Cordycepin (3′-deoxyadenosine) pentostatin (deoxycoformycin) combination treatment of mice experimentally infected with *Trypanosoma evansi*

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SUMMARY

The aim of this study was to evaluate the anti-trypanosomal effect of treatment with 3′-deoxyadenosine (cordycepin) combined with deoxycoformycin (pentostatin: inhibitor of the enzyme adenosine deaminase) *in vitro* by using mice experimentally infected with *Trypanosoma evansi*. *In vitro*, a dose-dependent trypanocidal effect of cordycepin was observed against the parasite. In the *in vivo* trials, the two drugs were used individually and in combination of different doses. The drugs when used individually had no curative effect on infected mice. However, the combination of cordycepin (2 mg kg^{-1}) and pentostatin (2 mg kg^{-1}) was 100% effective in the *T. evansi*-infected groups. There was an increase in levels of some biochemical parameters, especially on liver enzymes, which were accompanied by histological lesions in the liver and kidneys. Based on these results we conclude that treatment using the combination of 3′-deoxyadenosine with deoxycoformycin has a curative effect on mice infected with *T. evansi*. However, the therapeutic protocol tested led to liver and kidney damage, manifested by hepatotoxicity and nephrotoxicity.

Key words: trypanosomiasis, adenosine, adenosine deaminase inhibitor.

INTRODUCTION

Trypanosoma evansi is the aetiological agent of the disease known as '*Mal das Cadeiras*' or '*Surra*' in horses. However, this parasite has also been reported to affect domestic and wild mammals (Maudlin *et al*. 2004) and, in rare cases, humans (Powar *et al*. 2006). *Trypanosoma evansi* is the most widely distributed of the pathogenic African animal trypanosomes, affecting animals in Asia, Africa and Latin America and resulting in immeasurable economic losses (Luckins and Dwinger, 2004; Dobson *et al*. 2009). It is mechanically transmitted by haematophagous flies such as *Tabanus* spp. and *Stomoxys* spp. and/or vampire bats (Hoare, 1972). Infected animals showed typical clinical signs such as anaemia, oedema, weight loss and locomotor disturbance (Hoare, 1972; Maudlin *et al*. 2004).

Therapy for animal trypanosomiasis relies on the use of diminazene aceturate which is effective for treatment of the disease in cattle, buffalo, sheep, pigs and camels (Peregrine and Mamman, 1993; Sirivan *et al*. 1994). However, a single dose is not effective for horses, mules and dogs (Tuntasuvan *et al*. 2003; Colpo *et al*. 2005), resulting in lack of efficacy of these drugs (Tuntasuvan *et al*. 2003; Da Silva *et al*. 2008). In addition, the drugs used to treat *T. evansi* are considered hepatotoxic and nephrotoxic (Spinosa *et al*. 1999), and their prolonged use can cause worsening of the disease. Thus, it is important to investigate alternative therapies to improve the success of the treatment using new drugs, drug combinations and other components that could increase the curative efficacy. Accordingly, some studies have emerged suggesting new options of treatment for trypanosomiasis.

A recent study showed low therapeutic efficacy of the combination of cordycepin (3-deoxyadenosine) with the inhibitor of adenosine deaminase (ADA_1) in mice infected with *T. evansi* (Da Silva *et al*. 2011*a*). However, studies have shown efficacy of treatment with cordycepin in *T. brucei* infection in mice (Rottenberg *et al*. 2005; Vodnala *et al*. 2008, 2009). The efficacy of the treatment is related to the protection of cordycepin against the enzyme adenosine deaminase (ADA), which is responsible for the deamination of the analogue adenosine

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(Rottenberg *et al*. 2005; Vodnala *et al*. 2008, 2009). However, this protocol requires the combination of cordycepin with an inhibitor of ADA_1 and ADA_2 known as deoxycoformycin (Rottenberg *et al*. 2005), a different inhibitor from that used in the study with *T. evansi*.

The trypanocidal potential of cordycepin was noticed in experiments performed in the 1970s (Williamson, 1972; Williamson and Macadam, 1976). However, the administration of cordycepin did not result in a complete cure of infection (Aiyedun *et al*. 1973; Da Silva *et al*. 2011*a*). Cordycepin targets a vulnerable pathway in the trypanosomal metabolic economy in a way that is not targeted by currently available drugs (Rottenberg *et al*. 2005). Accordingly, the purine metabolism in trypanosomes and in other parasites presents a particular vulnerability because these parasites cannot engage in a new purine synthesis (James and Born, 1980; Hammond and Gutteride, 1984; Hassan and Coombs, 1988). Rather, they depend on the salvage pathway of nucleosides from the body fluids of the host (Agarwal *et al*. 1975). The inability of trypanosomes to engage in *de novo* purine synthesis has been exploited as a therapeutic target (Vodnala *et al*. 2009). Based on the aforementioned, we designed an experiment to evaluate the susceptibility of *T. evansi* in mice to treatment with a combination of 3′-deoxyadenosine and deoxycoformycin.

