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Clinical effectiveness of autohemotherapy as an adjuvant in the control of gastrointestinal nematodes in naturally infected sheep

Eficácia clínica da autohemoterapia como adjuvante no controle de nematódeos gastrointestinais em ovinos naturalmente infectados

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Abstract
This study aimed to evaluate the effects of autohemotherapy as an adjuvant in the control of gastrointestinal nematodes in sheep. Four experimental groups were formed: G1, 10 animals receiving autologous venous blood; G2, 10 animals receiving autologous venous blood and vermifuge containing levamisole; G3, 10 animals receiving only vermifuge containing levamisole; and G4, 10 animals as the control group receiving no treatment. We performed fecal egg count (eggs per gram, EPG) of strongyles, larval culture, hemogram, leukogram, and serum protein dosage prior to the start of treatment (D0), and on days 14 (D14) and 42 (D42). There was a significant decrease in the EPG of the groups receiving levamisole (G2 and G3) from D14 to the end of the experimental period. At the end of the evaluations, the mean EPG of G2 and G3 was significantly lower than that of G1 and G4. The most common nematode genus was *Haemonchus* (88%), and the least common was *Trichostrongylus* (1%). The Fecal Egg Count Reduction Test (FECRT) of G2 and G3 on D14 were 98.1% and 97.9%, respectively, however, in G1, the FECRT was zero on the two days when evaluation took place. G1 and G2 showed a significant increase in monocyte counts on D14 and D42. There was a significant increase in hematocrit and hemoglobin values in G2 and G3, however, a significant increase in the absolute value of red blood cells was observed only in G2. Two doses of autohemotherapy at 21-day intervals, administered alone or as an adjuvant to levamisole, is ineffective in controlling gastrointestinal nematodes in naturally infected sheep.
Keywords: autologous whole blood; FECRT; isotherapic; levamisole; small ruminant.

Resumo
Este estudo objetivou avaliar os efeitos da autohemoterapia

como adjuvante no controle de nematódeos gastrointestinais em ovinos. Quatro grupos experimentais foram formados: G1, 10 animais que receberam sangue venoso autólogo; G2, 10 animais que receberam sangue venoso autólogo e vermífugo contendo levamisol; G3, 10 animais que receberam somente vermífugo contendo levamisol; e G4, 10 animais do grupo controle, que não receberam tratamento. Realizamos contagem de ovos nas fezes (ovos por grama, OPG) de estrongilídeos, cultivo de larvas, hemograma, leucograma e dosagem de proteína sérica antes do início do tratamento (D0) e nos dias 14 (D14) e 42 (D42). Houve uma diminuição significativa no OPG dos grupos que receberam levamisole (G2 e G3) do D14 até o final do período experimental. Ao final das avaliações, o OPG médio de G2 e G3 foi significativamente menor do que G1 e G4. O gênero de nematódeo mais comumente encontrado foi *Haemonchus* (88%) e o menos foi *Trichostrongylus* (1%). O teste de Redução na Contagem de Ovos nas Fezes (RCOF) de G2 e G3 no D14 foi 98,1% e 97,9%, respectivamente, entretanto, no G1, o RCOF foi zero nos dois dias avaliados. G1 e G2 mostraram aumento significativo na contagem de monócitos em D14 e D42. Houve um aumento significativo nos valores do hematócrito e hemoglobina em G2 e G3, entretanto, um aumento significativo no valor absoluto de hemácias foi observado somente em G2. Duas doses de autohemoterapia em intervalos de 21 dias, administradas isoladamente ou como adjuvante do levamisole, não é eficaz no controle de nematóides gastrintestinais em ovinos naturalmente infectados.
Palavras-chave: sangue autólogo total; RCOF; isoterapia; levamisol; pequeno ruminante.

Introduction

The state of Paraná has 588,996 head of sheep and lambs. Approximately 14.9% of sheep and lambs in Brazil are in the south, and 3.0% are in Paraná[1]. There has been considerable interest in producing lambs for meat, originating from small and medium-sized creations that have a small number of sheep for maintenance. The sheep industry generates approximately R$ 96 million annually in Paraná[2].

