Amprolium-induced thiamine deficiency in mice: evaluation of a practical model by oral administration

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ABSTRACT

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INTRODUCTION

Neurological diseases are quite varied and have a great economic impact on livestock (GETHMANN et al., 2015). Among the metabolic disturbances of the central nervous system (CNS), there is polioencephalomalacia (PEM) triggered by thiamine deficiency (TD) (APLEY, 2015; SANT’ANA et al., 2009b; SEDAGHAT; JAVANBAKHT, 2014). PEM has been described in several regions involving ruminants (APLEY, 2015; SANT’ANA; BARROS, 2010). However, thiamine deficiency is also important for carnivores (Chastek’s palsy) (MOON; KANG; PARK, 2013) and humans (Beriberi, Wernicke’s encephalopathy, and Wernicke-Korsakoff syndrome) (NARDONE et al., 2013; VETRENO et al., 2012).

Thiamine deficiency produces energy metabolism disorders and several neurological dysfunctions (NARDONE et al., 2013). Thiamine is an essential cofactor for several enzymatic pathways (BROWN, 2014), including the citric acid cycle and pentose phosphate pathway, important in energy production, particularly in the CNS (MANZETTI; ZHANG; VAN DER SPOEL, 2014). Despite of its known crucial metabolic functions, the scarce knowledge of cell death mechanisms and lesions that occur due to TD-associated PEM, has stimulated the development of disease experimental models in animals (VETRENO et al., 2012). To this end, TD experimental models have been developed with laboratory rodents (NARDONE et al., 2013) and ruminants (NOGUEIRA et al., 2010; SANT’ANA et al., 2009a; SEDAGHAT; JAVANBAKHT, 2014).

Amprolium-induced PEM models in sheep (SANT’ANA et al., 2009a), bovine (NOGUEIRA et al., 2010), goats (SEDAGHAT; JAVANBAKHT, 2014), and buffaloes...
In the amprolium-induced TD model, 50-day postnatal Swiss male mice were used. The animals used in the study were kept, manipulated, and euthanized according to the ethics code of animal use in research, according to a protocol approved by the Universidade Federal do Tocantins Ethics Committee on Animal Use (CEUA-UFT, process 23101.000284/2014-13).

The mice were allocated into eight groups (n = 8 per group): control (Cont), deficient with amprolium A (TD-A), deficient with amprolium B (TD-B), and deficient with amprolium C (TD-C). All groups received tap water and commercial feed (Presence Ratos e Camundongos, Invivo, SP, Brazil) ad libitum. Groups A, B, and C received amprolium (Amprolbase, Farmabase, SP, Brazil) dissolved in drinking water at concentrations of 10, 20, and 30 mg/mL, resulting, respectively, in estimated doses of 1,250, 2,500 and 3,750 mg/kg, based on an average intake of 5 mL/water/day/animal (ANDRADE; PINTO; OLIVEIRA, 2002). The treatments continued for 20 or 80 days, with amprolium solutions changed every 24 hours. Throughout the treatment periods, weight gain, feed intake, and water consumption were monitored daily.

The animals were submitted to the open field and rotarod behavioural tests. The animals were habituated to the test room for 1 h 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 300-mm diameter circular arena (Bonther, SP, Brazil), with an acrylic base divided into 12 quadrants and a transparent acrylic cylindrical wall. In the tests (duration of 10 min) we evaluated distance travelled (quadrants outdone with the four limbs), faecal production, rearing, and grooming (CORDOVA et al., 2012). The tests were performed in two stages. The first (day zero) was performed on the first day of treatment and the second on the last day (day 20 or 80). The results obtained in the open field test are expressed as a percentage of the measurements of days 20 and 80 in relation to day zero (100%).

In the rotarod test (Insight Equipamentos, SP, Brazil) the animals were submitted to conditioning (training) and testing (CORDOVA et al., 2012). The conditioning was performed on the stationary cylinder for 30 s, followed by a 90-s period in the cylinder at a speed of 5 RPM. The animals that failed in this first stage were subsequently submitted to no more than two additional conditioning sessions. With this procedure, the animals presented similar baseline values in all groups. Thirty minutes after the last conditioning session, the animals were tested and the latency time to fall was recorded to determine the degree of motor coordination. The test consisted of two sessions in the rotarod with a maximum duration of 5 min, 30 min intervals between the sessions, starting at a speed of 5 RPM with an increase of 0.1 RPM/s, performed on the last day of treatment (day 20 or 80).

