ORIGINAL ARTICLE

WILEY

Amprolium exposure alters mice behavior and metabolism in vivo

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Funding information
Conselho Nacional de Desenvolvimento Científico e Tecnológico, Grant/Award Number: 470252/2013-8

Abstract

Background: Thiamine deficiency (TD) models have been developed, mainly using the thiamine analog pyrithiamine. Other analogs have not been used in rodents. We aimed to evaluate the effects and mechanisms of intraperitoneal (ip) amprolium-induced TD in mice. We also evaluated the associated pathogenesis using antioxidant and anti-inflammatory compounds (Trolox, dimethyl sulfoxide).

Methods: Male mice were separated into two groups, one receiving a standard diet (control animals), and the other a TD diet (deficient groups) for 20 days. Control mice were further subdivided into three groups receiving daily ip injections of saline (NaCl 0.9%; Cont group), Tolox (Tr group) or dimethyl sulfoxide (DMSO; Dmso group). The three TD groups received amprolium (Amp group), amprolium and Trolox (Amp+Tr group), or amprolium and DMSO (Amp+Dmso group). The animals were subjected to behavioral tests and then euthanized. The brain and viscera were analyzed.

Results: Amprolium exposure induced weight loss with hyporexia, reduced the behavioral parameters (locomotion, exploratory activity, and motor coordination), and induced changes in the brain (lower cortical cell viability) and liver (steatosis). Trolox co-treatment partially improved these conditions, but to a lesser extent than DMSO.

Conclusions: Amprolium-induced TD may be an interesting model, allowing the deficiency to develop more slowly and to a lesser extent. Amprolium exposure also seems to involve oxidative stress and inflammation, suggested as the main mechanisms of cell dysfunction in TD.

KEYWORDS
brain, dimethyl sulfoxide, thiamine deficiency, Trolox

1 | INTRODUCTION

Thiamine plays a central role in the metabolism of nervous tissue.[1] It is a cofactor for several enzymes, and the biosynthesis of cellular constituents,[2,3] and a crucial cofactor for the generation of mitochondrial ATP.[4]

Thiamine deficiency (TD) affects both humans and animals. In humans, TD is not considered rare, particularly in developing

countries, where malnutrition is widespread.[5] It also induces the classical manifestations of beriberi, Wernicke encephalopathy, and Wernicke-Korsakoff syndrome.[3,6,7] In animals, TD is related to food management problems or digestive disorders,[8,9] and is associated with the development of polioencephalomalacia (PEM) in ruminants (cattle, sheep, and goats),[8] and Chastek's paralysis in carnivores (canines and felines).[9,10]

The central nervous system (CNS) lesions induced by TD are extensive and varied, and occur in different structures; depending on the species involved, they may take diverse forms.[3,6,11] General TD-induced histopathological changes include neuronal degeneration and necrosis, *status spongiosus*, myelin degeneration, astrocytic swelling, gitter cell infiltration, inflammatory infiltrate, haemorrhage, and endothelial cell hypertrophy and hyperplasia.[3,12] In humans, the frequently affected areas are the thalamus, cerebellum, and brainstem.[13,14] In carnivores, the normally affected areas are the brainstem nuclei,[15] but in ruminants, PEM lesions are primarily located in the cerebral cortex.[11] In the traditional rat and mouse models of TD, the disorder largely mimics the disease that affects humans.[3,6]

Several potential mechanisms have been proposed to explain the development of neuropathology associated with TD, including changes in neurotransmitter metabolism, glucose utilization by neurons, oxidative stress, lactic acidosis, induction of apoptosis, changes in brain blood flow, inflammation, and excitotoxicity.[3,6,16,17] However, oxidative stress has been considered the primary cellular mechanism.[3]

The scarcity of knowledge about the mechanisms of cell degeneration and the need for a better understanding of the pathology associated with TD have stimulated the establishment of experimental models of the disease in laboratory rodents.[3] There are two well-defined models that are used for the study of TD.[3,6] The first method uses vitamin-free diets to deplete the body of thiamine (4 weeks). The second model generates TD more rapidly (9-12 days) by combining the thiamine-free diet with intraperitoneal (i.p.) injections of pyrithiamine. The second model, termed pyrithiamine-induced TD, faithfully mimics the neuropathology described for TD in humans,[18,19] but induces a sudden, severe, rapid, and difficult-to-control neurological disorder, which is infrequently fatal. Moreover, this model is very expensive. Another thiamine chemical analog, amprolium (significantly more accessible), is potentially useful for the induction of experimental TD,[20-23] but its effects on rodents are not well known. However, studies suggest that the in vivo action of amprolium in the induction of TD may be better than the widely used pyrithiamine model.[24]

Amprolium (1-([4-Amino-2-propyl-5-pyrimidinyl]methyl)-2-methyl-pyridinium chloride) is a coccidiostat widely used in animals.[25] Treatment with amprolium orally (PO) for 5 days is effective and safe.[26,27] However, high dosages or prolonged PO treatment induce PEM in ruminants[28] and metabolic and behavioral changes in laboratory rodents.[23] Amprolium acts as a thiamine antagonist, blocking vitamin transport across the blood-brain barrier,[29] uptake in the intestine,[30] and transport in the cell.[31]

In this study, we evaluated the effects of amprolium ip administration in mice, with respect to its behavioral, metabolic, and anatomopathological aspects. In addition, we evaluated the associated pathogenesis using antioxidant and anti-inflammatory compounds (Trolox, dimethyl sulfoxide). Thus, we aimed to perform an initial screening of amprolium in mice as a potential alternative model for induction of TD.