Small ruminants are affected by various sanitary problems, with gastrointestinal nematodes (GIN) being the main barrier to their development. Parasitism caused by GIN species is primarily due to the family Trichostrongylidae[3,4], and results in losses to the national economy[5].

There are several proposals for alternatives to control GIN, such as the selection of resistant breeds[6], phytotherapy and biological therapy[7,8,9], nematophagous fungi[10,11], vaccines against nematodes[12], and tannin-containing fodder[13].

Isotherapy was initially used in the 1930s by François Lamson, a French homeopath physician, for the treatment of intestinal parasitoses. This researcher used large dilutions of blood serum to treat amoebic dysentery, oxyuriasis, ascariasis, and teniasis. Autohemotherapy, which consists of removing blood from the animal and injecting it

intramuscularly, is considered as an isotherapic treatment by certain researchers. It is suggested that this method stimulates the endothelial reticle system and quadruples the number of macrophages in the organism. In 1924, it was confirmed that this treatment was valuable in numerous dermatoses, particularly in pruritic and furunculosis affections. This is the synthesis of the doctoral thesis conclusion entitled "A auto-hemoterapia nas dermatoses", written by Alberto Carlos David at the University of Porto in 1924. This thesis proves that the technique has been used since the first half of the nineteenth century and presents cases cured through its use[14].

In recent decades, autohemotherapy has been little known and practiced in the veterinary field by professionals and animal owners. There are few reports on this therapy in humans and animals in the literature. At the start of the last century, autohemotherapy has been indicated as a treatment for various dermatological conditions[15]. Subsequently, autohemotherapy has become a standard treatment for numerous dermatologic disorders, including urticaria and eczema, in Europe, North America, and Japan[16,17,18]. Currently, autohemotherapy has been used to treat dermatologic and other diseases, predominantly by alternative medical providers, particularly in Europe[19] and Latin America[20].

Autohemotherapy has been tested as a thrombocytopenia treatment in dogs[21], and as an adjuvant in the treatment of mastocytoma[22], canine parvovirus[23], and oral papillomatosis[24,25]. In production animals, the majority of reports are related to papillomatosis treatment in bovines and caprines[26,27,28,29]. The few reports regarding the use of autohemotherapy in humans have been related to the prevention of postoperative complications[30], and as adjuvants in the treatment of herpetic infections[31] and allergic diseases[32,33].

There are several reports on the clinical efficacy of autohemotherapy, however, it has been the target of considerable controversies, leading health professionals not to recognize it. It is an alternative therapy, without recognized scientific proof, and therefore, little research has been conducted in this area.

Therefore, this study aimed to evaluate the effects of autohemotherapy as an adjuvant in the control of GIN in sheep, in addition to evaluating the hematological profile of animals during treatment.

Material and methods

This project was approved on December 11, 2013 by the “Comitê de Ética do Uso de Animais” (CEUA) from the Universidade Estadual do Norte do Paraná, Brazil (reference number Secapee 1017/13).

Location, groups, experimental periods, and fecal examination

The study was conducted on a rural property in the municipality of Ribeirão Claro (23°11’38"”S 49°45’28”" O), north of Paraná State, between March and July, 2014. Forty adult ewes with EPG values ≥150 were selected, which corresponds to the minimum established EPG for resistance and anti-helminth efficiency tests in sheep[34].

Four experimental groups were formed after randomization based on the EPG values; G1 group, comprised of 10 healthy, non-pregnant (2–3 years old) ewes, mean EPG 3310, received autologous venous blood; G2 group, comprised of 10 healthy, non-pregnant

(2–3 years old) ewes, mean EPG 3240, received autologous venous blood and commercial vermifuge containing oral solution levamisole hydrochloride 5% (5 mg/Kg); G3 group, comprised of 10 healthy, non-pregnant (2–3 years old) ewes, mean EPG 3185, received only commercial vermifuge containing oral solution levamisole hydrochloride 5% (5 mg/Kg); and G4 group, control group, no treatment, comprised of 10 healthy, non-pregnant (2–3 years old) ewes, mean EPG 3580, received 10 mL of oral saline solution. All groups were treated simultaneously. The treatments (autohemotherapy and levamisole) were performed on day 0 (D0) and 21 days later. D0 was defined as the first day of treatment followed by two experimental periods at day 14 (D14) and day 42 (D42) (totaling three experimental periods). During this period, stool harvests were carried out for EPG counting using the technique described by Gordon and Whitlock[35] at a dilution of 1:25.