After the behavioural tests, the mice were euthanized and the viscera (liver, kidney, lung, and heart) and brain were collected for histopathological evaluation. The
samples, obtained during post-mortem examination, were conditioned in flasks containing 10% buffered formalin. After fixation, the samples were sent for histological processing. Briefly, they were dehydrated in increasing series of ethanol, diaphanized in xylol, and embedded in paraffin, followed by microtomy (thickness 5 μm) and staining by haematoxylin and eosin (HE). Histopathological analysis was performed using a Biophotika B20T trinocular optical microscope coupled to an ISH500 CMOS-5.0 digital colour camera (Tucson Photonics, Fujian, P. R. China). The images were projected on the monitor and captured by ISCapture v.3.6.7 software (Tucson Photonics, Fujian, P. R. China) in 40x objective.

Data are expressed as mean ± standard error (SE) and statistical significance was determined by analysis of variance, followed by Duncan’s post-hoc test, when appropriate (CORDOVA et al., 2012). Statistical significance was set at P ≤ 0.05. The data were processed using STATISTICA '98 Edition software (StatSoft, OK, USA).

RESULTS

To verify the amprolium intake in the established doses, the consumption of water per animal were measured daily. In the animals treated for 20 days (Table 1), we observed a significant reduction in water intake in the deficient groups at the end of treatment in relation to day zero (in total values, not considering normal daily losses), compared to the control group (TD-A, -5.03 mL ± 0.23; TD-B, -4.00 mL ± 0.69; Control, -2.03 mL ± 0.58, P = 0.020). On the other hand, in the 80 days treated animals, the TD-B group showed an increase in water intake at the end of the period, compared to the control group (TD-B, +1.23 mL ± 0.43; Control, -0.90 mL ± 0.20, P = 0.002). With the group variations consumptions at the end of the treatments, the water/animal/day intake was lower than expected for the species in some TD groups, considering the expected consumption of 5 mL/animal/day (ANDRADE; PINTO; OLIVEIRA, 2002). Considering the normal water losses through the drinking water system during the day, of approximately 25%, TD animals treated for 20 days had an average intake of 2.70 mL/animal/day. Thus, the animals ingested the calculated doses of amprolium about 565.1 mg/kg in the 20 days TD-A group (45.21% of the estimated dose) and 1,298.5 mg/kg in the 20 days TD-B group (51.94% of the estimated dose). In addition, in the TD-A group treated for 80 days, although there was no difference in the volume of water consumed compared to the control, the mean intake of amprolium was 862.5 mg/kg (69% of the estimated dose), considering the net consumption of the substance by increasing the animals’ body weight. However, the TD-B group treated for 80 days ingested 2,592 mg/kg (103.68% of the estimated dose). On the other hand, the animals of the TD-C group (30 mg/mL) did not ingest the water, developing dehydration in the first two days of the experiments, resulting in the elimination of the animals and discarding of the experimental group. The refusal of water by TD-C animals was probably due to the unpleasant taste of highly concentrated amprolium.

Table 1. Water consumption in the thiamine deficiency mouse model with oral amprolium.

<table>
<thead>
<tr>
<th></th>
<th>20-day treatment</th>
<th>80-day treatment</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Volume day zero (ml)</td>
<td>Volume day 20 (ml)</td>
</tr>
<tr>
<td>Control</td>
<td>8.60 ± 0.87</td>
<td>6.57 ± 1.44</td>
</tr>
<tr>
<td>TD-A</td>
<td>8.35 ± 0.88</td>
<td>3.32 ± 1.11</td>
</tr>
<tr>
<td>TD-B</td>
<td>7.90 ± 1.16</td>
<td>3.90 ± 0.47</td>
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*P ≤ 0.05 compared to control (n = 8). TD = thiamine deficiency.

The TD-A and TD-B mice showed lower body weight gain at the end of the treatments as compared to controls (Table 2), both after 20 (TD-A, 1.55 g ± 0.57; TD-B, 1.22 g ± 0.72, control, 4.33 g ± 0.51, P = 0.021), and 80 days (TD-A, 15.50 g ± 1.40; TD-B, 3.36 g ± 0.18; Control, 21.43 g ± 0.27, P = 0.001). However, only animals treated for 20 days had a reduction in feed intake at the end of the amprolium exposure period (TD-A, -1.63 g ± 0.32; TD-B, -2.01 g ± 0.25; Control, -0.27 g ± 0.08, P = 0.004, Table 3).

Table 2. Body weight gain in the thiamine deficiency mouse model with oral amprolium.

