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Amprolium-induced thiamine deficiency in female mice: Role of oxidative stress and inflammation

Deficiência de tiamina induzida por amprólio em camundongos fêmeas: Papel do estresse oxidativo e da inflamação

Deficiencia de tiamina inducida por amprolio en ratones hembra: Papel del estrés oxidativo y la inflamación

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Abstract
Experimental models of thiamine deficiency (TD) have primarily focused on male rodents, meaning that the effects of TD in females and the pathogenesis associated with neurological disorders remain unknown. This article aimed to present an investigation on the effects of TD with amprolium in female mice, evaluating metabolic and behavioral effects, as well as the modulation of ERK1/2 phosphorylation in the cerebral cortex and thalamus. Furthermore, we used the antioxidant Trolox and anti-inflammatory agent dimethyl sulfoxide to investigate the role of oxidative stress and neuroinflammation in this process. The animals were exposed to a thiamine-deficient diet with the additional administration of amprolium (60 mg/kg) for 20 days. After treatment, we observed a reduction in food consumption and animal body weight, with a decrease in motor coordination and exploratory activity, and, in parallel, an increase in the phosphorylation of ERK1/2, both in the cerebral cortex and thalamus in deficient animals. Deficient animals that received Trolox or dimethyl sulfoxide presented with attenuation of these effects, with the maintenance of motor coordination and total blockage of ERK1/2 activation. The results showed that female mice could be used as a valid TD model, compatible with other methods, showing important neurological changes. This study showed that in females, TD also involves mechanisms of oxidative stress and inflammation, responds positively, and can be used as a model animal.
Keywords: Cell signaling; MAPK; Behavior; Sex differences; Trolox; Dimethyl sulfoxide.

Resumo
Modelos experimentais de deficiência de tiamina (DT) têm se desenvolvido principalmente em roedores machos, fazendo com que os efeitos da DT em fêmeas e a patogênese associada a distúrbios neurológicos permanecem desconhecidos. O objetivo deste artigo foi apresentar uma investigação sobre os efeitos da DT com amprólio em camundongos fêmeas, avaliando efeitos metabólicos e comportamentais, bem como a modulação da fosforilação de ERK1/2 no córtex cerebral e no tálamo. Além disso, utilizamos o antioxidante Trolox e o anti-inflamatório

dimetilsulfóxido para investigar o papel do estresse oxidativo e da neuroinflamação nesse processo. Os animais foram expostos a uma dieta deficiente em tiamina com a administração adicional de amprólio (60 mg/kg) por 20 dias. Após o tratamento, observamos redução no consumo alimentar e no peso corporal dos animais, com diminuição da coordenação motora e da atividade exploratória e, paralelamente, aumento da fosforilação de ERK1/2, tanto no córtex cerebral quanto no tálamo dos animais deficientes. Animais deficientes que receberam Trolox ou dimetilsulfóxido apresentaram atenuação desses efeitos, com manutenção da coordenação motora e bloqueio total da ativação de ERK1/2. Os resultados mostram que camundongos fêmeas podem ser usados como um modelo válido de DT, compatível com outros métodos, apresentando alterações neurológicas importantes. Este estudo evidencia que, em fêmeas, a DT também envolve mecanismos de estresse oxidativo e inflamação, respondem positivamente e podem ser usadas como modelo animal.
Palavras-chave: Sinalização celular; MAPK; Comportamento; Diferenças sexuais; Trolox; Dimetilsulfóxido.

Resumen
Los modelos experimentales de deficiencia de tiamina (DT) se han centrado principalmente en roedores machos, lo que significa que los efectos de la DT en hembras y la patogénesis asociada con trastornos neurológicos siguen siendo desconocidos. El objetivo de este artículo fue presentar una investigación sobre los efectos de la DT con amprolio en ratones hembra, evaluando los efectos metabólicos y conductuales, así como la modulación de la fosforilación de ERK1/2 en la corteza cerebral y el tálamo. Además, utilizamos el antioxidante Trolox y el antiinflamatorio dimetilsulfóxido para investigar el papel del estrés oxidativo y la neuroinflamación en este proceso. Los animales fueron expuestos a una dieta deficiente en tiamina con la administración adicional de amprolio (60 mg/kg) durante 20 días. Tras el tratamiento, observamos una reducción del consumo de alimento y del peso corporal de los animales, con una disminución de la coordinación motora y la actividad exploratoria, y, paralelamente, un aumento de la fosforilación de ERK1/2, tanto en la corteza cerebral como en el tálamo en los animales deficientes. Los animales deficientes que recibieron Trolox o dimetilsulfóxido presentaron una atenuación de estos efectos, con el mantenimiento de la coordinación motora y un bloqueo total de la activación de ERK1/2. Los resultados mostraron que las hembras de ratón podrían utilizarse como un modelo válido de DT, compatible con otros métodos, mostrando importantes cambios neurológicos. Este estudio demostró que, en las hembras, la DT también implica mecanismos de estrés oxidativo e inflamación, responde positivamente y puede utilizarse como modelo animal.
Palabras clave: Señalización celular; MAPK; Comportamiento; Diferencias sexuales; Trolox; Dimetilsulfóxido.