To identify the genera of nematodes, larval culture (coproculture) was performed using the technique described by Roberts and Sullivan[36], with the L3 larvae recovered and identified using the criteria presented by Keith[37]. The percentage of fecal egg count reduction of nematodes of each identified genus in the culture of the larvae was calculated from these values using the Fecal Egg Count Reduction Test (FECRT) formula[38]:

FECRT = 1 - (EPG mean of treated on day x / EPG mean of the control on day x) × 100.

In the present study, FECRT was not used to estimate the resistance of the helminths to treatment, but to verify the percentage of reduction in the parasite load of animals and to identify the GIN species showing a more substantial decrease due to treatments.

Hematologic and Biochemistry analysis

Whole blood samples were collected from the jugular vein using an 18-gauge disposable needle. The first 4 mL of blood was dripped into a tube containing K3EDTA for hematology analysis, and the remaining 6 mL was transferred to a tube with a clot activator for serum chemistry analysis. All analyses were completed within 8 h of blood collection. Blood samples were centrifuged at 2000 × g for 10 min to extract the serum, which was frozen at -20ºC for further analysis.

Blood samples were analyzed using a BC-2800Vet-Mindray Auto Hematology (Mindray Headquartes, Shenzhen, China) system and multispecies software. The following parameters were measured: red blood cell (RBC) count, hemoglobin (Hb), hematocrit (HCT), white blood cell (WBC) count, and percentage and number of neutrophils, lymphocytes, monocytes, and eosinophils. Blood smear slides were prepared in the field immediately after collection prior to transfer to the tubes. The slides were manually stained using Wright's stain, and the WBC differential count was determined by counting 100 WBCs.

Total plasma protein (TPP) and albumin values were determined using the biuret and bromocresol green methods, respectively. The analyses were performed using the PKL PPC Automatic Chemistry Analyzer (Paramedical, Salerno, Italy) instrument. The globulin values were calculated by subtracting the TPP value from the albumin value.

Autohemotherapy:

Ten milliliters of blood was collected by jugular vein puncture of each animal in groups G1 and G2. After trichotomy and disinfection with 70% ethanol solution, the blood was immediately inoculated (autologous blood application) intramuscularly in the gluteal

region. Autohemotherapy was performed on D0 and D21.

Analyzes of data and statistical tests:

Statistical tests were performed using GraphPad Prism 5.0 (GraphPad Software Inc., CA, USA). The Wilcoxon test was used to compare the means of the EPGs of the treated and control groups. The comparison between the means of EPGs under the genera identified after the larvae growth was performed using the Mann–Whitney U test. Differences between the mean values of cell counts obtained by hemograms, leukograms, and plasma protein dosages were analyzed using a paired t-test. Statistical significance was set at p0.05.

Results and discussion

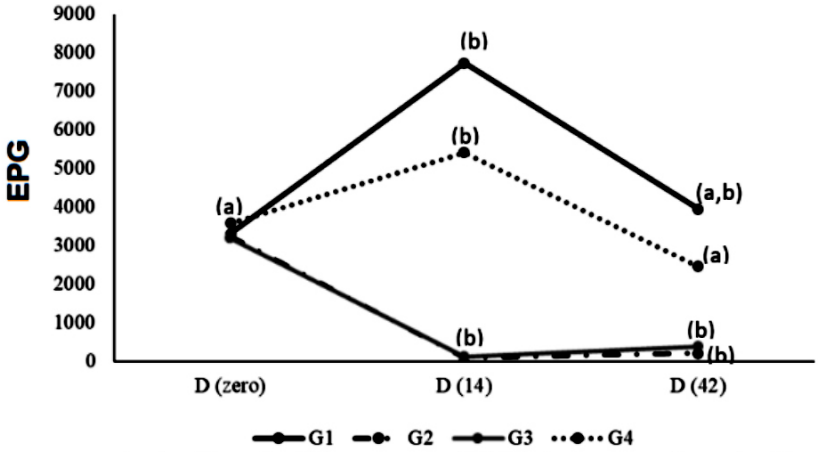
The mean EPG of both levamisole-treated groups treated (G2 and G3) was significantly lower ($p<0.005$) than that of the control group (G4) and the group treated only with autohemotherapy (G1). Although not significantly different, the mean EPGs of the animals in the autohemotherapy group (G1) were 40% (D14) to 60% (D42) higher than that of the control group (Table 1).