MATERIALS AND METHODS

Reagents

3′-deoxyadenosine (Cordycepin) was purchased from Sigma. Deoxycoformycin (Pentostatin; Tocris) was used as an inhibitor of ADA. Unless otherwise indicated, all reagents were diluted in PBS, aliquoted and stored at –20 °C until further use. The medium components used, except the antibiotics were purchased from Sigma.

Trypanosoma evansi *isolate*

Trypanosoma evansi was originally isolated from a naturally infected dog (Colpo *et al*. 2005). Two rats (R_1 and R_2) were intraperitoneally infected with blood cryopreserved in liquid nitrogen. The strain was later reactivated to obtain a large quantity of bloodstream forms of the parasite for subsequent infection of mice that formed the experimental groups and also for use in *in vitro* tests.

In vitro *tests*

Culture medium. The cultivation for *T. evansi* was adapted from the method reported by Baltz (1985). To prepare the culture medium, minimum essential medium (MEM) without glutamine (0·376 g), glutamine (0·016 g), sodium bicarbonate (0·088 g), glucose (0·04 g), HEPES free acid (0·238 g), non-essential amino acid solution (200 μL), penicillin (1596 U mL^{-1}) and estreptomicin (100 μg mL^{-1}) were used. The components were dissolved and homogenized in 30 mL of water, after adjustment of the pH to 7·1 with NaOH. The volume of the solution was then raised to 42 mL with sterile distilled water at an osmolarity of 0·30. Later, the culture medium was sterilized by filtration at 0·22 μm and stored in a refrigerator. On the day of testing, 10 mL were separated into a Falcon tube to which were added 1 μl mL^{-1} of 50 mM hypoxanthine (dissolved in 0·1 M NaOH) and 2 μl mL^{-1} of 1·2 mM 2-mercaptoethanol. Subsequently, the complete culture medium was equilibrated in a CO_2 incubator for 2 h (37 °C and 5% CO_2).

Acquisition of trypanosomes. For the *in vitro* tests, the trypomastigotes were obtained from rat No. 1 (R_1); when it showed high parasitaemia (10^7 trypanosomes μL^{-1}), it was anaesthetized under isoflurane anaesthesia to aid blood collection by intracardiac puncture. The samples were stored in EDTA tubes at 13 °C.

For separation of trypanosomes, 200 μl of blood were diluted in complete culture medium (200 μL), stored and centrifuged at 400 ***g*** for 10 min. The supernatant was removed and the trypanosomes were counted in a Neubauer chamber.

In vitro *assay*. The culture medium containing the parasites was mixed on microtitre plates (270 μL) followed by addition of cordycepin at concentrations of 0·5, 1·0, 5·0 or 10 mg mL^{-1}. The same concentrations of pentostatin were used in the tests that utilized a combination of cordycepin and pentostatin. For the control group, the highest concentration of PBS (diluent) was used. At 1, 3, 6 and 12 h after the onset of the experiment, counting of live parasites was performed in a Neubauer chamber. The tests were carried out in duplicate.

In vivo *trials*

Animals. A total of 49 female mice (mean age of 60 days) weighing approximately 25 ± 2·1 ***g*** were kept in cages (7 animals each), in a room with controlled temperature and humidity (25 °C; 70%). The animals were fed with commercial ration and received water *ad libitum*. All animals were submitted to an adaptation period of 10 days before the beginning of the experiment.

Experimental mice groups and trypanosomal infection. The mice were divided into 7 groups (A, B, C, D, E, F and G) of 7 animals each. Group A was not infected with the parasite and, thus, served as a

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negative control. Mice in groups B to G were infected intraperitoneally with $1{\cdot}4 \times 10^6$ trypanosomes in 0·1 mL of blood from rat No. 2 (R_2).

Parasitaemia evaluation. Parasitaemia was estimated daily by microscopic examination of blood smears from the mice. Each slide was prepared with mouse blood collected from the tail vein (Da Silva *et al*. 2006), stained by the Romanowsky method, and visualized at a magnification of 1000×.

Experimental design. Group A mice were the negative control and group B mice (infected with *T. evansi* and untreated) were the positive control. Mice in groups C to G were treated intraperitoneally at 24 h intervals for 3 days post-inoculation (p.i.), as follows: group C was treated with cordycepin (3′-deoxyadenosine) (2 mg kg^{-1}); group D was treated with pentostatin (deoxycoformycin) (1 mg kg^{-1}); group E was treated with pentostatin (2 mg kg^{-1}); group F was treated with the combination of cordycepin (2 mg kg^{-1}) and pentostatin (1 mg kg^{-1}); and group G was treated with the combination of cordycepin (2 mg kg^{-1}) and pentostatin (2 mg kg^{-1}). The effect of the treatment on the group was evaluated based on the parasitaemia levels, longevity, mortality of the mice and molecular analysis for *T. evansi* in infected mice that survived after treatment and thereby verify the trypanocidal effectiveness of the therapeutic protocol.

Collection of samples. On day 40 of the experiment, the survivor animals (in groups A, F and G) were anaesthetized with isoflurane in an anaesthetic chamber for collection of blood by cardiac puncture. The blood samples were collected in tubes without anticoagulant to obtain the serum. Thereafter, mice were euthanized in accordance with the recommendations of the Ethics Committee. The brains, livers and kidneys of the animals were removed for histological analysis.

