efficacies of 0 and 83%, respectively, indicating IVM resistance in both genera. In contrast, RBZ alone and IVM + RBZ combination showed high efficacies against *Cooperia* spp. and *Haemonchus* spp. (>90% FECRT). However, the combination was the only treatment that achieved 100% clinical efficacy against IVM-resistant *Haemonchus* spp. In fact, significant ($P < 0.05$) differences in the efficacy against *Haemonchus* spp. were

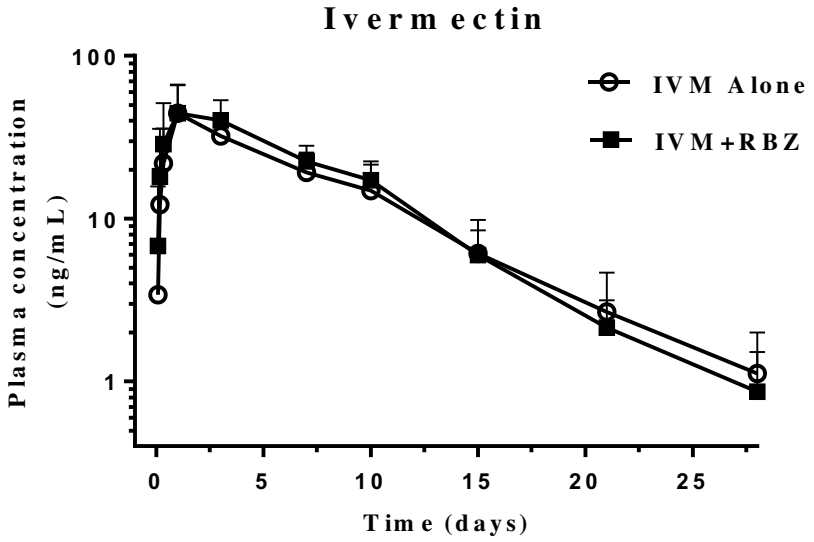

Fig. 1. Comparative mean (±SD) ivermectin (IVM) plasma concentration profiles obtained after its subcutaneous administration either alone (0.2 mg/kg) or co-administered with ricobendazole (RBZ) (3.75 mg/kg) to parasitized calves (n = 8).

Table 3
Nematode egg per gram counts (EPG, arithmetic mean, range) and reduction percentages of faecal egg counts (FECR) (undifferentiated) with its upper and lower confidence intervals 95%, after the subcutaneous administration of ivermectin (IVM, 0.2 mg/kg) and ricobendazole (RBZ, 3.75 mg/kg) given both separately and co-administered to naturally parasitized calves.

Experimental Group	EPG Counts (range)			FECRT[1] (CI)	
	Day 0	Day 15	Day 21	Day 15	Day 21
CONTROL	**791**[a] (420-1440)	**788**[a] (420-1960)	**724**[a] (300-2420)	-	-
IVM Alone	**739**[a] (420-1240)	**413**[a] (140-960)	**406**[a] (140-780)	**48%** (18-66)	**40%** (3-63)
RBZ Alone	**816**[a] (440-1240)	**47**[b] (0-140)	**43**[b] (0-120)	**94%** (89-97)	**94%** (89-97)
Combination IVM+RBZ	**809**[a] (440-1780)	**16**[b] (0-60)	**15**[b] (0-60)	**98%** (96-99)	**98%** (95-99)

EPG counts with different superscript letters are statistically different (P < 0.05).
[1] FECRT estimated according to Coles et al. (1992).

Table 4
Reduction percentages of faecal egg counts (FECR) with its upper and lower confidence intervals 95% for *Cooperia*, *Haemonchus*, *Ostertagia* and *Oesophagostomum* spp. (based on egg counts divided to genera using the proportion of each genus recovered as larvae from faecal larval cultures) after the subcutaneous administration of ivermectin (IVM, 0.2 mg/kg) and ricobendazole (RBZ, 3.75 mg/kg) given both separately and co-administered to naturally parasitized calves.