1. Introduction

To establish an effective treatment for a disease, it is important to understand its pathogenesis. Neurological diseases are commonly associated with metabolic disorders involving energy deficits and oxidative changes (Di Domenico et al., 2024; Wal et al., 2024). Indeed, thiamine deficiency (TD) has been implicated in several neurological disorders (Abdou & Hazell, 2015; Chandrakumar et al., 2019), showing associations with cellular and systemic changes including impaired neurotransmitter metabolism and glucose utilization, induction of lactic acidosis, oxidative stress, inflammation, and cell death (Abdou & Hazell, 2015; Hazell, 2009; Hazell & Butterworth, 2009). Thiamine acts as a cofactor in glycolysis, and its deficiency is related to impaired oxidative metabolism, which is associated with low adenosine triphosphate (ATP) levels in tissues with high-energy metabolism, such as the nervous tissues, leading to excitotoxicity and neurodegeneration (Hazell et al., 2013; Hazell & Butterworth, 2009; Vetreno et al., 2012).

In addition to metabolic disorders, inflammation is also relevant in many important neurological diseases, such as multiple sclerosis and Alzheimer's, in addition to TD itself (Bettendorff, 2023; Y. Liu et al., 2024; Tyczyńska et al., 2024). In TD, the inflammatory process includes edema, the formation of phagocytic vacuoles, changes in glial cell morphology, and increased microglial reactivity, resulting in the upregulation of inflammatory processes, their transcription genes, and pro-inflammatory molecules (Cassiano et al., 2022; Hazell & Butterworth, 2009; Vemuganti et al., 2006).

Oxidative stress is of significant importance in neurodegeneration and the induction of cell death due to the imbalance between the generation and neutralization of reactive oxygen species (ROS) (Gonsette, 2008; D. Liu et al., 2017; J.-Y. Wang et al., 2006). Oxidative stress and inflammation commonly occur in conjunction with neurological diseases (Calingasan & Gibson, 2000; Hazell & Butterworth, 2009; Vemuganti et al., 2006). Interestingly, studies have demonstrated that TD causes neurodegeneration through both processes, possibly synergistically determining changes in the modulation of cellular signaling

pathways, such as the mitogen-activated protein kinase (MAPK) pathway, resulting in neuronal death and neurological disorders (da Silva et al., 2024; Gomes et al., 2021; Medeiros et al., 2020).

In neurological disorders that trigger inflammation and oxidative stress in neurodegenerative processes, it is crucial to use neuroprotective antioxidants (such as Trolox) and anti-inflammatory substances (such as dimethyl sulfoxide; DMSO) to understand the pathogenesis of neurodegeneration, in addition to evaluating potential neuroprotective therapeutic substances (Cordova et al., 2012, 2013; Gomes et al., 2021; Medeiros et al., 2020; Moraes et al., 2018). Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) is an exogenous antioxidant analogous to vitamin E, which is soluble in water and is well known for its role in the pathogenesis of oxidative disorders (Barclay et al., 1995; Cordova et al., 2013; Diaz et al., 2007; Raspor et al., 2005; Satoh et al., 1997; Seker et al., 2020; Wongmekiat et al., 2007; Wu et al., 1990, 1991). Dimethyl sulfoxide (DMSO) is an organic compound with several pharmacological properties, including potent anti-inflammatory activity (Colucci et al., 2008; de Abreu Costa et al., 2017; Santos et al., 2003; Shimizu et al., 1997). DMSO is considered safe, with no toxicity at therapeutic doses, and is excreted via the respiratory and urinary tracts (Blythe et al., 1986; Brayton, 1986; Hucker et al., 1967; Jacob & de la Torre, 2009). The use of these compounds in the investigation of TD-induced neurodegeneration has revealed important aspects of its pathogenesis (Gomes et al., 2021; Medeiros et al., 2020; Moraes et al., 2018).

Experimental models of TD for *in vivo* studies in laboratory animals allow for an in-depth investigation of the anatomical, physiological, and cellular bases of the neurological disorders associated with this deficiency (Nardone et al., 2013). The most widely used TD model combines feeding mice with thiamine-free feed concurrent with the intraperitoneal injections (i.p.) administration of pyrithiamine (a thiamine pyrophosphokinase inhibitor) (Nardone et al., 2013; Vetreno et al., 2012). Thus, in pyrithiamine-induced TD, there is a stereotypical progression of neurological and behavioral signs that are mapped to changes in neuroanatomy and neurochemistry, mimicking the natural disease associated with TD (Hazell, 2009). However, some studies have used other thiamine analog antagonists, such as amprolium (da Silva et al., 2024; Greenwood & Pratt, 1985; Moraes et al., 2018; L. M. Pereira et al., 2017; Rindi et al., 2003). It has been suggested that the in vivo action of amprolium in inducing TD may be superior to that of the pyrithiamine model (Bunik et al., 2013), because of its ability to block the cellular uptake of vitamins, prevent intracellular diphosphorylation, and inhibit thiamine diphosphorylation. In this sense, amprolium could be considered as an analog that affects thiamine-dependent processes, rather than the thiamine diphosphate-dependent processes (Bunik et al., 2013). Recent studies have demonstrated significant behavioral and metabolic disturbances in thiamine-deficient mice treated with amprolium (da Silva et al., 2024; Moraes et al., 2018; L. M. Pereira et al., 2017).

