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Original Research

Metronidazole-Induced Sensorimotor Deficits in Rats Are Associated With Cerebral Cortex-Related Thiamine Dysmetabolism and Ameliorated by High-Dose Thiamine

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Abstract

Background: Metronidazole neurotoxicity is a well-known phenomenon, but the underlying mechanisms remain poorly understood and may be linked to disrupted thiamine metabolism. This study aimed to establish a rat model of metronidazole-induced neuropathology, characterize the associated physiological parameters, including the thiamine status in blood and cerebral cortex, and assess whether thiamine supplementation can correct the impairments. **Methods:** Dose-response relationships were estimated using oral metronidazole (200 or 400 mg/kg, n = 9 in each group) or saline (n = 10) for 29 days. Other rats were given oral metronidazole (400 mg/kg, n = 16) or saline (n = 16) for 39 days; half of each group additionally received intraperitoneal thiamine (100 mg/kg, n = 8) or saline (n = 8) from day 30 to 39. Sensorimotor function was assessed with the beam walking test, rotarod, and open field; electrocardiogram variability parameters were used for the assessment of autonomic nervous system functions. Thiamine diphosphate (ThDP) in whole blood was measured enzymatically, and thiamine derivatives in the cortex were quantified a week after discontinuation of metronidazole and/or thiamine fluorometrically. **Results:** Metronidazole at 400 mg/kg increased forelimb errors in the beam walking test on days 17/18 ($p = 0.03$), also increasing the number of defecation acts in the open field test ($p = 0.03$), whereas rotarod performance was unchanged. A dose-dependent elevation of blood ThDP levels was observed ($p < 0.0001$), while the levels of cerebral phosphorylated thiamine derivatives decreased ($p < 0.01$). Thiamine co-treatment reduced forelimb beam walking errors by 51% in metronidazole-treated rats ($p < 0.01$), also reducing errors by 43% in controls ($p = 0.04$). However, thiamine supplementation was ineffective in preventing the long-term blood and cerebral cortex alterations of thiamine pools induced by metronidazole. **Conclusions:** Metronidazole administration induces sensorimotor deficit and causes a stable reduction in cerebral cortex thiamine phosphates despite elevated blood ThDP. The latter indicates that the neurotoxicity was accompanied by thiamine deficiency in the cerebral cortex, but not in whole blood. High-dose thiamine mitigates the neurological signs, with no long-term effect on the alterations in thiamine pools.

Keywords: metronidazole; adverse effects; neurotoxic disorders; sensorimotor gait disorder; thiamine pyrophosphate; thiamine; thiamine deficiency; neuroprotection

1. Introduction

Metronidazole is a drug widely used in clinical practice for the treatment of infections caused by anaerobic microorganisms and protozoa [1]. Due to the nature of the diseases and the limited number of therapeutic alternatives, the drug is often administered in prolonged courses, which may lead to neurotoxicity [1,2,3]. The most frequent neurological manifestations are dysarthria, gait and coordination disturbances, altered mental status, and peripheral neuropathy [4,5]. These disorders are usually accompanied by characteristic magnetic resonance imaging (MRI) findings: lesions are located in the dentate nuclei of the cerebellum, the corpus callosum, and the red nuclei of the midbrain, less frequently involving the pons and medulla oblongata [4,6]. In most cases, withdrawal of the drug abrogates both clinical manifestations and MRI changes [7,8]. Despite the well-described clinical picture of metronidazole-induced neurotoxicity, its pathogenesis remains not well characterized [1].

One of the potential causes of this adverse effect is the disruption of thiamine-dependent metabolism. Thiamine diphosphate (ThDP, also known as thiamine pyrophosphate), the main biologically active form of vitamin B1, is a key coenzyme of energy metabolism and also participates in the regulation of certain cellular processes through non-coenzyme mechanisms [9,10,11,12]. The high energy demand of neurons makes nervous tissue particularly sensitive to ThDP deficiency [9]. Due to its structural similarity to thiamine, metronidazole can be used by bacterial thiaminases as a substrate to form a thiamine antagonist capable of inhibiting the ThDP-forming enzyme thiamine diphosphokinase [12,13,14]. Indeed, long-term administration of

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metronidazole may disturb thiamine-dependent metabolism in the central nervous system and cause neurological disorders [15].