Hepatic and renal function. The blood samples were centrifuged at 3500 ***g*** for 10 min to obtain serum. Liver function (alanine transaminase – ALT and alkaline phosphatase – ALP) and renal function (creatinine) were evaluated.

Histopathology. Evidence of drug-induced histopathological damage to the brains, livers and kidneys of *T. evansi*-infected mice treated with the drugs was investigated to detect the toxicity of the treatment. Samples of the organs were fixed in buffered formalin (10%), embedded in paraffin, sectioned at a thickness of 6 μm and stained with haematoxylin/eosin (H&E). The sections were qualitatively evaluated for differences in microarchitecture, organization and the presence of inflammatory cells. Measurements were made of the nuclear areas of hepatocytes and kidney corpuscles.

Molecular diagnosis. The brain and blood samples collected with EDTA (as anticoagulant) were preserved in ethanol for DNA extraction and posterior analysis using *T. evansi*-specific PCR (Ventura *et al*. 2002).

Statistical analysis

Data were submitted to analysis of variance (ANOVA) followed by Duncan test. The histological results were analysed by Student's *t*-test. Results were considered significant when $P < 0{\cdot}05$.

RESULTS

In vitro *tests*

The results showed a dose-dependent trypanocidal effect of cordycepin (Fig. 1A), pentostatin (Fig. 1B), and cordycepin and pentostatin combination (Fig. 1C) on *T. evansi* trypomastigotes. At 1 h post-treatment, a reduction of 48% and 55% in the levels of live trypomastigotes was observed at cordycepin concentrations of 5 and 10 mg mL^{-1}, respectively, when compared with the control group. The combination of cordycepin and pentostatin potentiated the trypanocidal effect. At 1 h after the beginning of the experiment there was a reduction of 69% (5 mg mL^{-1}) and 81% (10 mg mL^{-1}) in the number of live trypanosomes when compared with the control. At 3, 6 and 12 h post-treatment, a significant decrease ($P < 0{\cdot}001$) in the levels of live parasites was observed at all the concentrations used of cordycepin and the combination of cordycepin and pentostatin. No live parasites were detected at 6 h of incubation of the drug at concentrations of 10 mg mL^{-1} and 12 h of treatments with cordycepin eliminated the parasite, unlike in the control group (Fig. 1A). Tests using pentostatin showed no significant reduction in the number of live trypomastigotes in most groups, except in those treated with concentrations of 5 mg mL^{-1} (3, 6 and 12 h) and 10 mg mL^{-1} (1, 3, 6 and 12 h).

In vivo *tests*

Data of the *T. evansi*-infected groups are presented in Table 1. Parasitaemia in mice of groups B, C, D and E increased with time until death of the mice (Fig. 2). One mouse in group F presented with positive blood smears at day 21 p.i., while the other mice in the same group remained negative (Fig. 2). Mice in group G showed no parasitaemia, with longevity of 40 days p.i. (Table 1).

The mice that died during the experiment showed parasitaemia above 100 trypomastigotes per microscopic field (1000× magnification). In groups B, C, D