<table>
<thead>
<tr>
<th></th>
<th>20-day treatment</th>
<th>80-day treatment</th>
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<tbody>
<tr>
<td></td>
<td>Weight day zero (g)</td>
<td>Weight day 20 (g)</td>
</tr>
<tr>
<td>Control</td>
<td>42.43 ± 2.00</td>
<td>46.77 ± 1.49</td>
</tr>
<tr>
<td>TD-A</td>
<td>42.52 ± 0.87</td>
<td>44.07 ± 0.92</td>
</tr>
<tr>
<td>TD-B</td>
<td>43.82 ± 0.93</td>
<td>45.05 ± 0.69</td>
</tr>
</tbody>
</table>

*P ≤ 0.05 compared to control (n = 8). TD = thiamine deficiency.
Table 3. Feed consumption in the thiamine deficiency mouse model with oral amprolium.

<table>
<thead>
<tr>
<th></th>
<th>20-day treatment</th>
<th>80-day treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Consumption day zero (g)</td>
<td>Consumption day 20 (g)</td>
</tr>
<tr>
<td>Control</td>
<td>5.86 ± 0.81</td>
<td>5.59 ± 0.89</td>
</tr>
<tr>
<td>TD-A</td>
<td>6.08 ± 1.14</td>
<td>4.44 ± 0.82</td>
</tr>
<tr>
<td>TD-B</td>
<td>6.55 ± 0.94</td>
<td>4.54 ± 1.18</td>
</tr>
</tbody>
</table>

*a P ≤ 0.05 compared to control (n = 8). TD = thiamine deficiency.

In the open field behavioural test, no changes were observed in animals treated for 20 days (Figure 1A). However, the TD-B mice treated for 80 days (Figure 1B) showed a reduction of 39.27% in motor activity (distance) as compared to controls \((P = 0.010)\), a reduction of 20.06% in exploratory activity (rearing) \((P = 0.046)\), and a 90.83% increase in grooming frequency \((P = 0.0001)\). Similarly, animals treated for 20 days did not show changes in the rotarod test (Figure 1C). However, when exposed to amprolium for 80 days (Figure 1C), the animals showed a reduction in the latency to fall (TD-A, 161.67 s ± 9.73; TD-B, 156.50 s ± 2.93; Control, 187.00 s ± 5.48, \(P = 0.037\)).

Figure 1. Behavioural effects in the thiamine deficiency mouse model with oral amprolium.

The animals were tested after 20 and 80 days of treatment with amprolium added in drinking water (10 mg/mL, TD-A and 20 mg/mL, TD-B) in the open field and rotarod tests. (A) Open field of animals treated for 20 days. (B) Open field of animals treated for 80 days. (C) Rotarod of animals treated for 20 and 80 days. *P ≤ 0.05 compared to control \((n = 8)\). Source: author’s collection.

At necropsy, treated animals did not exhibit macroscopic changes. In addition, was not observed histological changes in the mice CNS, both in 20 (Figure 2) and in 80 days of treatment (Figure 3).
Figure 2. Brain histopathology in the thiamine deficiency mouse model with oral amprolium after 20 days of treatment.

The panel shows representative images of histological sections of the cerebellum, parietal cerebral cortex, striatum, hippocampus, and thalamus of mice treated with amprolium added in drinking water (10 mg/mL, TD-A and 20 mg/mL, TD-B). HE, obj. 40x. Scale bar = 20 μm. Source: author’s collection.
Figure 3. Brain histopathology in the thiamine deficiency mouse model with oral amprolium after 80 days of treatment.

The panel shows representative images of histological sections of the cerebellum, parietal cerebral cortex, striatum, hippocampus, and thalamus of mice treated with amprolium added in drinking water (10 mg/mL, TD-A and 20 mg/mL, TD-B). HE, obj. 40x. Scale bar = 20 µm. Source: author’s collection.

In parallel, we reevaluated the pathological aspects of the viscera (lung, heart, kidney, and liver) of treated animals. Histopathology showed that TD-A and TD-B animal groups had moderate proteinuria, both in 20 (Figure 4) and in 80 days of treatment (Figure 5). These results were determined by the finding of eosinophilic proteinaceous material in the renal tubular lumens. In addition, the kidneys of the TD-B group, treated for 80 days, presented moderate cellular swelling in the proximal tubules (Figure 5).
Figure 4. Histopathology of organs in the thiamine deficiency mouse model with oral amprolium after 20 days of treatment.