Experimental Group	FECR[1] Day 15 (CI)			
	Cooperia	*Haemonchus*	*Ostertagia*	*Oesophagostomum*
IVM Alone	**83%**[a] (73–89)	**0%**[a] (0–0)	**100%** (100–100)	**100%** (100–100)
RBZ Alone	**98%**[b] (97–99)	**97%**[b] (96–98)	**0%**[*] (0–0)	**100%** (100–100)
CombinationIVM + RBZ	**98%**[b] (96–99)	**100%**[c] (100–100)	**72%**[*] (38–87)	**100%** (100–100)

[1] FECR estimated according to Coles et al. (1992). FECR with different superscript letters are statistically different (P < 0.05).
[*] The low egg counts associated with *Ostertagia* spp. could be determined that the efficacy against this genus resulted unreliable.

observed between RBZ group and the combination. All the treatments achieved effective control against *Oesophagostomum* spp. The efficacy of RBZ alone and the combination against *Ostertagia* spp. was 0% and 72%, respectively. However, since the egg count allocated to this genus was too low, these results should be interpreted with caution.

The L_3 composition observed after faecal culture of pooled samples collected from the untreated control animals at 15 days post treatment was 80% *Cooperia* spp., 16% *Haemonchus* spp., 2% *Oesophagostomum* spp. and 2% *Ostertagia* spp. On the other hand, the animals treated with IVM showed only two nematode genera, with a predominance of *Haemonchus* spp. (74%) followed by *Cooperia* spp. (26%). This result, together with the clinical efficacy

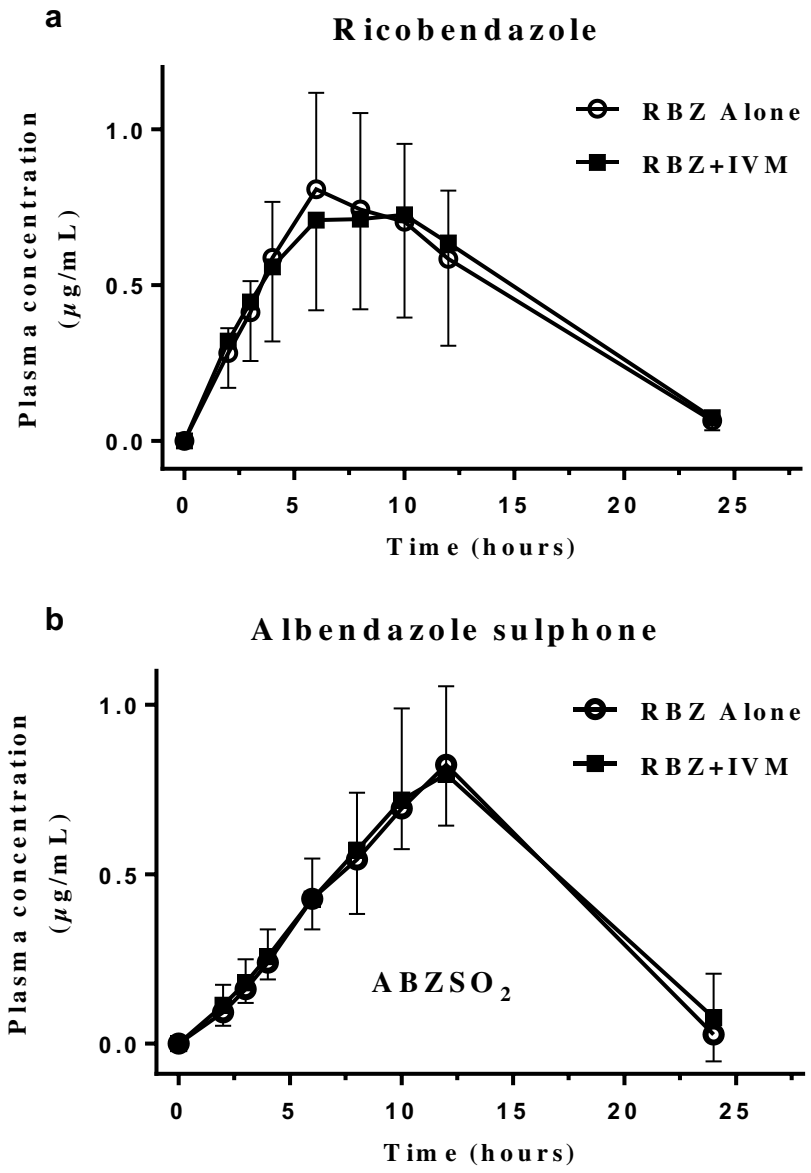

Fig. 2. (a) Comparative mean (±SD) ricobendazole (RBZ) plasma concentration profiles obtained after its subcutaneous administration either alone (3.75 mg/kg) or co-administered with ivermectin (IVM) (0.2 mg/kg) to parasitized calves (n = 8). (b) Comparative mean (±SD) albendazole sulphone ($ABZSO_2$) plasma concentration profiles obtained after the administration of ricobendazole (RBZ) either alone (3.75 mg/kg) or co-administered with ivermectin (IVM) (0.2 mg/kg), both given by the subcutaneous route to parasitized calves (n = 8).