Table 1. Means (± Standard Error of the Mean - SEM) of the EPGs of the animals of the G1 groups (autohemotherapy), G2 (autohemotherapy + levamisole), G3 (levamisole), and G4 (control, no treatment) at the start of treatment (D0), and on days 14 and 42

Groups	EPG		
	D (zero)	D (14)	D (42)
G1	3310 ±938	(a) 7731 ±1705	(a) 3950 ±1290
G2	3240 ±913	(b) 106 ±54	(b) 213 ±92
G3	3185 ±845	(b) 115 ±39	(b) 375 ±185
G4	3580 ±1117	(a) 5415 ±1562	(a) 2465 ±1366

Different letters represent significant differences ($p<0.05$) between groups on the same day (column), calculated using the Mann–Whitney U test.

Animals in the auto-hemotherapy (G1) and control (G4) groups had significant increases ($p=0.0078$ and 0.027, respectively) in the EPGs 14 days after treatment. These values decreased four weeks later, and returned to the same levels as those at the beginning of the experiment. The two levamisole-treated groups (G2 and G3) showed significant decreases ($p=0.0039$ and 0.0020, respectively) in their EPGs by the end of the evaluations (Figure 1).



Different letters represent significant difference ($p<0.05$) between days in the same group, calculated using the Wilcoxon test.

Figure 1. Evolution of means values of the EPGs of the animals in groups G1 (autohemotherapy), G2 (autohemotherapy + levamisole), G3 (levamisole), and G4 (control, no treatment) at the start of treatment (D0), and on days 14 and 42.

Our results demonstrated that, in the experimental model used, autohemotherapy did not contribute to a decrease in gastrointestinal nematode infection levels in infected sheep. The animals were administered two doses of autologous blood via intramuscular (IM) injection at 21-day intervals, however, other researchers who successfully used this treatment administered weekly applications for a longer period than that used in our study[22,25,29,39]. The present study is the first to evaluate the effect of autohemotherapy on the control of GINs in sheep.

The psychobiological effect of placebos that can cause an improvement or worsening of clinical symptoms in humans[40] is not observed in animals, because they have no discernment of the treatments they are submitted to. If any negative influence of the treatment occurred, it could be attributed to the stress of the physical restraint and the application of autologous blood, because in humans it causes pain[41]. The animals in the control group received saline solution orally, therefore, they were subjected to the same physical restraint as those in the other groups. Although the injection of autologous blood was likely painful, its physiological consequences have not yet been measured in the medium or long term. For this reason, we cannot explain the difference between the mean EPGs of the control group animal and those treated with autohemotherapy. For this investigation, animals in the control group received IM injections of saline solution to imitate the pain caused by autohemotherapy.

FECRT was performed to compare the reduction rates of egg counts in the feces of animals in the three treated groups, as well as to compare the reduction rates of trichostrongylides genera identified in the larval culture. We observed a FECRT of 98.1% and 97.9% in animals from G2 and G3, respectively, on D14, and FECRT of 91.4% and 84.8% in animals from G2 and G3, respectively, on D42. In the group treated only with autohemotherapy (G1), the FECRT was zero on the two days evaluated (Table 2).

Table 2. Fecal Egg Count Reduction Test of *Haemonchus*, *Trichostrongylus* and *Strongyloides* (FECRT %) of the animals in G1 (autohemotherapy), G2 (autohemotherapy + levamisole), and G3 (levamisole) on days 14 and 42

	G1		G2		G3	
	D14	D42	D14	D142	D14	D42
Haemonchus	0	0	97.4 %	83.1 %	97.1 %	69.6 %
Cooperia	0	0	0	0	0	0
Trichostrongylus	100%	100%	99.3 %	82.9 %	98.5 %	79.9 %
Strongyloides	85.5 %	100%	99.6 %	100%	100%	100%

The most frequently observed genus in larval cultures was *Haemonchus* (88%), followed by *Cooperia* (2%) and *Trichostrongylus* (1%). The genus *Oesophagostomum* was not found on any of the experimental days. The genus *Strongyloides* accounted for 9% of the nematodes identified by egg morphology in the Gordon and Whitlock examination.