Despite the model being extensively used for decades, TD studies with experimental rodent models have predominantly been conducted in male animals, and the manifestations of the disorder in female rodents are essentially unknown. More recently, there has been a significant trend of including both sexes in animal studies (Hughes, 2019) with research showing sex-based biological differences in several respects that may restrict the use of rodents of both sexes in research. The different behavioral manifestations of interference associated with sex are also well known (Altemus, 2006; W. Wang et al., 2018). Hormonal variations associated with the female reproductive phases can interfere with several behavioral aspects (Altemus et al., 2014). Similarly, differences in antioxidant resistance and inflammation between males and females have been well characterized (Netto et al., 2017; Rietjens et al., 2018). Indeed, several studies have demonstrated greater resistance to oxidative stress and neuroinflammation in females (Cole et al., 2016; Das et al., 2017; Fogle et al., 2011). However, depending on the study and experimental model, the use of both sexes may be valid, thereby optimizing the use of laboratory animals in scientific research (da Silva et al., 2024).

In the present study, we aimed to present an investigation on the effects of TD with amprolium in female mice, evaluating metabolic and behavioral effects, as well as the modulation of ERK1/2 phosphorylation in the cerebral cortex and thalamus. We tried to deepen our knowledge of the use of female rodents in experimental models of TD, investigate the

involvement of oxidative stress and inflammation in the neurological disorders of female mice deficient in thiamine with amprolium, and to evaluate whether there is any interference in the response to the experimental model previously used in males (da Silva et al., 2024; Moraes et al., 2018). To this end, we evaluated the modulation of extracellular signal-regulated protein kinases 1 and 2 (ERK1/2) phosphorylation in the central nervous tissue, as it is an important signaling pathway for various cellular functions which is sensitive to various stress factors, including TD (da Silva et al., 2024; Gomes et al., 2021), in addition to the use of behavioral tests, which are recognized as important in the evaluation of neurological disorders (Gomes et al., 2021; Medeiros et al., 2020; Moraes et al., 2018; Patti et al., 2005; Prut & Belzung, 2003).

2. Methodology

An experimental, laboratory, quantitative research was carried out (A. S. Pereira et al., 2018) using simple descriptive statistics using mean values, standard deviation (Shitsuka et al., 2014) and statistical analysis (Vieira, 2021) studying a total of 36 female mice.

2.1 Animals

A total of 36 female 50-day-old Swiss mice (*Mus musculus*) were provided by the Vivarium of the Universidade Federal do Norte do Tocantins (Araguaína, TO, Brazil). At weaning, the animals were randomly culled and housed by sex in open-top cages (3-4 per cage), maintained in an air-conditioned cabinet (23 ± 1 °C; humidity, 55 ± 5%;) on a 12 h light/dark cycle with tap water and food (AIN-93; PRAG Soluções Biociências, Jaú, SP, Brazil) provided *ad libitum*. All mice were maintained in the Institutional Experimental Pathology Laboratory under environmentally controlled conditions in accordance with the Universidade Federal do Tocantins Ethics Committee on Animal Use (CEUA-UFT), following the regulations of the National Council for Animal Experimentation Control (CONCEA) of the Ministry of Science, Technology, and Innovation, which follow international guidelines, including EU Directive 2010/63. All treatments and euthanasia by decapitation were performed in accordance with the Ethics Code of Animal Use in Research, and were approved by the CEUA-UFT (process 23.101.001.708/2019-63). All efforts were made to minimize animal suffering and to reduce the number of animals used. Euthanasia by decapitation is a necessary method to study intracellular signaling pathways, as commonly used anesthetics can interfere with response modulation (Salort et al., 2019). The sample size was selected based on pilot studies and previous experience (da Silva et al., 2024; Moraes et al., 2018). Nest building materials were provided, and all cages contained enrichment objects, such as tunnels. The animals were handled with sterile gloves, and the material for nests and bedding (autoclaved shavings) was monitored daily and changed every three days. The animals were also monitored daily for welfare control, to observe any possible important changes in food and water consumption as well as signs of pain, suffering, and distress.

2.2 Reagents

The primary antibodies used were: anti-phospho-ERK1/2 (Cat# M8159, RRID: AB_477245), anti-ERK1/2 (Cat# M5670, RRID: AB_477216), and anti-β-actin (Cat# A5441, RRID: AB_476744), and the secondary antibodies were: rabbit anti-mouse IgG-horseradish peroxidase (HRP)-conjugated (Cat# A9044, RRID: AB_258431) and goat anti-rabbit IgG-horseradish peroxidase (HRP)-conjugated (Cat# A9169, RRID: AB_258434) (Sigma-Aldrich, MO, USA). Acrylamide, Folin and Ciocalteu's phenol reagent, 3,3'- diaminobenzidine tetrahydrochloride (DAB), and ammonium persulfate were purchased from Sigma-Aldrich. Amprolium and 6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) were purchased from Santa Cruz Biotechnology (Dallas, TX, USA). Sodium dodecyl sulfate (SDS), N,N,N,N-tetramethylethylenediamine (TEMED), Tween® 20, Tris-HCl, bovine serum albumin, and dimethyl sulfoxide (DMSO) were purchased from Amresco (Solon, OH, USA). AIN-

93M (standard) and AIN-93TD (thiamine-deficient) chow were purchased from PRAG Soluções Biociências (Jaú, SP, Brazil). Glycine was obtained from Uniscience (Osasco, SP, Brazil) and all other reagents were of the highest analytical grade.