2 | METHODS

2.1 | Reagents

Amprolium, Trolox, and 2,3,5-triphenyl-tetrazolium chloride (TTC) were obtained from Santa Cruz Biotechnology (CA, USA). AIN-93M (standard) and AIN-93TD (thiamine deficient) chow were purchased from PRAG Soluções Biociências (SP, Brazil). Dimethyl sulfoxide (DMSO) was purchased from Amresco (OH, USA). HEPES was obtained from Uniscience (SP, Brazil). All other reagents were of the highest analytical grade.

2.2 | Animals and treatment

Male Swiss mice (50 days old) were used. They were maintained in an air-conditioned cabinet (23 ± 1°C) on a 12-hours light/dark cycle, with water and food available ad libitum. The animals were treated, manipulated, and euthanized according to the ethics code of animals used in research, approved by the Universidade Federal do Tocantins Ethics Committee on Animal Use (CEUA-UFT, process 23101.000284/2014-13). All efforts were made to minimize the number of animals used and animal suffering.

For TD induction, we induced dietary deficiency of thiamine in conjunction with ip injections of amprolium. The mice were separated into six groups (n = 6 per group) and subjected to different treatments (Table 1) for 20 days. Groups 1, 5, and 6 received the standard chow[32] (AIN-93M) throughout the 20-day period. Groups 2, 3, and 4 received a thiamine-deficient feed (AIN-93TD). The constituents of the TD diet were identical to those of the standard diet, except for the lack of vitamin. The groups received water and feed ad libitum, and daily ip injections of saline solution

TABLE 1 Experimental design and distribution of treatment groups of mice with amprolium-induced thiamine deficiency

Experimental group	Treatment
Control (Cont)	Saline
Deficient (Amp)	Amprolium
Deficient with Trolox (Amp+Tr)	Amprolium, followed by Trolox
Deficient with DMSO (Amp+Dmso)	Amprolium, followed by dimethyl sulfoxide
Control Trolox (Tr)	Trolox
Control DMSO (Dmso)	Dimethyl sulfoxide

Saline: NaCl 0.9% solution.
Amprolium, Trolox, and DMSO were dissolved or diluted in saline solution.

(NaCl 0.9%; control, Group 1), amprolium (80 mg/kg; deficient, Groups 2, 3, and 4) and/or Trolox (1 mg/kg; Groups 3 and 5) or DMSO (1 ml/kg, 10% solution in saline; Groups 4 and 6). The injected volumes were adjusted to 0.1 ml/10 g of body weight.[33-35] During the treatment period, weight gain and feed intake were monitored.

The dose of amprolium used was determined by preliminary internal tests (data not shown). A lower dose (60 mg/kg) administered for 20 days did not induce any change in the animals, whereas a higher dose (100 mg/kg) induced acute death without other changes in the animals. Thus, amprolium at 80 mg/kg was considered effective while not too aggressive, and improvements in the condition could be achieved with treatment within a reasonable time, as expected.

2.3 | Behavioral tests

The animals were subjected to the open field and rotarod behavioral tests, and were habituated to the test room for 1 hour before starting the tests, which were performed during the light phase of the circadian cycle (10 AM to 5 PM).

The open field tests were performed in a circular arena (Bonther, SP, Brazil). In the tests (duration: 10 minutes), we evaluated the distance travelled, fecal production, rearing, and grooming in two stages.[23,34] The first stage (day zero, basal behavior) was carried out on the first day of treatment and the second was carried out 24 hours after the last day (day 20). The results (means) are the measurements on day 20 expressed as a percentage of the measurements on day zero (100%) ± SE.

In the rotarod test (Insight Equipamentos Científicos, SP, Brazil), the animals were subjected to conditioning and testing.[23,36] The conditioning was performed on the stationary cylinder for 30 seconds, followed by a 90-second period on the cylinder at a speed of 5 rpm. The animals that failed in this first stage were subsequently subjected to no more than two additional sessions. A failure in the third session was exclusion criteria for the test sessions. After 30 minutes, the animals were tested and the latency time to fall was recorded. The test consisted of two sessions with a maximum duration of 5 minutes, and 30-minute intervals between the sessions, starting at a speed of 5 rpm and subsequent increase of 0.1 rpm/s, performed after the last day of treatment. The values are expressed in seconds ± SE.