There were no *Cooperia* larvae in the control group on D14 and D42; therefore, it was not possible to evaluate the FECRT of *Cooperia* in the treated groups. Although there was no reduction in egg counts of all nematodes in the animals treated with autohemotherapy (G1), genera *Trichostrongylus*, and *Strongyloides* quantified on D0 by coproculture were absent on D42 (Table 3).

Table 3. Fecal Egg Count (EPG) of *Haemonchus*, *Trichostrongylus, Cooperia* and *Strongyloides* of the animals in G1 groups (autohemotherapy), G2 (autohemotherapy + levamisole), G3 (levamisole), and G4 (control, no treatment) at the start of treatment (D0), and on days 14 and 42

EPG GENUS	G1			G2			G3			G4		
	D0	D14	D42	D0	D14	D42	D0	D14	D42	D0	D14	D42
Haemonchus	3145	7393	3832	3240	96	204	3058	107	368	3222	3635	1208
Cooperia	66	151	119	0	3	2	127	6	0	179	0	0
Trichostrongylus	99	0	0	0	1	6	0	2	8	179	151	37
Strongyloides	0	188	0	0	6	0	0	0	0	0	1630	1220

The animals in the groups treated with autohemotherapy did not show significant changes in the leukogram, except for an increase in monocytes on D14 ($p = 0.0085$) and D42 ($p = 0.0011$) after treatment. Although the same was observed in the other groups, there was a 400% increase in monocytes in G1, and 97.5, 217, and 256% in G2, G3, and G4, respectively, at the end of the experimental period (D42) (data not shown). Pavão et al.[47] also found no changes in neutrophil and lymphocyte counts, however, a slight increase (1–4%) was observed in monocytes of mice infected with *Trypanosoma cruzi* and subjected to autohemotherapy.

The acquired immune response against gastrointestinal nematode infections in sheep has been associated with the activity of Th2CD4+ lymphocytes, eosinophilia, and an increased number of inflammatory cells in the mucosa, such as eosinophils, mast cells, and leukocytes[42]. Neutrophils, eosinophils, and antigen-presenting cells also participate in innate immunity against *Strongyloides stercoralis* larvae. Moreover, eosinophils have been shown to have the ability to chemotaxis to the parasite microenvironment, kill the parasite, and then present antigens to T cells to induce adaptive immunity to infection[43,44,45].

Levamisole acts on the immune system in a similar way to thymopoietin, a hormone produced in the thymus, stimulating the action of T cells, the response to antigens, interferon production, increasing the phagocytic activity of macrophages and neutrophils, and stimulating cell-mediated, lymphokine production, and the function of suppressor cells[47]. It is related to immunomodulatory activity in immunocompromised individuals and increased efficacy in vaccinations[48,49]. Qureshi et al.[50] and Mojzisova et al.[49] demonstrated increased activity and proliferation of lymphocytes in buffaloes and dogs treated with levamisole. However, in our study, we did not observe any changes in neutrophil and lymphocyte counts in levamisole-treated animals. Holsback et al.[8] reported a significant increase in lymphocyte counts of sheep seven days after levamisole treatment at the same dose used in this study, and attributed this increase to the immunomodulatory effect of levamisole.

There was a significant increase in HCT and Hb values only in levamisole-treated animals (Table 4). However, a significant increase ($p = 0.012$) in the absolute value of RBCs was observed only in G2 animals. Holsback et al.[8] reported a return to normal RBC, Hb, HCT, and TPP values in levamisole-treated sheep. All ten ewes in G1 had varying degrees of anemia at the start of the experiment[51], however, by the end of the experiment, only four ewes had counts below 8 million RBCs/μL of blood (data not shown), although the mean value was below the minimum normal value for the species (Table 4). Bambo et al.[24] and Faria et al.[52] evaluated the effect of autohemotherapy in dogs and observed no significant increase in RBC counts, HCT, and Hb after multiple treatment sessions.