2.3 Treatments

A dietary TD model with amprolium injections was used as the model in this study (da Silva et al., 2024; Moraes et al., 2018). The mice were divided into six groups (n = 6 per group), and subjected to different treatments (Table 1). The animals were randomly assigned to each treatment group, and treatments were performed at the same time each day (2:00 p.m.). The three control groups received saline solution (Cont group, 0.9% NaCl), Trolox antioxidant (Tr Group, 1 mg/kg), or anti-inflammatory DMSO (Dmso group, 1 ml/kg), and three deficient groups received amprolium alone (Amp group, 60 mg/kg) or amprolium associated with the neuroprotectors Trolox (Amp+Tr group) or DMSO (Amp+Dmso group). All substances were administered intraperitoneally (i.p.) for 20 consecutive days. The injected volumes were adjusted to 0.1 ml/10 g of body weight (Cordova et al., 2012). The control groups (Cont, Tr, and Dmso) received the standard AIN-93M diet (PRAG Soluções Biociências, SP, Brazil) throughout the treatment, in accordance with the American Institute of Nutrition (Reeves et al., 1993). The deficient groups (Amp, Amp+Tr, and Amp+Dmso) received thiamine deficient diet AIN-93TD (PRAG Soluções Biociências, SP, Brazil) throughout the treatment period. The constituents of the thiamine-deficient diet were identical to those of the standard diet, except for the lack of vitamin. The animals received water and food *ad libitum*, and their body weight and food consumption were monitored daily.

Table 1 - Experimental design and distribution of the treatment groups among female 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. Source: Research data (2025).

2.4 Behavioral analysis

For behavioral tests, the animals were tested on a rotarod and in an open-field arena. Before testing, animals were habituated for 1 h in a sound- and light-attenuated room (30 lx). The tests were performed during the light phase of the circadian cycle (10 AM to 5 PM).

The rotarod test (Insight Equipamentos Científicos, SP, Brazil) was performed to assess motor coordination. The animals were subjected to conditioning (training) and testing (Aguiar Jr. et al., 2009; Medeiros et al., 2020; Moraes et al., 2018; L. M. Pereira et al., 2017). Conditioning was performed on a stationary cylinder for 30 s, followed by a 90-s period with the cylinder rotating at a speed of 5 rpm. Animals that failed the first stage were subsequently subjected to no more than two additional conditioning sessions. Failure in the third session was set as the exclusion criterion for the test sessions. All animals were properly conditioned. Next, the animals were tested and the latency to fall was recorded to determine the degree of motor coordination (30 min after the conditioning session). The test comprised two sessions in the rotarod with a maximum duration

of 5 min with a 30 min interval between the sessions, starting at a speed of 5 rpm with an increase of 0.1 rpm/s, and performed 24 h after the last day of treatment. The results are expressed as standard deviation (SD) in seconds.

The open-field test was used to study motor and exploratory behaviors (Patti et al., 2005). The tests were performed in a 300-mm diameter circular arena (Bonther, SP, Brazil), with an acrylic base divided into 12 quadrants and a transparent acrylic cylindrical wall. In the 10-min tests, we evaluated the distance traveled (quadrants outdone with the four limbs), rearing (number of times the animal stood on hind legs), and grooming (number of times the mouth or paws were on the body and the head). The tests were performed in two stages: the first (day zero, basal behavior) on the first day of treatment (before drug application), and the second 24 h after the last day of treatment. The results were expressed as a percentage of the measurements on the last day relative to day zero (100%) ± SD (Cordova et al., 2012; Medeiros et al., 2020; Moraes et al., 2018; L. M. Pereira et al., 2017).

2.5 Western blotting

The frontal cerebral cortex and thalamus were dissected, mechanically homogenized in 300 µl of sample buffer (200 mM Tris, 40 mM EDTA, 4% SDS, pH 6.8), and immediately boiled for 5 min. A sample dilution solution (1:4 v/v; 40% glycerol, 50 mM Tris, and minimal bromophenol blue) and β-mercaptoethanol (at a final concentration of 5%) were added to each sample. Protein content was estimated by assessing absorbance at 750 nm, and the concentration was calculated using a bovine serum albumin (BSA) standard curve (Peterson, 1977). The samples (60 µg of total protein) were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (VERT-i10® electrophoresis system, Loccus, SP, Brazil) using 10% gels (Cordova et al., 2004, 2012). Proteins were transferred to nitrocellulose membranes (1.2 mA/cm^2; 1.5 h) using a wet-blotting apparatus (WEST-i10®, Loccus) (Gomes et al., 2021). The membranes were then blocked for 1 h with 2% bovine serum albumin in Tris-buffered saline (TBS; 10 mM Tris, 150 mM NaCl, pH 7.5). ERK1/2 total and phosphorylated forms, as well as β-actin, were detected by overnight incubation with specific antibodies diluted 1:10,000 (P-ERK1/2 and β-actin) or 1:40,000 (T-ERK1/2) in TBS-T (10 mM Tris, 150 mM NaCl, 0.1% Tween® 20, pH 7.5) containing 2.5% BSA. Thereafter, membranes were incubated with anti-rabbit or anti-mouse peroxidase-linked secondary antibodies (1:5,000) for 1 h, and the reactions were developed using DAB chromogen (Gomes et al., 2021). All blocking and incubation steps were followed by three washes (5 min each) with TBS-T. The optical density (OD) of the bands was quantified using ImageJ version 1.52k software (RRID: SCR_003070; National Institutes of Health, Bethesda, MD, USA). The phosphorylation level of ERK1/2 was determined as the ratio of the OD of the phosphorylated band to that of the total band, and the data were expressed as a percentage of the control (100%) (Cordova et al., 2012; Gomes et al., 2021; Medeiros et al., 2020). The values are presented as the mean ± SD. β-actin was used as the control for protein loading.