results, demonstrated the presence of *Haemonchus* and *Cooperia* strains resistant to IVM. Whereas *Ostertagia* spp., *Cooperia* spp. and *Haemonchus* spp. survive RBZ treatment (77%, 17% and 6%, respectively); only *Cooperia* spp. and *Ostertagia* spp. were recovered from faecal cultures obtained from the IVM + RBZ treatment group (72% and 28%, respectively).

The excretion of eggs to the pasture (reported as the sum of the 15 animals included in each group) is shown in Table 5. Although no significant differences were found in terms of efficacy, the excretion of eggs to the pasture was found to be much lower after the combined treatment. The IVM group had the highest sum of eggs excreted (15,927,168), followed by RBZ group (2,021,762), and IVM + RBZ group (629,763).

4. Discussion

Drug combinations are known to be successful in treatments in different fields of human and veterinary medicine. The advantages of drug combinations in cancer chemotherapy (Goldin and Mantel, 1957) and tuberculosis (Conn et al., 1959) were demonstrated over 50 years ago. Nematodicidal drug combinations could be a valid strategy to optimize parasite control. Due to the increasing anthelmintic resistance problem in the ruminant livestock production systems, anthelmintic combinations would enable effective control of nematodes, in particular by delaying the emergence of resistance and controlling parasite populations with existing resistance (Geary et al., 2012). The use of anthelmintic combinations has been amply explored in sheep (Anderson et al., 1988; Entrocasso et al., 2008; McKenna, 2010; Geurden et al., 2012; Suarez et al., 2014), but much less is known regarding the management of anthelmintic resistance in cattle nematodes (Sutherland and Leathwick, 2011). The perception that resistance in cattle parasites would develop very slowly is clearly incorrect and anthelmintic resistance must be considered a threat to animal productivity in grazing cattle (Sutherland and Leathwick, 2011; Leathwick and Besier, 2014). The challenge ahead for cattle producers will be to maximize

Table 5
Pattern of pasture eggs excretion in different experimental groups. The total number of nematode eggs excretion per day was estimated based on the weight of each calf, amount of faeces excreted per day and the egg per gram counted (EPG).

ExperimentalGroups (n = 15)	Mean Body weight (kg)	Mean amount of faecesexcreted per animal/day (g)[1]	Total amount of faeces excreted pergroup/day (kg)	Day 15		Day 21	
				EPG (mean)	Total numberof eggsexcreted perday[2]	EPG (mean)	Totalnumberof eggs excreted per day[2]
UntreatedCONTROL	**179**[a]	**2754**[a]	**41.3**[a]	**788**[a]	**31 934 902**[a]	**724**[a]	**28 482 324**[a]
IVM Alone	**178**[a]	**2734**[a]	**38.3**[a]	**413**[a]	**15 927 168**[a]	**406**[a]	**15 600 357**[a]
RBZ Alone	**183**[a]	**2856**[a]	**42.8**[b]	**47**[b]	**2 021 762**[b]	**43**[b]	**1 856 479**[b]
CombinationIVM + RBZ	**182**[a]	**2816**[a]	**42.2**[b]	**16**[b]	**629 763**[b]	**15**[b]	**607 996**[b]

Values with different superscript letters are statistically different (P<0.05).
[1] Faeces excreted per animal/day estimated according to Stromberg, (1997).
[2] Sum of eggs excreted per day by the 15 animals included in each experimental group.

the advantages of resistance management from the experience gained in small ruminants, like the widespread acceptance of combined anthelmintics as a resistance management tool (Leathwick and Besier, 2014). However, unfavourable PK or PD interactions between constituent actives or excipients are possible and must be considered. The present study assessed the PK and PD interaction after the combined use of IVM and RBZ in cattle under natural field conditions.