2.4 | Cell viability assay

We used the TTC reduction method to evaluate the cellular viability in the CNS. TTC is reduced to an insoluble pigment (red formazan) by mitochondrial dehydrogenases, and thus, the amount of pigment formed is directly proportional to cellular metabolic activity.[37]

After the behavioral tests, the mice were euthanized by decapitation, and their brains removed at 4°C and sliced at a thickness of 500 μm using a cutting matrix for mouse brains. Subsequently, the slices were preincubated in HEPES-saline buffer (124 mM NaCl, 4 mM KCl, 1.2 mM MgSO$_4$, 25 mM HEPES, 12 mM glucose, and 1 mM CaCl$_2$, pH 7.4) at room temperature for 30 minutes and then incubated in the same buffer for 30 minutes with 1% TTC at 37°C.[35,38] After incubation, the buffer was removed, and the slices were washed with saline (0.9% NaCl) and then fixed in 4% paraformaldehyde solution. The slices were then scanned, and cell viability was determined by measuring the optical density (O.D.) of the chromogen produced in the areas of interest on the cut surface of the slices—cerebral cortex, striatum, thalamus, and hippocampus—using Image J software (National Institute of Health, MD, USA) (Figure 1). The data are expressed as percentages relative to the control (considered 100%) and the values are expressed as means ± SE.

2.5 | Histopathological analysis

The viscera (liver, kidney, lung, and heart) and the brains were isolated during post mortem examination and fixed in 10% buffered formalin. Afterward, they were routinely processed and stained with hematoxylin and eosin (HE). Histopathological analysis was performed using a Bioptika B20T trinocular optical microscope coupled to an ISH500 CMOS-5.0 digital color camera (Tucsen Photonics, Fujian, P. R. China). The images were projected on the monitor and captured by ISCapture v.3.6.7 software (Tucsen Photonics, Fujian, P. R. China), while using a 40× objective.

For the histopathological analysis, two semi-quantitative methods were used: the determination of the mean value of changes (MVC) and the degree of tissue changes (DTC). The MVC calculation is based on the incidence of lesions,[39] with the assignment of numerical values for each animal: grade 1 (absence of lesions), grade 2 (occurrence of focal lesions), and grade 3 (occurrence of diffuse lesions). The DTC was used to determine the intensity of the
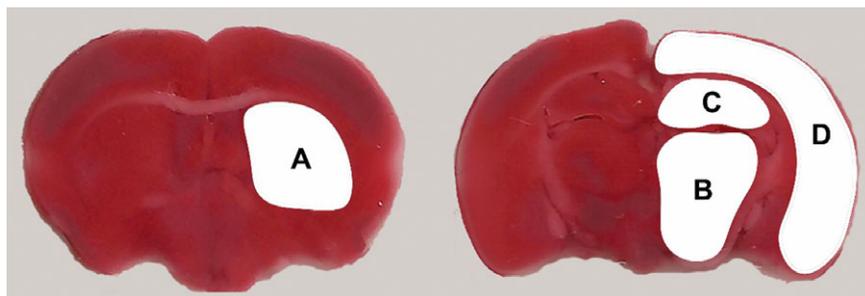

FIGURE 1 Schematic representation of cell viability in mouse brain slices, evaluated by the colorimetric method of TTC reduction. The area of interest in the encephalic structures was delimited and evaluated by O.D. of the produced red chromogen. The striatum (A), thalamus (B), hippocampus (C), and cerebral cortex (D) were analyzed

changes,[40] and each lesion was classified into progressive degrees for tissue function impairment: stage I (mild lesions that do not impair the functions), stage II (moderate lesions that compromise the functions), and stage III (marked lesions with severe impairment of functions, possibly becoming irreversible) (Table 2). The DTC value was calculated for each animal using the formula: DTC = $(1 \times \Sigma I) + (10 \times \Sigma II) + (100 \times \Sigma III)$, where ΣI, ΣII, and ΣIII correspond to the number of changes at stages I, II, and III, respectively. DTC values between 0 and 10 indicate normal functional tissue activities, those between 11 and 20 indicate mild functional impairment, those between 21 and 50 indicate moderate functional impairment, and those between 51 and 99 indicate damage, while values above 100 indicate irreversible damage.

2.6 | Statistical analysis

Data are expressed as means ± standard error (SE) and statistical significance was determined by analysis of variance, followed by Duncan's post hoc test, wherever appropriate.[34] Statistical significance was set at $P \leq 0.05$. The data were processed using STATISTICA '98 Edition software (StatSoft, OK, USA).

TABLE 2 Histopathological classification of liver lesions of mice with amprolium-induced thiamine deficiency

Histological change	Stage
Microvesicular cytoplasmic degeneration (lipid), focal	I
Cellular edema	I
Cellular hypertrophy	I
Nuclear hypertrophy	I
Nuclear contour deformity	I
Cellular atrophy	II
Breakdown of hepatocyte cords	II
Microvesicular cytoplasmic degeneration (lipid), diffuse	II
Macrovesicular cytoplasmic degeneration (lipid), focal	II
Hydropic degeneration, moderate	II
Nuclear vacuolation	II
Absence of a nucleolus	II
Eccentric nucleus	II
Vascular congestion	II
Cholestasis	II
Macrovesicular cytoplasmic degeneration (lipid), diffuse	III
Hydropic degeneration, accentuated	III
Chromatin condensation	III
Nuclear fragmentation	III
Absence of nucleus	III
Cytoplasmic eosinophilia	III
Cell rupture	III
Fibrosis	III
Ductal proliferation	III

Adapted from Poleksic & Mitrovic-Tutundzic.[40]

3 | RESULTS

3.1 | Effects on feed intake and weight gain

Animals that received the TD diet in association with amprolium (Amp group) showed a significantly lower body weight gain (Table 3) compared with the control group ($P = 0.001$). Co-treating with the protective substances Trolox and DMSO did not prevent the reduction of weight gain in these animals (Amp+Tr, $P = 0.912$; Amp+Dmso, $P = 0.038$; compared with Amp group). However, Trolox and DMSO per se did not influence the weight gain (Tr, $P = 0.746$; Dmso, $P = 0.512$; compared with control).

