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Original Article

Diminazene Aceturate Liposomes: Morphometric and Biochemical Liver, Kidney, and Spleen of Rats Infected with *Trypanosoma evansi*

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ABSTRACT

The aim of this study was to evaluate the effect of treatment with liposomal (L-DMZ) and conventional (C-DMZ) diminazene aceturate formulations on hepatic and renal functions of rats, experimentally infected with *Trypanosoma evansi*. For this purpose, 72 Wistar rats (*Rattus norvegicus*) were divided into six groups (A, B, C, D, E, and F). Each group was subdivided into two other subgroups in order to assess the biochemical and histological results on days 7 and 40 post-treatment (PT). Treatments were carried out based on two different therapeutic protocols: L-DMZ and C-DMZ at 3.5 mg/kg^{-1}, single dose (groups C and D), and five successive doses within intervals of 24 h (groups E and F). Groups A and B corresponded to uninfected and infected (without treatment) animals, respectively. Sample collections were held on days 7 and 40 PT for the assessment of hepatic [alkaline phosphatase (AP), alanine transferase (ALT), albumin, gamma glutamil transferase (GGT) and renal functions (creatinine and urea). Additionally, the histology of fragments of liver, kidney, and spleen was performed. Animals in group B showed a significant increase in AP, GGT, ALT, and urea when compared with group A. On day 7 post-inoculation (PI), the biochemical analysis showed a reduction ($P < 0.05$) of AP and GGT, while the levels of urea were increased in groups C, D, E, F. On day 40 PT, ALT was increased in these same groups when compared with group A. In histopathology, changes in liver samples were observed on day 7 PT in groups D and F, especially regarding the area and density of the hepatocytes. Renal analysis exhibited changes in glomerular space, glomerular, and corpuscular areas in group E. Therefore, these results allowed us to conclude that the treatment with L-DMZ and C-DMZ led to variable biochemical changes, which defined the functions of the liver and kidneys of treated animals, since the main histopathology alterations were observed in animals treated with liposomes, at their higher dosages. Thus, treatments with L-DMZ and C-DMZ in five consecutive doses were effective although being followed by liver toxicity.

Introduction

Diminazene aceturate (DMZ) is a drug commonly used in Brazil for the treatment of trypanosomosis in domestic animals [27,31]. However, DMZ presents reports of resistance and ineffectiveness against different isolates [10], and it is considered toxic to camels, dromedaries, dogs, and horses [20,34]. This toxicity is attributable to its residual period, not to mention the fact that it easily spreads among various tissues and organs [26].

In this context, the aim of this study was to evaluate the biochemical and histological parameters of rats experimentally infected with *T. evansi* and treated with DMZ, conventional and encapsulated in liposomes, in order to evaluate the toxicity of these formulations. A recent study has shown that L-DMZ has curative

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effectiveness in treating rats experimentally infected with *T. evansi* [25].

The use of liposomes has improved the selectivity for alterations of the pharmacokinetic behavior of drugs (release, metabolism, and excretion). This feature favors the therapy-reducing toxicity, biodistribution, and delivery of the active ingredient to the target organ or tissue. Liposomes are used as an alternative transport system in order to reduce systemic toxicity, mainly because these molecules modify the pharmacokinetics and biodistribution of drugs [21].

Material and Methods

Reagents

The reagents used for the preparation of culture medium, except for antibiotics and diminazene aceturate (DMZ), were obtained from Sigma Chemical Co (St. Louis, MO, USA). DMZ (Ganazeg®) was purchased from Novartis, São Paulo, Brazil. Soybean phosphatidylcholine (100%) was obtained from Idealfarma (São Paulo, Brazil), while polysorbate 80 was supplied by Henrifarma (São Paulo, Brazil), and cholesterol was donated by Cristália (São Paulo, Brazil). Potassium phosphate monobasic and sodium phosphate dibasic were purchased from Vetec (Rio de Janeiro, Brazil), and ethyl acetate was obtained from F. Maia (São Paulo, Brazil). HPLC grade methanol and acetonitrile were acquired from Tedia (São Paulo, Brazil).

Liposomes Preparation

Liposomes were produced in batches of 100 mL, in triplicate, employing the method of evaporation in reverse phase [23], as described by [25].