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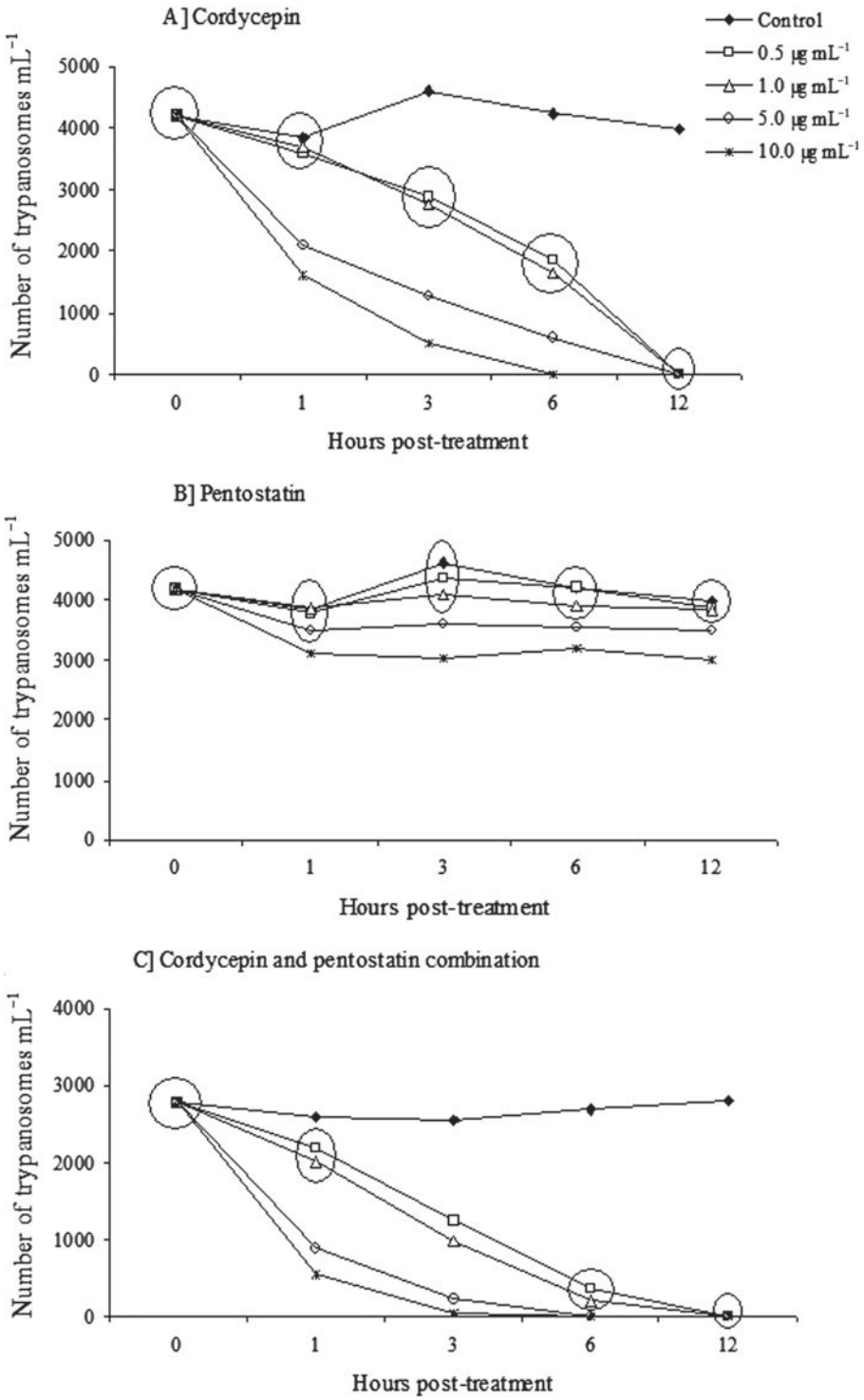

Fig. 1. Dose–response effect of cordycepin (A), pentostatin (B) and cordycepin and pentostatin combination (C) on the viability of *Trypanosoma evansi in vitro* compared with the control group. Means followed by the same letters within a column (circles) are not statistically different among themselves at 5% probability by Tukey's test.

and E all mice died, while in group F only 1 mouse died. Death did not occur in mice in groups A and G within 40 days post-treatment.

Hepatic and renal function. Results of the biochemical analyses of samples obtained from mice in groups A, F and G performed at the end of the experiment (day 40 p.i.) are presented in Fig. 3. A significant increase in the levels of alanine aminotransferase was found in the sera of mice treated with the combination of the two drugs, groups F and G (Fig. 3A). In the same groups a similar increase in alkaline phosphatase levels was observed (Fig. 3B). Serum creatinine levels in the mice did not show significant change compared with the control group (Fig. 3C).

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Table 1. Mean and standard deviation of the pre-patent period, longevity, mortality and success of therapy using treatment with cordycepin (3′-deoxyadenosine) combined with pentostatin (deoxycoformycin) in mice experimentally infected with *Trypanosoma evansi*

(Means followed by the same letters in the same column do not differ significantly in the test of Duncan. *Considered a therapeutic success for drug-treated mice that survived for 40 days and remained negative for the parasite by examination of their blood smears and PCR.)

Groups $n=7$	Treatment	Pre-patent period (days)	Longevity (days)	Mortality (no. dead/ no. in group)	*Therapeutic success (%)
A	Negative control: non-infected	–	$40{\cdot}0^{a}$ ($\pm 0{\cdot}0$)	0/6	–
B	Positive control: infected and untreated	$4{\cdot}86^{ab}$ ($\pm 0{\cdot}38$)	$8{\cdot}86^{c}$ ($\pm 0{\cdot}38$)	6/6	0·0
C	Treated with 2 mg kg^{-1} of cordycepin	$7{\cdot}1^{a}$ ($\pm 1{\cdot}51$)	$12{\cdot}1^{b}$ ($\pm 3{\cdot}08$)	6/6	0·0
D	Treated with 1 mg kg^{-1} of pentostatin	$4{\cdot}1^{ab}$ ($\pm 1{\cdot}21$)	$8{\cdot}14^{c}$ ($\pm 0{\cdot}90$)	6/6	0·0
E	Treated with 2 mg kg^{-1} of pentostatin	$3{\cdot}5^{b}$ ($\pm 0{\cdot}82$)	$8{\cdot}0^{c}$ ($\pm 0{\cdot}0$)	6/6	0·0
F	Cordycepin (2 mg kg^{-1}) + pentostatin (1 mg kg^{-1})	$37{\cdot}6^{bc}$ ($\pm 6{\cdot}7$)	$38{\cdot}1^{a}$ ($\pm 5{\cdot}67$)	1/6	83·3
G	Cordycepin (2 mg kg^{-1}) + pentostatin (2 mg kg^{-1})	$0{\cdot}0^{c}$ ($\pm 0{\cdot}0$)	$40{\cdot}0^{a}$ ($\pm 0{\cdot}0$)	0/6	100·0