In parallel with the decrease in body weight gain, the Amp group showed a reduction in feed intake (Table 4) relative to the control group ($P = 0.004$). In the deficient animals receiving Trolox or DMSO, no effects on the reduction of feed consumption were observed, compared with the Amp group (Amp+Tr, $P = 0.382$; Amp+Dmso, $P = 0.525$). Similarly, Trolox and DMSO per se did not interfere with the feed intake of the animals (Tr, $P = 0.997$; Dmso, $P = 0.665$; compared with control).

3.2 | CNS cell viability assay

In the CNS cell viability assay of mice with amprolium-induced thiamine deficiency, the animals from the Amp group showed a decrease of 9.99% in cell viability within the cerebral cortex ($P = 0.00007$, compared with control; Figure 2). Trolox (Amp+Tr group) partially reduced the fall in cell viability in the cerebral cortex of the deficient animals, with a 5.51% reduction in viability with respect to the control ($P = 0.007$, with respect to the Cont group; $P = 0.021$, with respect to the Amp group). On the other hand, DMSO (Amp+Dmso group) significantly blocked the fall in cell viability in the cerebral cortex ($P = 0.001$). In addition, no changes were observed in the cell viability within the hippocampus, striatum, and thalamus.

TABLE 3 Body weight gain of mice with amprolium-induced thiamine deficiency

Group	Weight (g) Day zero	Weight (g) Day 20	Weight gain (g)
Cont	39.96 ± 0.99	45.17 ± 1.30	5.21 ± 0.49
Amp	41.08 ± 0.90	41.02 ± 1.10	−0.06 ± 1.03*
Amp+Tr	39.49 ± 1.41	39.59 ± 1.77	0.11 ± 1.10*
Amp+Dmso	39.51 ± 1.88	36.25 ± 1.91	−3.26 ± 1.51*
Tr	40.78 ± 0.70	46.49 ± 0.98	5.71 ± 0.58
Dmso	39.14 ± 1.55	45.43 ± 1.35	6.29 ± 1.48

The mice in different treatment groups were provided with AIN-93M chow and saline (control, 0.9% NaCl), AIN-93DT chow and amprolium (Amp), AIN-93DT chow and amprolium with Trolox (Amp+Tr), AIN-93DT chow and amprolium with DMSO (Amp+Dmso), AIN-93M chow and Trolox (Tr), and AIN-93M chow and DMSO (Dmso). The results represent the means ± SE of weight gain (in grams) during the experimental period, derived from six independent replicated. The results were analyzed using ANOVA followed by Duncan's test.

*$P \leq 0.05$ compared to control.

3.3 | Anatomopathological evaluation

During the anatomopathological evaluations, we initially investigated the brains of the mice with amprolium-induced thiamine deficiency. Surprisingly, we did not observe any morphological changes in the CNS in any of the treatments used (Figure 3A), despite the fall in cellular viability observed in the cerebral cortex (Figure 2). We evaluated all the encephalic structures, with an emphasis on the hippocampus, cerebral cortex, striatum, cerebellum, and thalamus. On the other hand, at the gross pathology level of animal viscera, we observed a moderate increase in blanching of the liver in the animals of the Amp group (Figure 3B). In the Amp+Tr and Tr groups, the livers were slightly pale. Histopathology revealed that the animals of the Amp group developed macrovesicular hepatic steatosis, with a

TABLE 4 Feed intake of mice with amprolium-induced thiamine deficiency

	Consumption (g) Day zero	Consumption (g) Day 20	Variation (g)
Cont	5.19 ± 0.30	4.02 ± 0.20	−1.17 ± 0.28
Amp	5.77 ± 0.27	2.83 ± 0.28	−2.95 ± 0.41*
Amp+Tr	5.19 ± 0.30	2.74 ± 0.27	−2.46 ± 0.28*
Amp+Dmso	5.13 ± 0.41	1.83 ± 0.16	−3.30 ± 0.29*
Tr	5.69 ± 0.42	4.52 ± 0.09	−1.17 ± 0.48
Dmso	5.43 ± 0.36	4.50 ± 0.07	−0.93 ± 0.29