2.6 Statistical analysis

Data are expressed as the mean ± SD, while statistical significance was determined by analysis of variance, followed by Duncan's posthoc test, when appropriate (Cordova et al., 2012; da Silva et al., 2024; Medeiros et al., 2020). Statistical significance was set at $P \leq 0.05$. The data were processed using STATISTICA '98 Edition software (RRID: SCR_014213, StatSoft, Tulsa, OK, USA).

3. Results

3.1 Weight gain and feed consumption

A reduction in body weight was observed in thiamine-deficient female mice treated with amprolium (Amp group)

compared to the control group (Cont group; P = 0.002) after 20 days of treatment (Table 2). The same profile was observed in deficient females treated with Trolox (Amp+Tr group; P = 0.0001 compared to the control) and DMSO (Amp+Dmso group; P = 0.0004 compared to the control). In the control groups treated with neuroprotective substances, the use of Trolox and DMSO did not change the body weight of the animals (Tr group, P = 0.570; Dmso group, P = 0.156, relative to the Cont group).

Table 2 - Body weight gain of female mice with amprolium-induced thiamine deficiency, treated or untreated with Trolox or DMSO.

	Weight (g) Day zero	Weight (g) Day 20	Weight gain (g)
Cont	35.68 ± 1.68	41.17 ± 2.40	5.48 ± 1.22
Amp	33.77 ± 0.29	30.60 ± 0.35	-3.17 ± 0.17 [a]
Amp+Tr	35.30 ± 5.10	27.65 ± 3.75	-7.65 ± 1.35 [a]
Amp+Dmso	37.80 ± 3.90	32.20 ± 2.54	-5.60 ± 2.00 [a]
Tr	39.30 ± 4.30	46.05 ± 4.75	6.75 ± 0.45
Dmso	31.75 ± 3.15	33.95 ± 3.25	2.20 ± 0.10

Female mice in different treatment groups were provided with AIN-93M chow and saline (control, 0.9% NaCl), AIN-93TD chow and amprolium (Amp), AIN-93TD chow and amprolium with Trolox (Amp+Tr), AIN-93TD chow and amprolium with DMSO (Amp+Dmso), AIN-93M chow and Trolox (Tr), and AIN-93M chow and DMSO (Dmso). The results represent the mean ± SD of weight gain (g) during the experimental period, derived from six independent replicates. The results were analyzed using ANOVA followed by Duncan's test. *a* Denotes P ≤ 0.05, compared with the control. Source: Research data (2025).

Regarding feed consumption (Table 3), amprolium-deficient females showed a marked reduction in consumption compared to the control group (P = 0.004). Similar results were observed in deficient animals treated with Trolox (Amp+Tr group, P = 0.007 relative to the control group) or DMSO (Amp+DMSO group, P = 0.002 relative to the control group). The use of Trolox and DMSO (groups Tr and Dmso) did not alter the feed consumption of these animals (Tr group, P = 0.628; Dmso group, P = 0.116, relative to the Cont group).

Table 3 - Feed intake of female mice with amprolium-induced thiamine deficiency, treated or not with Trolox or DMSO.

	Consumption (g) Day zero	Consumption (g) Day 20	Variation (g)
Cont	5.00 ± 0.55	3.80 ± 0.55	-1.20 ± 0.34
Amp	4.55 ± 0.34	2.02 ± 0.46	-2.53 ± 0.21 [a]
Amp+Tr	4.55 ± 0.36	2.18 ± 0.44	-2.37 ± 0.13 [a]
Amp+Dmso	4.99 ± 0.20	2.27 ± 0.10	-2.72 ± 0.16 [a]
Tr	5.03 ± 0.32	4.01 ± 0.51	-1.02 ± 0.19
Dmso	4.78 ± 0.30	4.24 ± 0.24	-0.55 ± 0.42

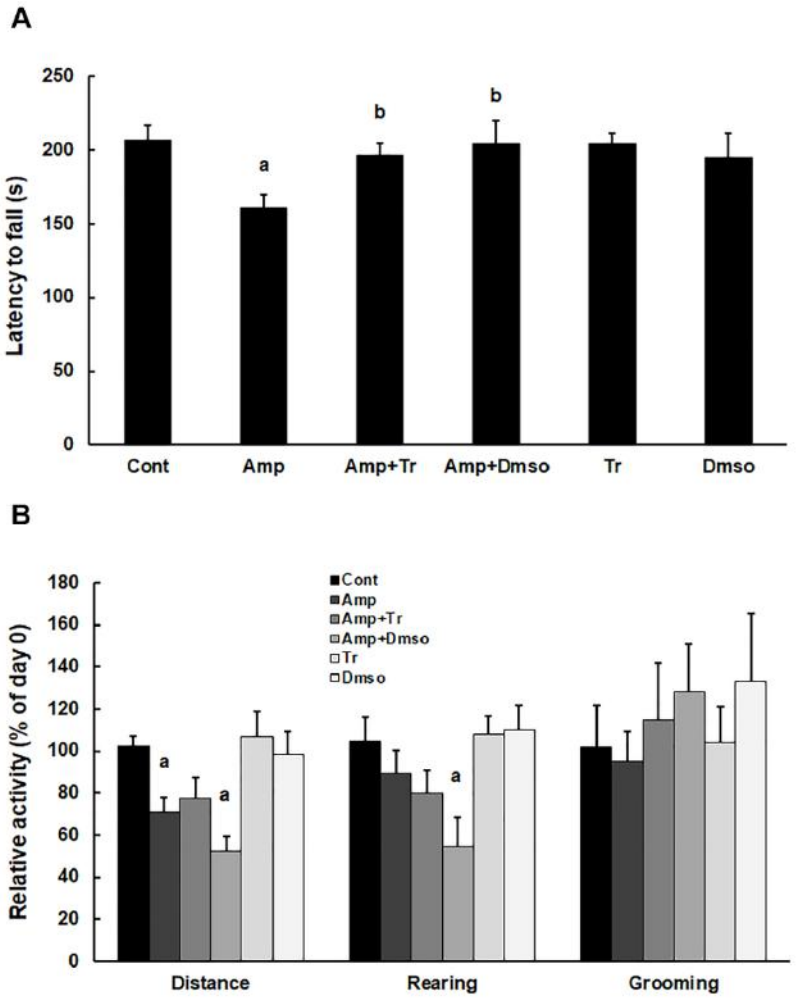
The female mice in different treatment groups were treated with AIN-93M chow and saline (control, 0.9% NaCl), AIN-93TD chow and amprolium (Amp), AIN-93TD chow and amprolium with Trolox (Amp+Tr), AIN-93TD chow and amprolium with DMSO (Amp+Dmso), AIN-93M chow and Trolox (Tr), and AIN-93M chow and DMSO (Dmso). The results represent the mean ± SD of feed consumption (g) during the experimental period, derived from six independent replicates. The results were analyzed using ANOVA followed by Duncan's test. *a* Denotes P ≤ 0.05, compared with the control. Source: Research data (2025).