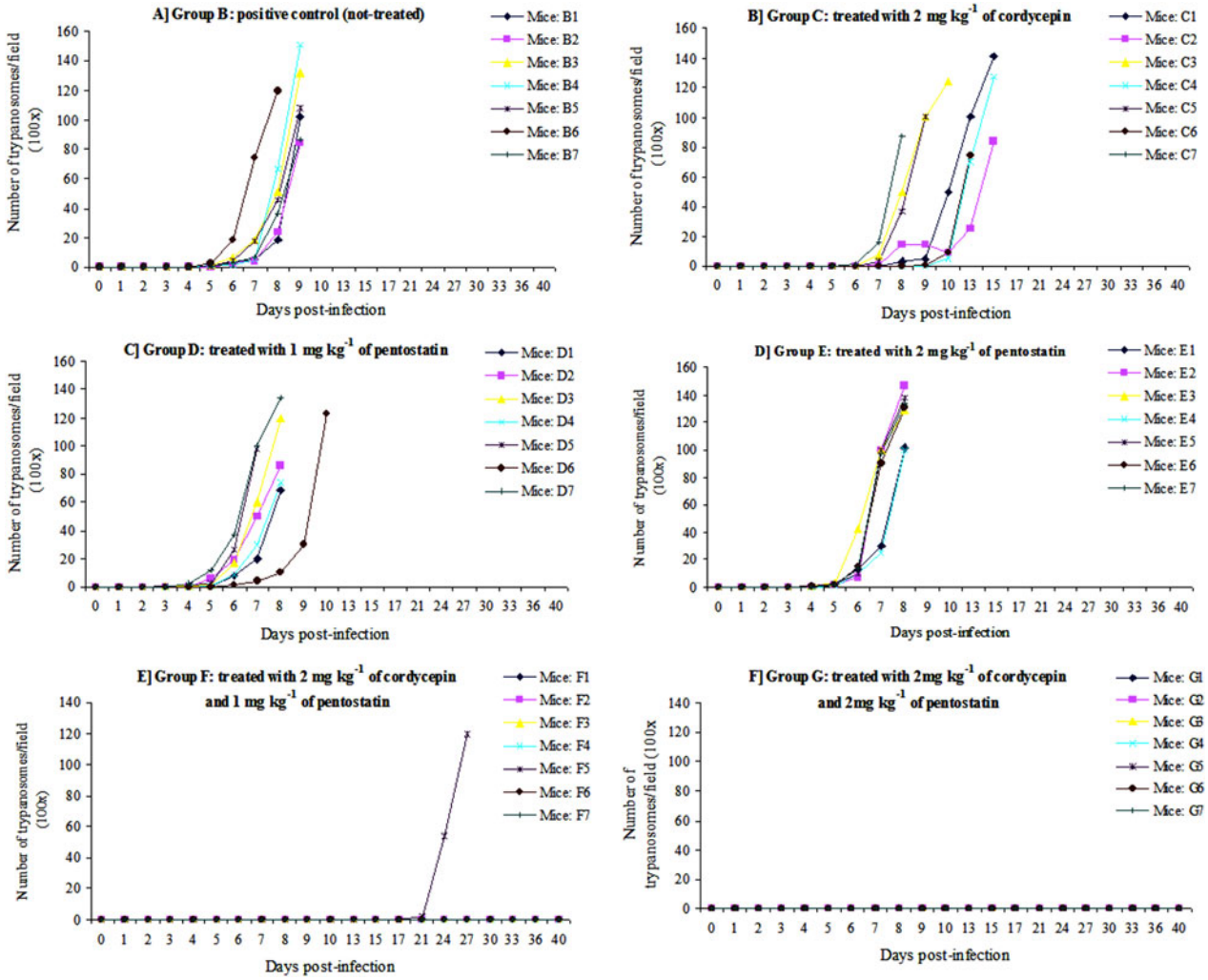

Fig. 2. Parasitaemia of *Trypanosoma evansi*-infected mice at day 40 post-inoculation (p.i.) in groups B to G. The treatment started at day 1 p.i. (3 doses at 24-h intervals).

Histopathology. The liver indicated strong nuclear and cytoplasmic tumefaction and necrotic degeneration. Furthermore, intense congestion of the sinusoids by leukocytes was observed. Thus, the microarchitecture of the organ was lost. There were statistically significant differences ($P=0{\cdot}0001$)