The panel shows representative images of histological sections of the heart, liver, lung, and kidney of mice treated with amprolium added in drinking water (10 mg/mL, TD-A and 20 mg/mL, TD-B). HE, obj. 40x. Scale bar = 20 μm. Source: author’s collection.
DISCUSSION

The model applied in this work was based on amprolium PO administration dissolved in drinking water. This method allows the spontaneous and natural intake of the substance, mimicking the amprolium therapeutic administration technique in domestic animals for coccidiosis treatment, including its known adverse effect: induction of TD by prolonged treatment. To verify the amprolium intake at the established doses, we measured the daily consumption of water per animal. We observed a significant reduction in water intake in the groups treated for 20 days. Thus, these animals ingested calculated doses of amprolium lower than those estimated (approximate reduction of 50%). This variation of water intake between the TD groups treated for 20 or 80 days suggests gradual adaptation to the substance, with the ingested volume/animal restored at the beginning of the period (first third of the treatment). Our model showed that very high concentrations of amprolium in drinking water (30 mg/mL, TD-C group) prevented spontaneous consumption.

In both treatment periods, TD animals presented lower body weight gain. However, only TD animals treated for 20 days showed a reduction in feed consumption.
Despite the development of anorexia in TD animals, through the modulation of signalling pathways associated with central regulation of food intake (LIU et al., 2014) and by neuroendocrine circuits changes (BA, 2012), we believe that in our study the reduction of ingestion (and, in this case, weight gain) at TD groups treated for 20 days may have been caused by the reduction in water intake, below the volume considered normal for the species (ANDRADE; PINTO; OLIVEIRA, 2002). However, the results pertaining to the animals treated for 80 days positively indicated TD induction in the mice. Our results corroborate data obtained from other species, such as the decrease in weight gain parallel to the maintenance of water and feed intake. Studies have shown that amprolium-induced weight loss precedes clinical signs in sheep (SPICER; HORTON, 1981). In addition, adverse effects (weight loss and cerebrocortical necrosis) have not been demonstrated with the administration of amprolium at therapeutic doses (YOUNG et al., 2011). The loss of body weight has also been observed in other dietary TD rodent models (NAKAGAWASAI et al., 2004) and in humans with Wernicke-Korsakoff syndrome (SAAD et al., 2010). However, it is not entirely clear how TD causes a reduction in body weight gain, but studies have shown that TD rodents develop a reprogramming of homeostasis, increment, and set point of body weight (BA, 2012).

In the open field behavioural evaluation, only the TD-B mice treated for 80 days showed reductions in motor and exploratory activities and increased grooming frequency. Similarly, animals treated for 80 days also demonstrated a reduction in the latency to fall in the rotarod test. These data suggest that mice treated with amprolium PO developed neurological disorders, similar to that observed in other TD models. Studies have reported quite wide alterations in TD rodents, such as cognitive, learning, and memory dysfunctions (NAKAGAWASAI et al., 2004; PIRES et al., 2007; PITKIN; SAVAGE, 2004), and motor activity impairments (FERREIRA-VIEIRA et al., 2016). Additionally, these behavioural dysfunctions were correlated with changes in cholinergic, GABAergic, and glutamatergic neurotransmission (FERREIRA-VIEIRA et al., 2016; FREITAS-SILVA et al., 2010; PITKIN; SAVAGE, 2004). However, in these studies, the models used were based on dietary TD, with or without parallel administration of pyrithiamine. In our model, we maintained the ad libitum diet with standard commercial feed (containing thiamine) in conjunction with amprolium administration in drinking water. Studies have shown that amprolium acts in the body in a potent and identical way to pyrithiamine (DUDEJA et al., 2001; BIZON-ZYGMANSKA et al., 2011; BUNIK; TYLICKI; LUKASHEV, 2013). Thus, the data suggest that our model had a positive effect on TD induction. Unexpectedly, post-mortem evaluation revealed no changes in the CNS. These findings suggest that the neurological changes observed in behavioural tests in our amprolium TD model (animals treated for 80 days) are mild and exclusively functional, possibly involving neurochemical aspects, insufficient to induce degenerative processes or cell death. Studies have shown that TD rodents progressively develop endothelial cell hypertrophy and hyperplasia, astrocytic swelling, status spongiosus, neuronal degeneration and necrosis, myelin degeneration, gitter cell accumulation, and inflammatory infiltrate. These lesions are usually located in the thalamic nuclei, mammillary bodies, cerebellum, tectum, periaqueductal grey matter, and cerebral cortex (VETRENO et al., 2012). However, it is interesting to note that most of the rodent studies performed to date have used a different model for TD induction (pyrithiamine-induced) (NARDONE et al., 2013; VETRENO et al., 2012). This model induces an accelerated and stereotyped progression of neurological and behavioural clinical signs that have been mapped to specific neuroanatomical and neurochemical changes, mimicking the pathology described for human and carnivore TD (HAZELL; BUTTERWORTH, 2009). In addition, studies have shown that both behavioural and anatomopathological differences occur between the models of simple diet deficiency of thiamine and in the pyrithiamine-induced TD model. In the non-thiamine diet model, the progression of behavioural changes evolve slowly, with the first changes appearing on the ninth day, becoming more evident on the 25th day, with changes in emotional behaviour, muricide, and forced swimming test disturbances (VETRENO et al., 2012). In addition, vitamin administration after this period does not suppress these behavioural changes (NAKAGAWASAI, 2005). On the other hand, in the pyrithiamine-induced TD model, signs such as weight loss appear until the 10th day, progressing to ataxia (13th -15th day), opisthotonus, and seizures; which appear until the 16th day of treatment and progress to the animals’ death, unless thiamine is administered (VETRENO et al., 2012). In this case, the later changes are accompanied by marked CNS lesions (ANZALONE et al., 2010). In our model, we used amprolium PO, a compound known to cause TD in animals, but not studied in relation to TD experimental induction in laboratory rodents. Despite this, studies suggest that amprolium would be the ideal element for the induction and evaluation of thiamine deficiency (BUNIK; TYLICKI; LUKASHEV, 2013).