The mice in different treatment groups were treated with AIN-93M chow and saline (control, 0.9% NaCl), AIN-93DT chow and amprolium (Amp), AIN-93DT chow and amprolium with Trolox (Amp+Tr), AIN-93DT chow and amprolium with DMSO (Amp+Dmso), AIN-93M chow and Trolox (Tr), and AIN-93M chow and DMSO (Dmso). The results represent the means ± SE of feed consumption (in grams) during the experimental period, derived from six independent replicates. The results were analyzed using ANOVA followed by Duncan's test.
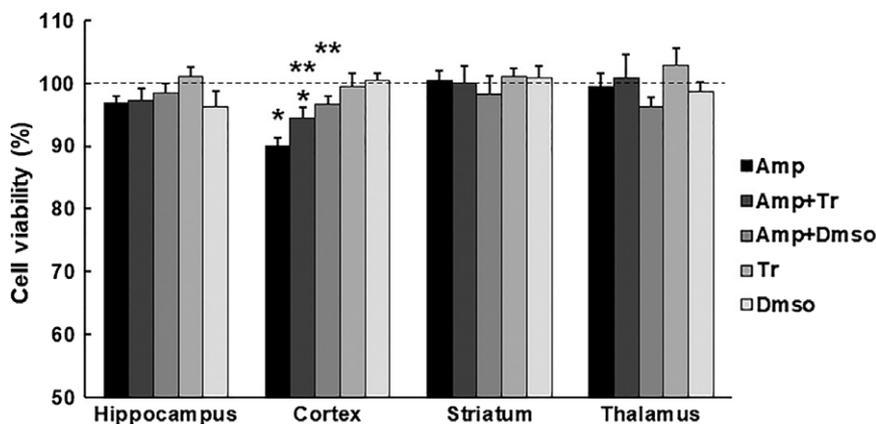*$P \leq 0.05$ compared to control.

focal-to-diffuse distribution (Figure 3C). Interestingly, similar conditions were seen in the Amp+Tr and Tr animals but with microvesicular steatosis instead (Figure 3C). On the other hand, in the Amp+Dmso and Dmso groups, we observed very discrete hepatic steatosis, signaled by the presence of sparse hepatocytes with small, focally distributed cytoplasmic vacuoles (Figure 3C).

The histological findings reflected MVC elevation (Figure 4A) in the Amp (2.67 ± 0.21, $P = 0.0001$), Amp+Tr (2.50 ± 0.22, $P = 0.0002$), and Tr groups (2.33 ± 0.21, $P = 0.0008$), compared with control (1.17 ± 0.17). However, despite the similar MVC among these groups, when compared with the Amp group (61.83 ± 17.71), Trolox significantly reduced the intensity of the lesions, as shown by the liver DTC values of 20.17 ± 4.83 ($P = 0.001$) in the Amp+Tr group and 21.00 ± 3.67 ($P = 0.0009$) in the Tr group (Figure 4B). Interestingly, the liver DTC values of the Amp+Dmso (4.00 ± 2.08, $P = 0.988$) and Dmso groups (4.17 ± 1.85, $P = 0.979$) were similar to that of the Cont group (3.83 ± 1.96). In the other viscera (kidney, lung, and heart) no morphological changes were observed (data not shown).

3.4 | Behavioral effects of amprolium exposure

In the open field test, the TD mice (Amp group) showed a 48.98% reduction in distance travelled ($P = 0.00007$) and a 47.71% reduction in rearing activity ($P = 0.0001$), with respect to the control (Figure 5A). Trolox (Amp+Tr group) did not attenuate these effects in mice, with a 34.27% reduction in distance travelled relative to the control ($P = 0.003$; $P = 0.176$, compared with Amp group) and a rearing decrease of 35.93% relative to the control ($P = 0.005$; $P = 0.207$ compared with Amp group). Similarly, the DMSO (Amp+Dmso group) did not reduce distance travelled, with a 34.22% decrease relative to the control ($P = 0.003$; $P = 0.201$ compared with Amp group). However, it conferred partial protection during rearing ($P = 0.049$, relative to the
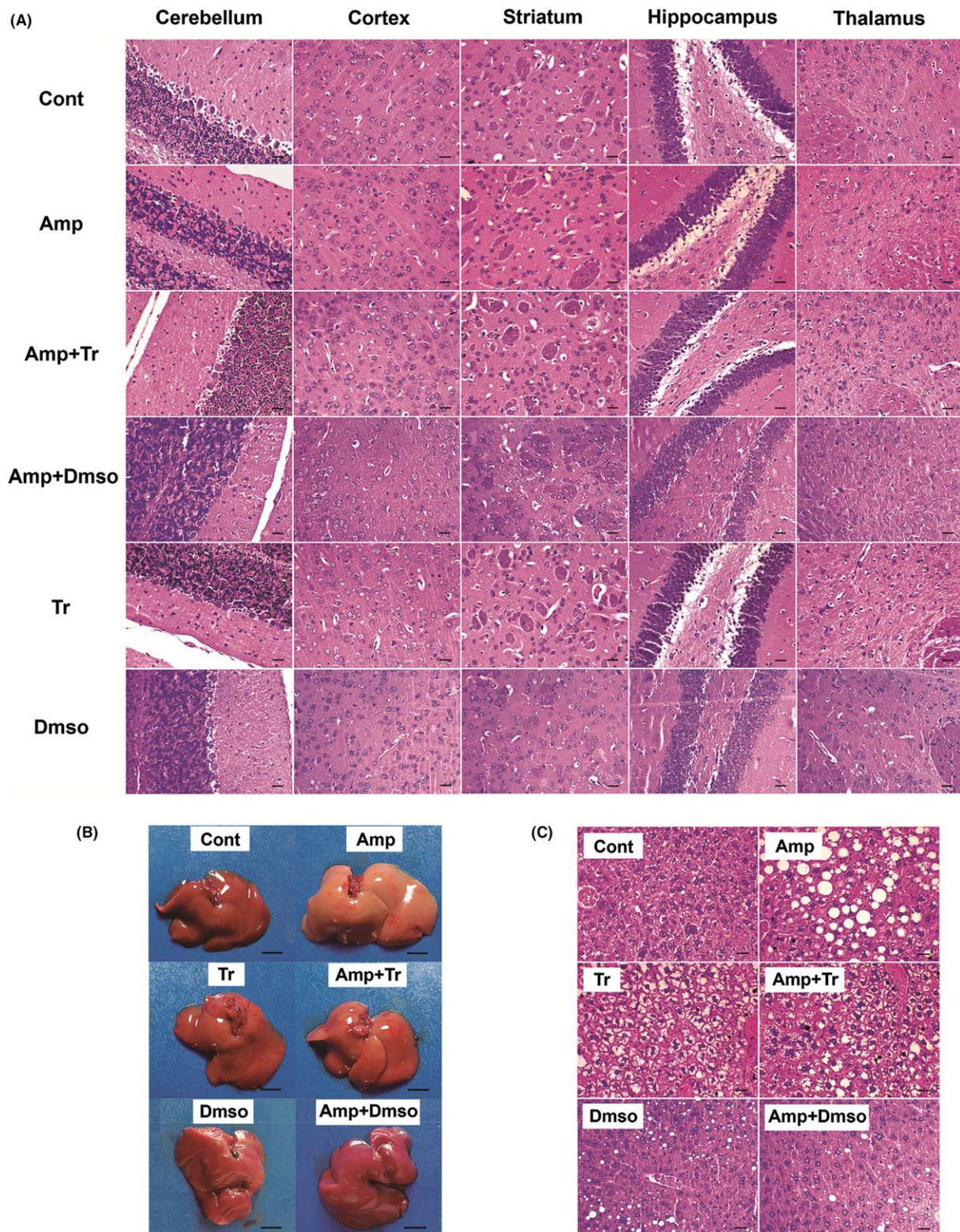