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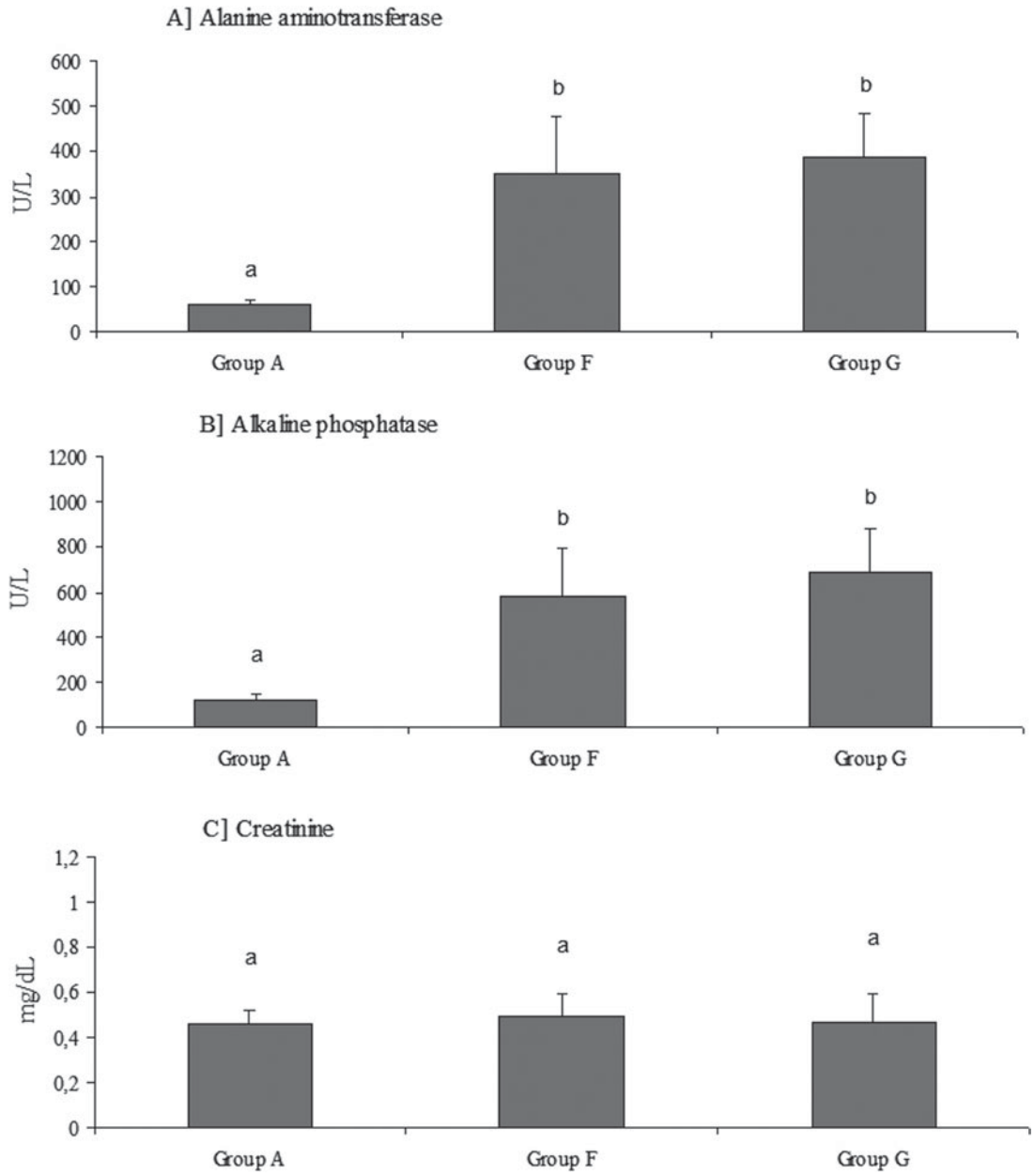

Fig. 3. Alanine aminotransferase, alkaline phosphatase, and creatinine in mice infected with *Trypanosoma evansi* treated with cordycepin combined with pentostatin (group F and G) compared with those not infected (group A).

between the nuclear areas of the hepatocyte of the control and infected animals (Fig. 4). In the renal tissue, a significant increase in the glomerular area ($P=0{\cdot}041$) of the infected animals was observed (Fig. 5A). No changes were observed in the brain tissues of animals studied and treated with cordycepin and pentostatin combination (Fig. 5B).

PCR. The molecular analysis was performed to confirm the effectiveness of the therapeutic protocol used, since parasitaemia was not detected in the survivor animals in groups F and G. Specific PCR assays from blood and brain of these animals were negative for the presence of *T. evansi*. Therefore, treatment was 83·3 and 100% effective for groups F and G, respectively (Table 1).

Committee on ethics and animal welfare

The experimental procedures used in this study were approved by the Animal Welfare Committee of Universidade Federal de Santa Maria (UFSM), under number 026/2012.

DISCUSSION

In this study, a dose-dependent effect of cordycepin against *T. evansi* was observed *in vitro* in contrast to the relative susceptibility expressed *in vitro* to the same drug by *T. congolense, T. vivax, T. brucei rhodesiense, T. evansi, T. brucei brucei, T. cruzi* and *Leishmania* sp. in previous studies (Williamson, 1972; Aiyedun *et al*. 1973; Williamson and Macadam, 1976; Maser *et al*. 2001; Rottenberg