3.2 Behavioral analysis

In the rotarod test (Figure 1A), thiamine-deficient females (Amp group) showed a shorter latency time to fall (161.08 s ± 8.9, P = 0.043) compared to the control group (Cont = 206.60 s ± 10.10) after 20 days of treatment. However, the concomitant use of Trolox and DMSO reversed the effects of TD in the animals (Amp+Tr group, 197.00 s ± 8.17, P = 0.032; Amp+Dmso group, 204.83 s ± 14.98, P = 0.041, in relation to the amp). In addition, Trolox and DMSO per se did not influence motor coordination.

In the open field test (Figure 1B), Amp-deficient females (Amp group) showed a 28.91% reduction in the distance covered compared with the control group (P = 0.025). The association with Trolox or DMSO did not reverse this effect (Amp+Tr group, P = 0.599; Amp+Dmso group, P = 0.136, compared to the Amp group). Interestingly, in relation to exploratory activity, we observed a reduction in activity only in the DMSO-treated deficient group (Amp+Dmso group, P = 0.012, compared to the Cont group). The Trolox and DMSO controls (Tr and Dmso groups) showed no changes in locomotor and exploratory activities. Additionally, no changes in grooming parameters were observed in any group.

Figure 1 - Results of the behavioral evaluation of female thiamine deficiency model mice induced with amprolium.