FIGURE 2 Brain cell viability of mice exposed to amprolium-induced thiamine deficiency. The panel shows the cell viability of the different encephalic regions of mice treated for 20 d with AIN-93M chow and saline (control, 0.9% NaCl), AIN-93DT chow and amprolium (Amp), AIN-93DT chow and amprolium with Trolox (Amp+Tr), AIN-93DT chow and amprolium with DMSO (Amp+Dmso), AIN-93M chow and Trolox (Tr), and AIN-93M chow and DMSO (Dmso). The slices were incubated with TTC for 30 min before they were scanned and the optical densities (O.D.) of the brain regions were measured by specific software. The results represent the means ± SE of the percentages of O.D. relative to the control (considered 100%), derived from six independent replicates. The results were analyzed using ANOVA followed by Duncan's test.
*$P \leq 0.05$ compared with the control; **$P \leq 0.05$ relative to the Amp group

FIGURE 3 Anatomopathological evaluation of mice exposed to amprolium. A, The treatments did not induce morphological changes in the cerebellum, cerebral cortex, striatum, hippocampus, and thalamus of mouse brain in the thiamine deficiency model induced with amprolium. B, Thiamine-deficient mice with amprolium showed pale livers, but the change in the color of the livers was attenuated by Trolox and DMSO. C, Thiamine-deficient mice developed diffuse macrovesicular liver steatosis. Trolox co-treatment ameliorated the condition to microvesicular steatosis, whereas DMSO prevented development of steatosis. Trolox per se also induced microvesicular steatosis. Mice were treated for 20 d with AIN-93M chow and saline (control, 0.9% NaCl), AIN-93DT chow and amprolium (Amp), AIN-93DT chow and amprolium with Trolox (Amp+Tr), AIN-93DT chow and amprolium with DMSO (Amp+Dmso), AIN-93M chow and Trolox (Tr), and AIN-93M chow and DMSO (Dmso). Staining with HE; magnification 400× . Scale bars: 20 μm (A, C) and 0.5 cm (B)
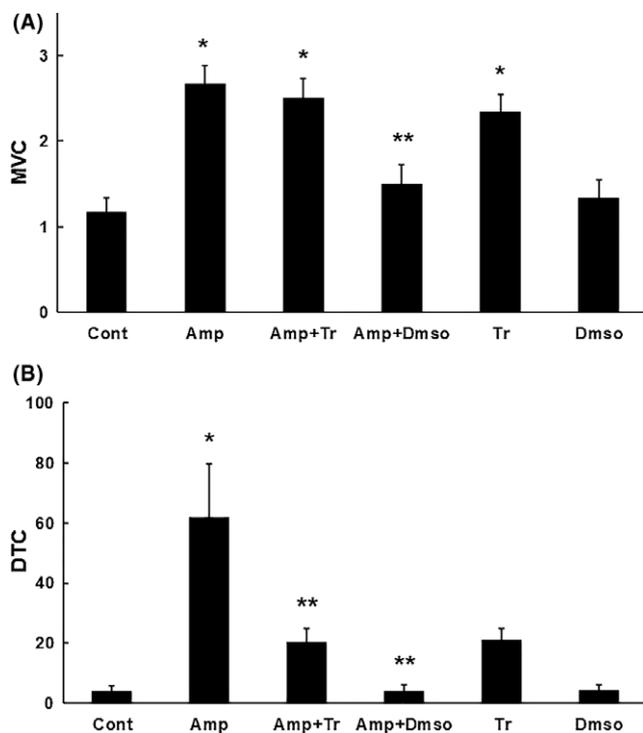
FIGURE 4 Histopathological evaluation of mouse liver in the thiamine deficiency model induced with amprolium. The panel shows the determination of the mean value of changes (MVC; A, and the degree of tissue changes (DTC; B, of mice treated for 20 d with AIN-93M chow and saline (control, 0.9% NaCl), AIN-93DT chow and amprolium (Amp), AIN-93DT chow and amprolium with Trolox (Amp+Tr), AIN-93DT chow and amprolium with DMSO (Amp+Dmso), AIN-93M chow and Trolox (Tr), and AIN-93M chow and DMSO (Dmso). Values represent the means ± SE derived from six independent replicates. The results were analyzed using ANOVA followed by Duncan's test. *P ≤ 0.05 compared with the control; **P ≤ 0.05 relative to the Amp group
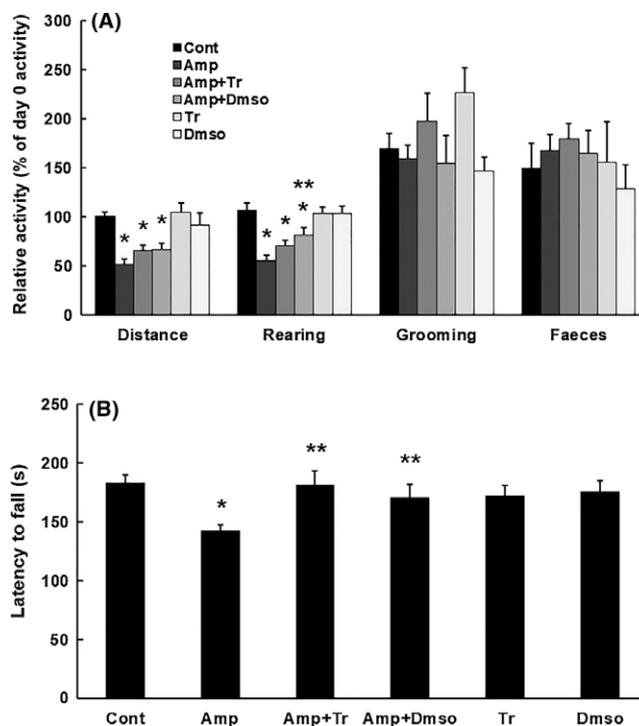