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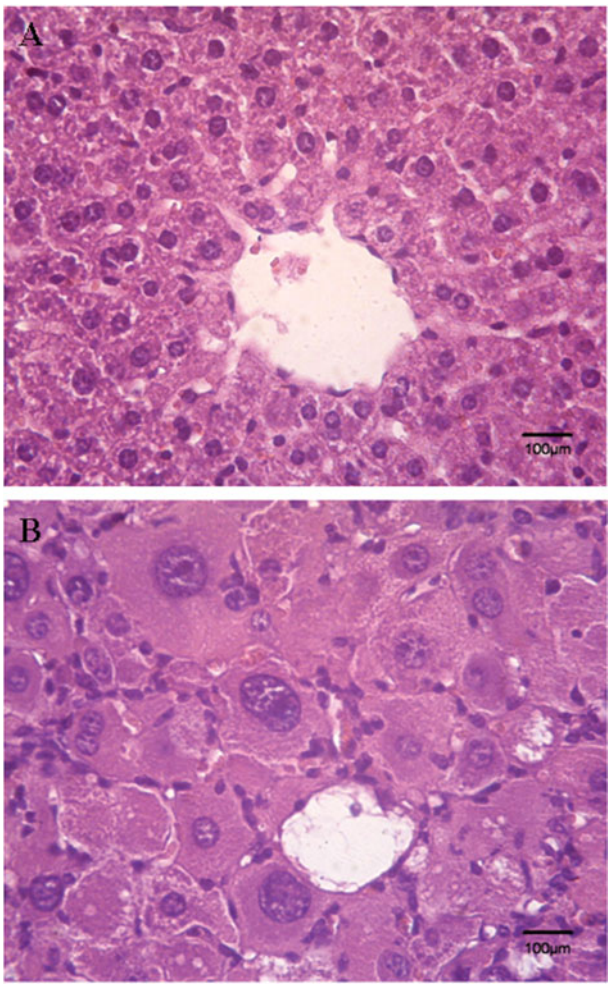

Fig. 4. Histological section of liver of healthy mice (A). Photomicrographs of liver sections of infected mice with *Trypanosoma evansi* and treated with the cordycepin and pentostatin combination showing a strong nuclear and cytoplasmic tumefaction and necrotic degeneration in the hepatocytes (B), haematoxylin-eosin stain.

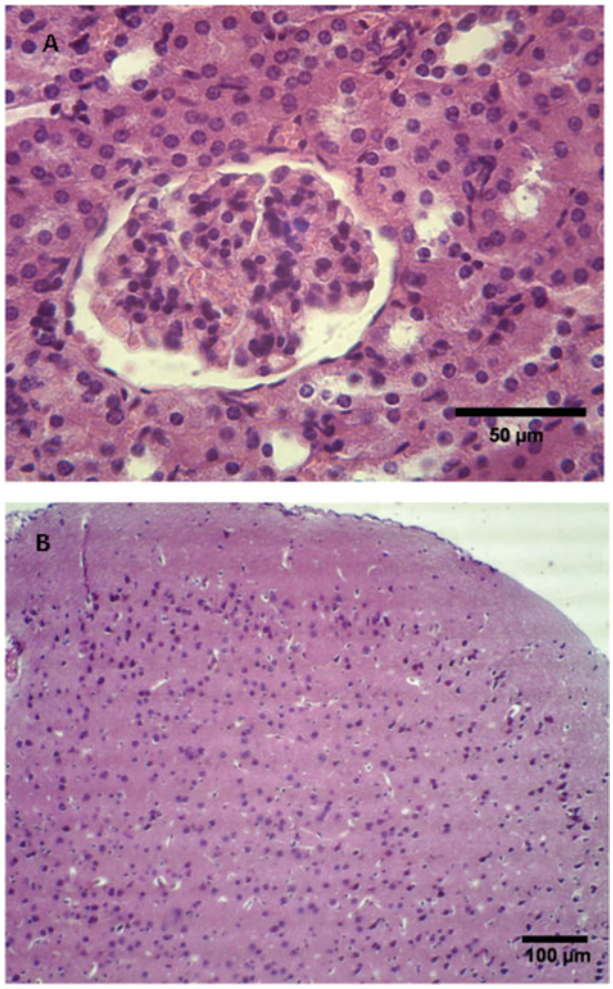

Fig. 5. Mice infected with *Trypanosoma evansi* and treated with the cordycepin and pentostatin combination. Photomicrographs showing the renal tissue that showed a significant increase in glomerular area (A), and cortical region of the brain without histological alterations, despite parasite infection and treatment (B).

et al. 2005; Vodnala *et al*. 2009; Da Silva *et al*. 2011*a*). The susceptibility of *T. evansi* to this adenosine analogue is likely to be related to the parasite's inability to engage in *de novo* purine synthesis (James and Born, 1980; Hammond and Gutteridge, 1984; Hassan and Coombs, 1988). Our study showed that the ADA inhibitor exerts trypanocidal activity, *in vitro*. This effect was, however, seen only in the first evaluation post-treatment at 1 h, which can be explained by the short half-life of the inhibitor (McConnell *et al*. 1978; Major *et al*. 1981). Deoxycoformycin can be unstable in acid and neutral media but relatively stable in the vicinity of pH 9 (Bzowska *et al*. 1985). However, in the *in vitro* tests, the culture medium was pH 7·1, which may have rendered the molecule pentostatin unstable over time and thereby reduced its trypanocidal action. But, the reduction in the number of parasites in the first hour of study suggests that the inhibitor (pentostatin) acts on the ADA present in *T. evansi* (Da Silva *et al*. 2011*b*) and thus kills the parasite. The use of inhibitors, such as anticholinesterase, explains the mechanism of action of some pesticides such as organophosphates (Santos *et al*. 2007).