Trypanosoma evansi Isolate

T. evansi was originally isolated from a dog naturally infected [7], kept cryopreserved under laboratory environment. Initially, two Wistar rats (R1 and R2) were infected intraperitoneally with blood (cryopreserved in liquid nitrogen) in order to obtain an inoculum for all the experimental animals.

Animals and Experimental Design

Our experimental animals were composed of 72 male Wistar rats (*Rattus norvegicus*), 60 days old, and weighing an average of 170 g (±6.3 g). The animals were kept in cages with controlled temperature, humidity, and light (23 °C, 70% RH, and 12 h light/dark). They received food and water *ad libitum* daily through an adjustment period of 10 days. During this period, the rats were evaluated in their physical and parasitological aspects, receiving antiparasitic drugs (pyrantel pamoate and praziquantel).

The animals were divided into six groups (A, B, C, D, E, and F), and each group was once again divided into two other subgroups for biochemical and histological analysis to be performed on days 7 (subgroups A1, B1, C1, D1, E1, F1) and 40 (subgroup A2, B2, C2, D2, E2, F2) after treatment.

T. evansi Inoculation

Animals of groups B, C, D, E, and F were inoculated intraperitoneally with 0.2 mL of fresh blood, obtained from rats previously infected with *T. evansi*. It represented a dose of approximately 10^6 parasites/animal.

Infection Evaluation

Parasitemia was evaluated daily until the first sample collection (day 7) and every two days until the second period (day 40). It was held through blood smears, stained through Romanowsky technique, and microscopically observed at a magnification of 1000× [32].

Treatments

The treatments were held as follows: Groups C and E were treated with liposomal diminazene aceturate (L-DMZ). Group C was given 3.5 mg/kg^{-1} as a single dose and group E was given the same dose but for five consecutive days. Groups D and F were treated with conventional diminazene aceturate (C-DMZ). Group D was administered 3.5 mg/kg^{-1} as a single dose and Group F received the same dose for five consecutive days. Groups A and B did not receive any treatment, representing the negative control and positive control, respectively.

The treatment started within 24 h post-inoculation (PI) when the animals were showing low parasitemia (0 to 1 trypomastigotes/field). L-DMZ and C-DMZ were administered intraperitoneally.

Sample Collection

Blood samples were taken from animals in subgroups A1, B1, C1, D1, E1, and F1 on day 7 and on day 40 in subgroups A2, B2, C2, D2, E2, and F2. The animals were anesthetized with isoflurano, and immediately after the anesthesia induction, by cardiac puncture, 5 mL of blood was collected in order to obtain serum samples. After this procedure, the animals were euthanatized. The liver, spleen, and kidneys were removed for the collection of fragments for histology and biochemical tests to evaluate tissue lesion. All the collection procedures followed the ethic and welfare requirements for animal experimentation.

Biochemical Analysis

In order to assess hepatic and renal functions, we performed the analysis of enzymatic activity and assessments of catabolite in all groups. The activity of alanine transferase (ALT), gamma glutamil transferase (GGT), levels of albumin, as well as the catabolite urea and creatinine were assessed through a semiautomatic analyzer (TP Analyzer Plus®, Thermoplate-China), using commercial kits (Diagnostic Labtest® SA, Lagoa Santa, MG, Brazil). All the tests were performed in triplicate.

Histopathological and Morphometry Analysis

Histological analyses were carried out in order to investigate possible tissue damage in animals infected and treated with L-DMZ and C-DMZ. Samples of liver, kidney, spleen, and brain were fixed in formaldehyde solution (10%), for 24 h, dehydrated in alcohol series, diaphanized in xylene, and embedded in paraffin. The blocks were trimmed providing sample sections of 6 por μm thick in microtome Easy Path EP-31-20094. The slides were stained with hematoxylin–eosin (HE), and subsequently photographed at 40× (liver), 10× (kidney), and 4× (spleen) in five random fields for morphometry.

Morphometry was performed using the software ImagePro Plus®. On each slide, several random fields were photographed (05 in the liver, 12 renal corpuscles in 06 fields in the kidney and spleen). The Image-Pro Plus software was used to measure the different morphometric parameters studied, namely, cell density and hepatocyte nuclear area in the liver, corpuscular, capsular, and glomerular area in the kidney and spleen.