Table 4. Medium ± standard error (σX) of red blood cells (RBC), hemoglobin (Hb), hematocrit (HCT), leukocytes, and total plasma protein (TPP) of the animals in G1 groups (autohemotherapy), G2 (autohemotherapy + levamisole), G3 (levamisole), and G4 (control, no treatment) at the start of treatment (D0), and on days 14 and 42

	RBC (9-15 x 10^6)	Hb (g/dL) (9.0 – 15.0)	HCT (%) (28-40%)	LEUKOCYTES (4-12,000)	TPP (5.4-9.0)
G1					
D zero	6.6 ± 0.32 (a)	9.2 ± 0.49 (a)	28 ± 1.26 (a)	6,572 ± 581.7 (a)	5.89 ± 0.20 (a)
D14	7.2 ± 0.70 (a)	8.8 ± 0.68 (a)	26 ± 1.72 (b)	7,811 ± 958.4 (a)	5.78 ± 0.22 (a)
D42	6.7 ± 0.84 (a)	9.4 ± 1.15 (a)	26 ± 2.95 (b)	6,181 ± 523.6 (a)	5.79 ± 0.36 (a)
G2					
D zero	7.2 ± 0.28 (a)	10.0 ± 0.47 (a)	30 ± 1.04 (a)	8,078 ± 933.9 (a)	6.56 ± 0.24 (a)
D14	8.5 ± 0.96 (a)	11.2 ± 0.28 (a,b)	32 ± 1.06 (a)	5,661 ± 831.9 (a)	6.56 ± 0.18 (a)
D42	9.4 ± 0.52 (b)	12.3 ± 0.71 (b)	35 ± 1.44 (b)	7,450 ± 800.6 (a)	6.38 ± 0.14 (a)
G3					
D zero	8.2 ± 0.32 (a)	10.6 ± 0.48 (a)	32 ± 1.06 (a)	7,300 ± 373.8 (a)	6.60 ± 0.16 (a)
D14	7.4 ± 0.71 (a)	12.0 ± 0.19 (b)	35 ± 0.56 (b)	7,595 ± 561.9 (a)	6.50 ± 0.22 (a)
D42	8.5 ± 0.55 (a)	11.6 ± 0.50 (a,b)	32 ± 1.34 (a,b)	7,145 ± 968.5 (a)	6.24 ± 0.14 (a)
G4					
D zero	7.7 ± 0.57 (a)	11.0 ± 0.58	31 ± 1.33 (a)	6,665 ± 550.9 (a)	6.10 ± 0.18 (a)
D14	7.4 ± 0.79 (a)	9.4 ± 0.72	28 ± 2.08 (b)	7,210 ± 769.4 (a)	6.10 ± 0.23 (a)
D42	7.1 ± 0.43 (a)	9.5 ± 0.93	28 ± 2.15 (b)	7,381 ± 586.2 (a)	5.75 ± 0.33 (a)

* Different letters represent significant difference ($p<0.05$) between days in the same group (column), calculated by "paired t-test."

We observed varying and unrelated hemogram, leucogram, TPP, EPG, and FECRT values, therefore, we cannot infer any benefits or drawbacks of autohemotherapy on GIN control in sheep. We believe that the animals of the two non-treated groups (G1 and G4) were favored with the lower loads of contamination in the pastures probably generated by the treated animals with levamisole, since all animals were housed in the same paddocks during the 42-day duration of this study.

Conclusions

In this study, it was concluded that treatment with two doses of autohemotherapy at 21-day intervals, administered alone or as an adjuvant to levamisole, was not able to control parasitic infections caused by GIN in naturally infected sheep, and did not influence myeloid and lymphoid lineage cells and blood proteins, and therefore produced no beneficial or harmful effects on sheep.

Despite reports of clinical improvement in treated animals, this study did not identify any improvement in animals during the 42-day evaluation period. A longer study period and injections at shorter intervals may clarify the benefits of autohemotherapy in the control of worms in sheep.

Conflict of interests

The authors declare that there is no conflict of interest.

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