Pentostatin is an ADA inhibitor that can prevent degradation of cordycepin, and thus a combination of the two drugs has been used for the treatment of certain malignant tumours in humans, such as leukaemia and melanoma (Adamson *et al*. 1977). This combination has also been effective against *T. brucei* (Rottenberg *et al*. 2005; Vodnala *et al*. 2009); in this study, we observed that the highest dose used proved 100% effective against *T. evansi*. However, cordycepin when used alone was not curative *in vivo*, but rather increased the longevity of the treated animals. This was probably because of the rapid deamination of cordycepin (3′-deoxyadenosine) to 3′-deoxyinosine, which can then be inactivated by the trypanosomal enzymes inosine and deoxyinosine hydrolases (Rottenberg *et al*. 2005). Based on the *in vitro* results, we believe that the addition of pentostatin prevents the degradation of the

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adenosine analogue, and thus the combination of the two drugs produced a brief trypanocidal action. However, when cordycepin was used alone, it had no apparent effect on the parasitaemia and in the longevity of mice.

The anti-trypanosomal activity of cordycepin against *T. brucei* was noted to be enhanced by the addition of the ADA inhibitor EHNA (Williamson and Scott-Finnigan, 1978). However, in contrast, this combination failed to induce a curative effect in 42·5% of mice infected with *T. evansi* (Da Silva *et al*. 2011*a*). The combination of adenosine analogue 3′-deoxyadenosine and coformycin or deoxycoformycin produced a curative effect in *T. brucei* infection in mice (Rottenberg *et al*. 2005) as was similarly observed in the *T. evansi*-infected mice in our study. The difference in curative efficacy may be related to specificity of the ADA inhibitor, i.e. the inhibitor EHNA is specific for the isoform ADA_1, since the other two inhibitors (coformycin or deoxycoformycin) are capable of inhibiting both ADA_1 and ADA_2. It should be remembered that tissue extracts contain predominantly ADA_1, which is supposed to be derived mainly from injured tissues. ADA_2 is found in serum and derived from stimulated T cells (Greiger and Nagy, 1986; Franco *et al*. 1997).

The administration of cordycepin and coformycin/deoxycoformycin eliminated parasites in the brain parenchyma when administered after *T. brucei brucei* penetration into the brain (Rottenberg *et al*. 2005). This is a highly desirable outcome, because the difficulty of chemotherapy in *T. evansi* infections has been attributed to survival of trypanosomes in cerebrospinal fluid as trypanocidal drugs do not cross the blood–brain barrier or do so in insufficient doses to cure infection (Jennings *et al*. 1977; Spinosa *et al*. 1999). Therefore, treatment with cordycepin can be a viable alternative for animals of high economic or sentimental value due to the high costs of cordycepin and ADA inhibitors in Brazil.

Apparently the mice of this study showed no clinical signs suggestive of intoxication. However, in the biochemical tests an increase in liver enzymes associated with histological lesions in the liver of animals in groups F and G was observed. The treatment also caused low intensity, histological renal lesions without impairment of the renal function given that the creatinine levels remained within normal limits. Thus, any application of the promising treatment results observed from our studies should be made cautiously to avoid untoward effects. Effects of toxicity of cordycepin in combination with the ADA inhibitor 2-deoxycoformycin in beagle dogs were also observed as well as when only the inhibitor was used. But the dogs which received only cordycepin showed no drug-related toxicities (Rodman *et al*. 1997). In a future study, we plan to investigate whether toxicity may be related to the ADA inhibitor.

Data from this study did not detect resistance of the parasite to cordycepin combined with the inhibitor except in 1 of the 7 mice of group F in which infection relapsed (and which died later). However, it has been reported that *T. b. brucei* developed resistance to cordycepin upon prolonged culture with low doses of the compound; such resistant parasites showed diminished virulence and reduced growth *in vivo* (Vodnala *et al*. 2009). In other studies, the cordycepin-resistant parasites showed a genetic defect in TbAT1 and surprisingly without cross resistance to other trypanocidal drugs (Maser *et al*. 1999; Koning *et al*. 2004). Therefore, cordycepin combined with an ADA inhibitor may be an optimal treatment option when there is resistance to other anti-trypanosomal drugs. Results from our studies suggest curative efficacy of a combination of 3′-deoxyadenosine and deoxycoformycin in mice experimentally infected with *T. evansi*. Note that *in vitro* and *in vivo* the cordycepin and pentostatin combination potentiated the trypanocidal effect against *T. evansi*. Future studies are necessary to evaluate the effects of the treatment on haematological and biochemical parameters, enzymatic purinergic system, as well as to verify if pentostatin has the ability to inhibit the ADA of *T. evansi*.

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