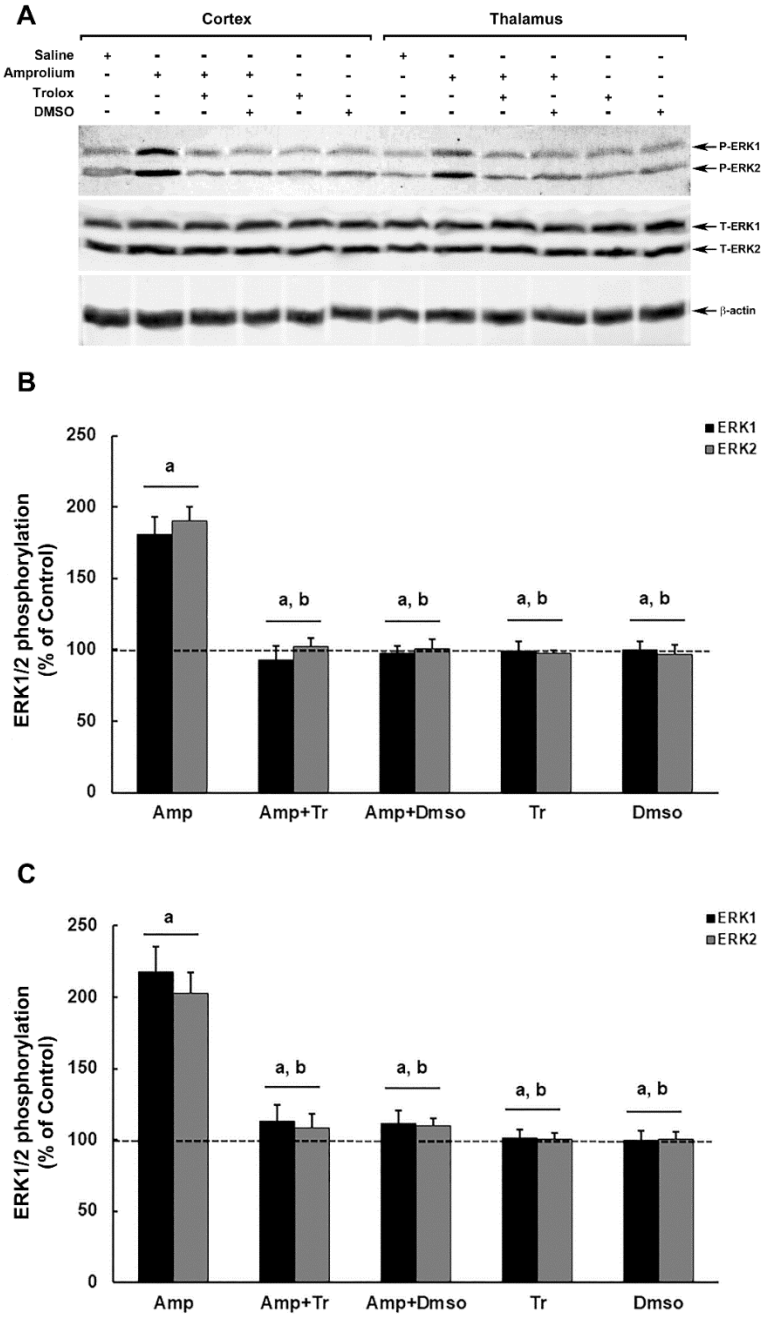


The animals performed the rotarod (A) and open field (B) tests after treatment for 20 d with AIN-93M chow and saline (control, 0.9% NaCl), AIN-93TD chow and amprolium (Amp), AIN-93TD chow and amprolium with Trolox (Amp+Tr), AIN-93TD chow and amprolium with DMSO (Amp+Dmso), AIN-93M chow and Trolox (Tr), and AIN-93M chow and DMSO (Dmso). Values represent the means ± SD of the evaluations performed on day 20 as a percentage of those performed on day zero (basal value, 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. *a* denotes P ≤ 0.05 compared with the control, *b* P ≤ 0.05 compared with the Amp group. Source: Research data (2025).

3.3 Evaluation of ERK1/2 modulation

When evaluating the role of oxidative stress and inflammation in the modulation of cell signaling pathways in the central nervous system (CNS) of thiamine-deficient female mice, we observed an interesting effect on the ERK1/2 pathway (Figure 2). In both the cerebral cortex (Figure 2B) and thalamus (Figure 2C), deficient animals (Amp group) exhibited an increase in ERK1/2 phosphorylation of approximately 100% ($P < 0.0001$ compared to controls). However, in both deficient groups treated with Trolox (Amp+Tr group) or DMSO (Amp+Dmso group), we observed complete suppression of the increase in ERK1/2 phosphorylation ($P < 0.0001$, in relation to the Amp group), both in the cerebral cortex and thalamus, highlighting the strong participation of oxidative stress and inflammation in the modulation of this pathway in deficient females. Treatment with Trolox (Tr group) or DMSO (Dmso group) did not alter ERK1/2 phosphorylation.

Figure 2 - Modulation of ERK1/2 phosphorylation in the brain of female mice in the thiamine deficiency model induced with amprolium.



The panel shows the representative immunoblotting (A) and quantification of ERK1/2 phosphorylation in the cerebral cortex (B) and thalamus (C) from female mice treated for 20 d with AIN-93M chow and saline (control, 0.9% NaCl), AIN-93TD chow and amprolium (Amp), AIN-93TD chow and amprolium with Trolox (Amp+Tr), AIN-93TD chow and amprolium with DMSO (Amp+Dmso), AIN-93M chow and Trolox (Tr), and AIN-93M chow and DMSO (Dmso). Total and phosphorylated forms of ERK1/2 and β-actin were detected using specific antibodies, and the reaction was developed using DAB chromogen. The level of phosphorylation was determined as a ratio of the OD of the phosphorylated band over the OD of the total band. The data are expressed as the percentage of the control (considered 100%). Values are presented as the mean ± SD derived from six independent replicates. The results were analyzed using ANOVA followed by Duncan's test. *a* denotes $P \leq 0.05$ compared with the control, *b* $P \leq 0.05$ compared with the Amp group. Source: Research data (2025).

4. Discussion

The biological effects of TD (Manzetti et al., 2014) have been widely investigated rodent models (Nardone et al., 2013; Vetreno et al., 2012). Although pyrithiamine is the most commonly used thiamine analog in animal models, other antagonists, such as oxythiamine, amprolium, and 3-deazathiamine (Bunik et al., 2013; Dudeja et al., 2001; Greenwood & Pratt, 1985; Rindi et al., 2003; Tylicki et al., 2018), have also been used in in vitro models (Chornyy et al., 2007; X. Wang et al., 2007). In *in vivo* models, the association of an inadequate diet with antagonists is interesting, as it accelerates the progression and appearance of neurological signs in animals, which can be observed from the 10th day of thiamine deficiency induction (Vetreno et al., 2012). However, in this study, we chose to intraperitoneally administer amprolium, an analog of thiamine that is more readily available. Unlike pyrithiamine, amprolium acts primarily extracellularly, competitively inhibiting the transport of thiamine into the cell, thus generating cellular deficits even when nutrients are still present in the body (Tylicki et al., 2018). Amprolium has been widely used in TD models in ruminants (Nogueira et al., 2010; Sant'Ana et al., 2009; Sedaghat & Javanbakht, 2014; Tanwar et al., 1994); however, recent studies on TD in laboratory rodents have shown that the use of amprolium is viable, showing manifestations of behavioral and metabolic changes characteristic of TD (Moraes et al., 2018; L. M. Pereira et al., 2017) within a reduced exposure time (da Silva et al., 2024; Moraes et al., 2018).

Mice are commonly used in *in vivo* experiments. Experiments investigating the neurological effects of TD in predominantly male animals are no exception. To optimize the use of laboratory animals, it is necessary to analyze the effects of TD induction on the nervous tissue of both sexes, and to investigate whether differences occur, whether this makes the use of females infeasible, or whether the use of males is simply a paradigm. The aspects considered most critical for the use of females are related to behavior (Altemus, 2006; W. Wang et al., 2018) and oxidative stress (Netto et al., 2017; Rietjens et al., 2018), which are possibly related to hormonal variations during the reproductive cycle (Altemus et al., 2014), in addition to greater antioxidant resistance when compared to males (Cole et al., 2016; Das et al., 2017; Fogle et al., 2011).