Apart from the pathological evaluation of the CNS, the changes were restricted to the kidneys of TD animals. Histopathology revealed moderate proteinuria in TD mice, in addition to moderate cellular swelling in the proximal tubules of the TD-B group treated for 80 days. These data indicate that prolonged treatment with amprolium PO may affect renal cellular metabolism and
initiate a nephrosis process with glomerular and tubular lesions.

Although it was observed different results in relation to the model applied in other species (ruminants), the preliminary data of our study suggest that this model may prove advantageous in comparison to the classic form of TD induction in rodents (with pyrithiamine) in some respects. The animals presented lower body weight gain and psychomotor behavioural changes, particularly when exposed for a longer period (80 days), even in the absence of morphological lesions in the CNS. Interestingly, animals treated for 80 days presented these changes without decreasing feed and water intake, suggesting that the effects induced by amprolium PO are determined by metabolic and neurological modifications in the animals. It is important to emphasize that the absence of morphological lesions in the nervous tissue does not exclude the possibility of important neural functional disorders, since significant damages can be produced by specific changes in the cell signalling systems, without occurrence of neurodegeneration or necrosis (CORDOVA et al., 2012). In our model, mice developed metabolic and behavioural changes on a diet with commercial balanced feed ad libitum (which contained thiamine in the formulation), suggesting that amprolium induced vitamin deficiency, even if presumably subtle. In the studies of TD induction with amprolium PO in bovines (NOGUEIRA et al., 2010), sheep (SANT’ANA et al., 2009a), and goats (SEDAHGAT; JAVANBAKHT, 2014), the mean time for onset of neurological signalling was varied, ranging from 22 to 59 days, often culminating to the acute death of the animals. Despite this, some animals failed to develop clinical signs and lesions in the CNS (NOGUEIRA et al., 2010). In our model, the mice did not develop typical neurological signs of TD for the species (paralysis, lateral decubitus, opisthotonus, seizures), but showed detectable and homogeneous behavioural psychomotor disorders (80 days of treatment). These differences (ruminants vs. rodents) suggest species-specific characteristics, particularly in pharmacological aspects, including variations in amprolium intestinal absorption (EMEA, 2001). Despite this, the milder manifestation of TD by mice with amprolium PO shows the potential of using the model to study the disorder’s pathogenesis, since it eliminates the risk of early and sudden animal death, which makes it difficult to analyse specific time points of disease progression.

CONCLUSION

The model of oral administration of amprolium in mice, dissolved in drinking water, is probably valuable for studying the appearance of behavioural and metabolic changes, without being aggressive, which would result in the rapid development of serious injuries. It is a simple and feasible experimental model of TD induction, optimized for use with laboratory animals.

CONFLICT OF INTEREST STATEMENT
Farmabase played no role in the study design or in the collection, analysis, and interpretation of the data, nor in the decision to submit the manuscript for publication. None of the authors has any financial or personal relationships that could inappropriately influence or bias the content of the paper.

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