IVM and RBZ are two of the most widely used anthelmintic drugs in Argentina that may be combined to treat GI nematodes in cattle. The short persistence of RBZ would not prevent the longer-duration component from selecting for resistance during a period of suboptimal concentrations at the tail of the IVM elimination curve; but this situation is not different from what would be experienced if that constituent active was used alone (Geary et al., 2012). In the current study, no adverse PK interactions were observed after the combined subcutaneous administration of IVM and RBZ in calves. Scarce information is available on the PK interactions of co-administered anthelmintic drugs in cattle. Alvarez et al. (2008) found that some type of PK interaction between ABZ and IVM occurs after their co-administration to lambs. Additionally, a drug to drug PK interaction was also observed after the combined administration of IVM + ABZ + LEV (Suarez et al., 2014). These reports showed that the interaction between co-administered drugs may induce changes on the PK behavior of either molecule in lambs. Although PK interactions between nematodicidal drugs have been less studied in cattle, Leathwick et al. (2016) found a significantly greater bioavailability of ABM after the oral ABM + LEV combination than after the single-active oral administration in cattle. In contrast, the same study did not find a difference between single and combined treatments in the concentrations of LEV in plasma (Leathwick et al., 2016). Similarly, no significant PK changes were observed in the current study for either RBZ/metabolites or IVM after their SC co-administration in cattle. Furthermore, Cromie et al. (2006) did not find differences in the plasma PK profile of IVM and closantel administered by SC injection to cattle, either alone or as combined formulation. Similarly, no PK interactions were observed after the combined subcutaneous administration of RBZ and LEV in calves (Canton et al., 2015). Thus, the observed PK data (no adverse interactions after the combined treatment) agree with those previously reported for other anthelmintic combinations in cattle, indicating that the PK of each molecule is not influenced by the presence of the other.

Additionally, it seems that administering RBZ alone or in combination with IVM would provide an equivalent therapeutic response, as non significant differences in the overall clinical efficacy (FECR) were found between both treatments. IVM alone failed to control *Haemonchus* spp. and *Cooperia* spp., showing efficacies of 0 and 83%, respectively (Table 4). Although *Cooperia* spp. is known to be the most frequent genus involved in IVM resistance in Argentina (Anziani et al., 2001; Fiel et al., 2001; Suarez and Cristel, 2007), the findings of the present study are in agreement with those of other studies carried out in Argentina, in which both genera were found to be resistant to IVM (Anziani et al., 2004; Fiel et al., 2015). It has been suggested that on farms where resistant nematode populations are present, the use of drug combinations may be an alternative to improve chemical control (Anderson et al., 1988; Geary et al., 2012; Bartram et al., 2012). Although published information in cattle is scarce, some preliminary results indicate that the combination of macrocyclic lactones and LEV was highly effective in minimizing the survival of resistant nematodes (Smith, 2014; Leathwick et al., 2016). Even though the current trial showed no significant improvement in FECR between the RBZ alone and the RBZ + IVM (Table 3), the observed clinical efficacy for the combination was almost as expected, i.e. additive anthelmintic effects between the two drugs (Bartram et al., 2012). An additive effect occurs when the combined effect of two drugs equals the sum of their independent activities measured separately (Entrocasso et al., 2008). This effect has been demonstrated in some trials done in sheep with anthelmintic combinations (Anderson et al., 1991, 1988; Mckenna, 1990b; Entrocasso et al., 2008). In the current study, the marked reduction in the total nematode egg counts 15 days after treatment support the high efficacy of RBZ after its administration alone or together with IVM (94% and 98%, respectively). However, it is important to highlight the efficacies against the different genera (Table 4). The efficacy against *Cooperia* spp. was 83% (IVM), 98% (RBZ) and 98% (IVM + RBZ), while the efficacy against *Haemonchus* spp. was 0% (IVM), 97% (RBZ) and 100% (IVM + RBZ). Remarkably, the combination was the only treatment that achieved 100% clinical efficacy against IVM-resistant *Haemonchus* spp. These results are similar to those from a recent field study in cattle, in which efficacies of 78% (IVM), 99% (RBZ) and 99% (IVM + RBZ) against *Cooperia* spp., and 42% (IVM), 99% (RBZ) and 100% (IVM + RBZ) against *Haemonchus* spp. were observed (Canton et al., 2015). Once again, the combination was the only treatment that achieved 100% clinical efficacy against IVM-resistant *Haemonchus* spp. This means that the combined group achieved higher efficacy against resistant parasite populations than did either of the component anthelmintics used alone, with fewer resistant parasites surviving treatment.