FIGURE 5 Behavioral evaluation of mice in the thiamine deficiency model induced with amprolium. The animals performed open field (A) and rotarod (B) tests after treatment for 20 d with AIN-93M chow and saline (control, 0.9% NaCl), AIN-93DT chow and amprolium (Amp), AIN-93DT chow and amprolium with Trolox (Amp+Tr), AIN-93DT chow and amprolium with DMSO (Amp+Dmso), AIN-93M chow and Trolox (Tr), and AIN-93M chow and DMSO (Dmso). Values represent the means ± SE of the evaluations performed on day 20 as a percentage of those performed on day zero (basal, considered 100%) for the open field test, and the latency to fall (in seconds) for the rotarod test, derived from six independent replicates. The results were analyzed using ANOVA followed by Duncan's test. *P ≤ 0.05 compared with the control, **P ≤ 0.05 compared with the Amp group

Cont group; P = 0.038, with respect to Amp group). Grooming and disposal of fecal boli were not altered by the treatments (Figure 5A).

In the rotarod test (Figure 5B), animals from the Amp group demonstrated a decrease in latency time to fall, yielding a mean of 142.29 ± 4.80 seconds, against the control group mean of 183.11 ± 7.24 seconds (P = 0.006). Interestingly, both Trolox (Amp+Tr group) and DMSO (Amp+Dmso group) reversed the effects of decreased motor coordination observed in the Amp animals, with mean values of 181.50 ± 11.69 seconds (P = 0.008) and 170.50 ± 10.81 seconds (P = 0.035), respectively. Trolox and DMSO per se did not influence motor coordination.

4 | DISCUSSION

The biological effects of TD[41] have been widely studied using rodent models.[3,6] Although pyrithiamine is the most commonly used thiamine analog in animal models, other antagonists, such as oxythyamine, amprolium, and 3-deazathiamine,[20,24,29,30,42] have been used in in vitro models,[43,44] but very little is known about their effects

in vivo. We evaluated for the first time the effects of amprolium administration by ip injection in mice. Amprolium, a chemical analog of thiamine, is significantly inexpensive and accessible, and is widely used in ruminant models of TD.[21,22,45,46]

The first manifestations observed were the decrease in weight gain and feed intake by the TD mice. These effects are interesting because studies show that TD induces anorexia, increases the resting energy expenditure, and results in a generalized loss of body weight.[47,48] Hyporexia is reversed within 3 days of restoration of thiamine in the diet,[48,49] suggesting that thiamine has a crucial physiological role in the programming of body weight homeostasis, increment, and setpoint regulation.[48] When we combined TD treatments with Trolox or DMSO, we did not observe any changes to the reduction in body weight gain and food consumption. Protective effects of Trolox on animal weight gain have been demonstrated,[34] but other mechanisms are probably also involved.