In this study, the first manifestation observed in the deficient females was a reduction in body weight and feed consumption. These manifestations are very important because, first, they occurred identically to the effect previously observed in male mice (Moraes et al., 2018) and, second, the effects of inducing anorexia by TD and increasing resting energy expenditure are known, resulting in a generalized loss of body weight (Bâ, 2012; M. Liu et al., 2014), demonstrating that females manifest the effect of TD in the same manner as males. Hyporexia was reversed within 3 days of restoring dietary thiamine levels (Bâ, 2012; Ke et al., 2003), indicating that thiamine plays a crucial physiological role in programming body weight homeostasis, increment, and set-point regulation (Bâ, 2012). However, as observed in this study, the use of neuroprotective substances (Trolox and DMSO) did not reverse weight loss and reduction in food consumption observed in female mice. This effect was identical to that observed in male animals (Moraes et al., 2018), indicating that thiamine plays an important role in regulating feed consumption and body weight gain but is not entirely dependent on oxidative stress and inflammation. The protective effects of Trolox on weight gain in animals have also been demonstrated (Cordova et al., 2012); however, other mechanisms are likely involved.

In contrast, the effects of Trolox and DMSO were observed in the motor coordination test (rotarod), with maintenance of the latency time for falling in deficient females that received the substances, highlighting the influence of inflammation and oxidative stress on the motor activity of the animal. However, in the open-field test, no reversal of the effects of TD was observed when Trolox and DMSO were used. Deficient female mice showed reduced locomotor and exploratory activities, with a slight improvement in the distance covered by Trolox during the test. Interestingly, a similar pattern was observed in TD induction experiments in male mice (Moraes et al., 2018). Animals deficient in thiamine present oxidative stress and inflammation in the CNS, which can interfere with the metabolism of neurotransmitters (Abdou & Hazell, 2015), resulting in behavioral changes (Carvalho et al., 2006; Ferreira-Vieira et al., 2016). However, our findings suggest that oxidative stress and inflammation are not the only mechanisms involved in behavioral disorders.

Additionally, despite the known neurobiological differences between males and females, these factors did not prevent the detection of neural changes in females in our amprolium TD model. Notably, the animals showed evident changes in the modulation of ERK1/2 phosphorylation, with a complete reversal of the effect observed when deficient females received Trolox or DMSO in parallel, both in the thalamus and cerebral cortex. This finding is important as oxidative stress and inflammation are considered the primary mechanisms associated with the neurodegenerative processes in TD (Vetreno et al., 2012), and are directly related to the activation of MAPKs (Irving et al., 2000; Kaminska, 2005; Kyriakis & Avruch, 2012). Even considering the differences between males and females (Altemus, 2006; Netto et al., 2017; Rietjens et al., 2018; W. Wang et al., 2018) under different biological conditions (Altemus et al., 2014; Cole et al., 2016; Das et al., 2017; Fogle et al., 2011), oxidative stress and inflammatory responses are most likely involved in the activation of ERK1/2, as the effect of TD on ERK was abolished by Trolox and DMSO. This suggests that TD-mediated ERK1/2 activation occurs via mechanisms dependent on ROS and inflammatory mediators. Neuroinflammation is an important component of the pathogenesis of TD. Some regions of the brain are more vulnerable to TD because of the increased transcription of genes related to pro-inflammatory cytokines, in addition to the occurrence of damage to the mitochondrial membrane and its function, resulting in an increase in ROS related to oxidative stress (Hazell et al., 2013). In our study, the possible involvement of inflammation and oxidative stress in female mice was observed, both in the cerebral cortex and thalamus; using Trolox (antioxidant) and DMSO (anti-inflammatory), there was a total blockade of increased phosphorylation of ERK1/2.

5. Conclusion

In this study, we highlighted the relevance of the dietary induction model of thiamine deficiency associated with the administration of amprolium developed by our group, which is efficient at observing important neural changes in mice. Compared with the TD model with pyrithiamine, amprolium-induced deficiency, in addition to being involved in oxidative stress and inflammation, induces the process less aggressively, and has a much lower acquisition cost, favoring its adoption in future research. Furthermore, female mice showed significant metabolic, behavioral, and neural changes when subjected to the model, including reduced food consumption, weight gain, behavioral changes, and modulation of the MAPK ERK1/2 signaling pathway. Associated with this, females demonstrated positive responses to the use of tools such as the antioxidant Trolox and the anti-inflammatory DMSO, similar to what was observed in previous studies with males, indicating that both sexes could be used in experimental models of inducing thiamine deficiency, thus optimizing the use of laboratory animals in scientific research.

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Availability of data and materials

Data can be made available upon reasonable request.

Authors' contributions

All listed authors meet the authorship requirements. MPS, CASC, and FMC conceived and designed the experiments. MPS, FWBL, AGSM, JPN, and FMC performed the experiments. MPS, CASC, and FMC performed the experiments and wrote the manuscript. All the authors have read and approved the final manuscript.

Ethics approval

All procedures performed in studies involving animals were in accordance with the ethical standards of the institution or practice at which the studies were conducted (Universidade Federal do Tocantins Ethics Committee on Animal Use, CEUA-UFT, permit number 23.101.001.708/2019-63).

Competing interests

The authors declare that there are no conflicts of interest.

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