The pre-existing level of resistance to one of the anthelmintics in the combination may be a likely explanation for the lack of greater efficacy of the combined treatment. Likewise, Suarez et al. (2014) observed similar nematode control after the use of either a triple combined treatment (LEV + ABZ + IVM) or IVM alone against

multiple resistant *H. contortus*. In sheep, a population of *H. contortus* not effectively controlled in the field by four anthelmintics (ABM + LEV + ABZ + closantel) administered concurrently in a fixed commercial formulation has also been described (Baker et al., 2012). One of the most important prerequisite criteria to maximize the ability of multiple active formulations by managing existing resistance and slowing its further development is the pre-existing levels of resistance to each of the anthelmintics in the combination. Ideally, the use of nematodicidal combinations may be a valid strategy if the efficacy of each of the anthelmintic molecules approaches 100% (Bartram et al., 2012). This would be the ideal situation to use a nematodicidal combination, but today anthelmintic resistance is unfortunately widespread in Argentina. Therefore, a scenario where the nematode population is susceptible to IVM is not representative of the real situation in most of the commercial cattle farms. As indicated in modeling studies (Dobson et al., 2011; Leathwick, 2012; Leathwick et al., 2012) the key to achieving success with the use of anthelmintic combinations would require there administration before significant resistance (efficacy < 70%) to one or more of the active components develops.

Additionally, the excretion of eggs to the pasture was found to be much lower following the combined treatment. As shown in Table 5, the highest number of excreted eggs per day was exhibited by the group treated only with IVM (a total sum of almost 16 million), followed by the RBZ group (2 million), and finally the IVM + RBZ treated calves (0.6 million). Therefore, the total number of eggs excreted was reduced to 49.9% (IVM alone), 6.3% (RBZ alone) and 1.8% (IVM + RBZ) compared to the untreated control group (almost 32 million). From an epidemiological perspective, animals treated with RBZ excreted 3.2 times more eggs to the pasture than did those treated with the combination. In conclusion, the pasture would have higher contamination after treatments with RBZ alone than with RBZ + IVM. Thus, the epidemiological advantage observed after the combined treatment is not negligible and should be taken into account. Yet, its epidemiological importance in grazing systems in a context characterized by continuous re-infections remains to be established. A rational use of combined treatments is a promising tool, but in the absence of an adequate level of refugia of unselected parasites, the use of combined anthelmintics has the potential to select for the development of multiple drug resistant nematodes, hence reducing the range of anthelmintic options (Leathwick and Besier, 2014). Furthermore, Leathwick (2013) found that increasing the proportion of the population in refugia always slowed the development of resistance, as did using combinations in preference to an annual rotation.

Overall, the IVM-RBZ combination has a therapeutic additive effect in cattle under the current natural field conditions. In fact, the combination was highly effective at controlling the IVM-resistant *Haemonchus* spp. Moreover, the excretion of eggs to the pasture was remarkably found to be much lower after the combined treatment. Thus, the epidemiological relevance of the eggs excreted following treatment should be highlighted, but further studies are required to determine its real impact on pasture contamination. In natural field conditions, the potential therapeutic advantages of combined anthelmintic treatments should be cautiously assessed, especially considering that once resistance to at least one of the active ingredients in a dual combination has developed, the value of drug combinations in significantly increasing the clinical efficacy is arguable (Entrocasso et al., 2008; Leathwick et al., 2012). Therefore, combinations should only be a part of an integral package of resistance management strategies (Leathwick and Besier, 2014; Leathwick et al., 2015). The results of the current trial confirm that understanding the resistance status on individual farms is essential for parasite control. The presence of resistant nematodes, the level of animal infection, and the degree of pasture contamination are important aspects that should be taken into consideration before implementing nematodicidal combinations on commercial cattle farms. This is the first report on pharmaco-therapeutic assessment of the IVM + RBZ anthelmintic drug combination under a real cattle farm situation.

Conflict of interest statement

There are no potential conflicts of interest associated with this study.

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