In the behavior tests, we observed a reduction in locomotor and exploratory activities (open field) and motor coordination (rotarod) in

the Amp group. Interestingly, Trolox failed to reverse the effects in the open field test, whereas DMSO improved exploratory activities. However, co-treatment with both Trolox and DMSO blocked the loss of coordination, resulting in similar latency times to the control. TD animals exhibit oxidative stress and inflammation in the CNS, which may interfere with neurotransmitter metabolism,[50] with the consequent onset of behavioral changes.[51,52] However, our findings suggested that oxidative stress and inflammation were apparently not the only mechanisms involved in behavioral disorders. The disparity of actions of the antioxidant Trolox is intriguing, but other studies have also reported its controversial biological consequences.[53] DMSO is a potent anti-inflammatory agent (besides having free radical scavenger activity), used mainly in the treatment of traumatic cerebral edema and ischemic injury.[54,55] This could explain the effects of DMSO, with a probable anti-inflammatory action in the early stages of amprolium-induced TD.

Many studies indicate that, in rodents, TD-induced CNS lesions are primarily found in the thalamic nuclei.[3] Interestingly, we observed an effect of amprolium on cell viability only in the cerebral cortex. Despite this, we did not observe any morphological changes. Compared with the behavioral changes, ip amprolium-induced neural lesions are apparently mild and functional, yet not sufficient to induce degenerative processes or cell death.[34] It is important to note that TD studies in rodents performed to date used pyrithiamine, whereas in this study, we used amprolium. Amprolium is known to cause TD and PEM in domestic animals, but has not been used in laboratory rodents, although studies indicate that it is ideal for the induction and evaluation of the deficiency.[24] Added to the disparity between the neurological manifestations of amprolium and pyrithiamine, there is a difference in the treatment times. Rodents induced with pyrithiamine begin to present morphological lesions in the encephalon between 9 and 10 days after treatment.[3,6] We maintained a continuous administration of amprolium for up to 20 days, without observing any detectable morphological alterations, despite the occurrence of metabolic and behavioral manifestations. The difference in the speed for the establishment of changes between these compounds could be related to their different biological activities at the cellular level. Pyrithiamine directly inhibits the activity of thiamine diphosphate-dependent enzymes and inhibits thiamine transformation in thiamine diphosphate,[42] whereas amprolium blocks the activity of thiamine transporters (ThTr 1 and 2) and organic cation transporter proteins (OCT 1 and 2).[56] Recently, we evaluated the effects of oral administration of amprolium in mice.[23] In this model, effects became evident only after 80 days of exposure, with interference in feed intake, body weight gain and behavioral changes. In this study, the animals remained on commercial standard diet containing thiamine. These data suggest that amprolium also acts by reducing the intestinal absorption of the vitamin.[30] The absence of obvious clinical neurological manifestations with amprolium confirms the suitability of these TD models, since TD frequently manifests subclinically.[57]

Interestingly, co-treatment with Trolox and DMSO led to a partial reduction in cortical cell viability loss with Trolox, and total protection in animals that received DMSO. Studies have reported high concentrations of reactive oxygen species (ROS) in the thalamus and cerebral cortex of TD animals.[58] However, recent studies showed that inflammatory mechanisms overlap with those that increase oxidative stress.[59] Thus, taking into account the known antioxidant and anti-inflammatory activities of Trolox and DMSO, our findings suggested at least a partial involvement of these processes in amprolium-induced TD.

The histopathology results revealed that the Amp group developed macrovesicular hepatic steatosis, while, interestingly, co-treatment with Trolox produced a moderate amelioration of the condition, manifesting as microvesicular steatosis. The histopathological aspect of steatosis has functional meaning, since the microvesicular form reflects an acute stage and the macrovesicular reflects the progression of the condition.[60] Thus, it appears that Trolox has delayed the development of steatosis in deficient animals. Previous studies have also demonstrated important protective effects of Trolox in stress to the liver.[61,62] However, despite promoting functional improvements in hepatocytes, Trolox also failed to restore morphological changes in other models.[62] On the other hand, DMSO completely prevented the development of hepatic steatosis, evidencing a potent cytoprotective effect.[63-65] Studies have shown that oxidative stress and inflammation are directly related to the development of hepatic steatosis.[66,67] Changes to the liver suggest that amprolium-induced TD modifies the body's energy metabolism,[47,48] inducing hyporexia and weight loss, and overloading the liver with fatty acids, in a negative energy balance, as expected for the condition.

In this study, we report, for the first time, the metabolic, behavioral, and anatomopathological changes induced by ip amprolium injection in mice. Amprolium-induced deficiency also appears to involve oxidative stress and inflammation. The slow and less aggressive induction of TD by amprolium may prove useful and serve as a suitable model for assessing the progressive changes that occur in TD. In addition, amprolium has a much lower acquisition cost, potentially favoring its adoption in future research.

CONFLICT OF INTEREST

None.

AUTHOR CONTRIBUTIONS

All listed authors meet the requirements for authorship. JOM, LMP, and FMC conceived and designed the experiments; JOM, LMP, SDCR, RCNM, and FMC performed the experiments. JOM, CASC,

and FMC performed the experiments and wrote the main manuscript text. All authors have read and approved the manuscript.

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