Another proposed mechanism of metronidazole neurotoxicity is the development of oxidative stress. The interaction of metronidazole with catecholamines generates nitrate radicals that reduce oxygen to superoxide anion radical and hydrogen peroxide. The increase in reactive oxygen species triggers lipid peroxidation of cell membranes, ultimately leading to intramyelinic edema [8,16,17]. However, the disruption of thiamine-dependent metabolism itself may exacerbate oxidative stress because, for example, ThDP is a cofactor of transketolase, the rate-limiting enzyme of the pentose phosphate pathway responsible for maintaining cellular antioxidant defense [18,19]. Additionally, ThDP-dependent 2-oxoglutarate dehydrogenase (OGDHC) and pyruvate dehydrogenase (PDHC) complexes can produce reactive oxygen species at a high level in a highly regulated manner, such as glutathione- or ThDP-dependent ones [20,21,22]. Thus, impaired ThDP-dependent metabolism can be considered both a part of the mechanism of metronidazole neurotoxicity and also a factor that enhances oxidative stress and damage to nervous tissue.

The link between metronidazole use and altered thiamine metabolism is further supported by both experimental and clinical data. A histological study of metronidazole-induced encephalopathy causing changes in gait and loss of balance in rats has shown symmetrical CNS lesions in the brainstem and cerebellar nuclei comparable to those caused by thiamine deficiency [4,23]; cortical involvement has also been reported [24,25]. Recently, prolonged metronidazole administration was reported to induce a decrease in serum thiamine, although the reported values remain questionable [26]. In patients, the partial similarity of MRI findings in metronidazole-induced encephalopathy and Wernicke's encephalopathy, which develops as a result of thiamine deficiency, provides further evidence of the relationship between metronidazole neurotoxicity and impaired thiamine-dependent metabolism [27,28]. Clinical cases of metronidazole-induced encephalopathy have also been described in which regression of neurological symptoms was observed after parenteral administration of high-dose thiamine [29,30].

Therefore, to further investigate the mechanisms of metronidazole-induced neurotoxicity, the present study carried out a comprehensive assessment of neurological impairments developing during prolonged drug administration, examined the effect of thiamine on their severity, and characterized changes in thiamine status in the blood and cerebral cortex.

2. Materials and Methods

2.1 Materials

Unless otherwise specified, chemical reagents were purchased from Macklin Biochemical (Shanghai, China). The following additional reagents were used: thiamine hydrochloride (Sisco Research Laboratories Pvt. Ltd., Mumbai, India), ThDP (Solarbio, Beijing, China), potassium ferricyanide (purity ≥99%; Sigma-Aldrich, Steinheim, Germany), acetonitrile of gradient HPLC grade (PanReac AppliChem, Darmstadt, Germany), isobutyl alcohol of analytical grade (Komponent-Reaktiv, Moscow, Russia). Milli-Q grade water was used throughout the analysis. The mixture of phosphopentoses as well as yeast transketolase apoenzyme for blood ThDP determination was prepared according to a previously published protocol [31].

2.2 Animals, Experimental Design, and Drug Administration

Animal experiments were conducted in compliance with the Guide for the Care and Use of Laboratory Animals, which follows the European Union Directives 86/609/EEC and 2010/63/EU, and were approved by the Bioethics Commission of Lomonosov Moscow State University (Protocols No. 139-a-2, May 19, 2022 and No. 139-3-a, December 4, 2025).

The study was performed in two independent experimental series. Male Wistar rats (RRID: RGD_13508588; age 13 ± 1 weeks, body weight 305 ± 15 g) were obtained from the Educational and Research Center for Wildlife Rehabilitation "Chashnikovo ABS". The series consisted of 28 and 32 animals, respectively. Rats were housed four to five per cage (555/4K, 580 × 375 × 200 mm) with free access to tap water and standard rodent chow (laboratorkorm.ru), following a two-week acclimatization period.

In series 1, designed to assess the dose-dependency of metronidazole effects (Fig. 1), animals were randomly assigned to three groups that received intragastric administration of either metronidazole at 200 mg/kg (n = 9), metronidazole at 400 mg/kg (n = 9), or saline (n = 10) for 29 days. Survival was 100% in all groups. In series 2, designed to investigate whether thiamine can reverse the metronidazole effects (Fig. 1), metronidazole at 400 mg/kg (n = 16) or saline (n = 16) was also intragastrically administered to rats for 39 days. From day 30 to 39, half of the animals in each group additionally received daily intraperitoneal injections of thiamine (100 mg/kg) or saline. This resulted in four groups (n = 8 each): control, control + thiamine, metronidazole, and metronidazole + thiamine. One rat in the control + thiamine group died on day 32 of the experiment. Subsequent necropsy did not determine the cause of death. Of note, a similar metronidazole treatment regimen in rats has been described as prolonged in the literature in the case of 60-day or 8-week treatment [26,32], with a 30-day period sometimes included [33]. Such time is comparable to our own experimental period of 29 or 39 days (Fig. 1). We use the term "repeated" administration for a shorter period as a contrast to the single-dose administration.

Metronidazole was administered as an aqueous solution via intragastric gavage, and thiamine hydrochloride

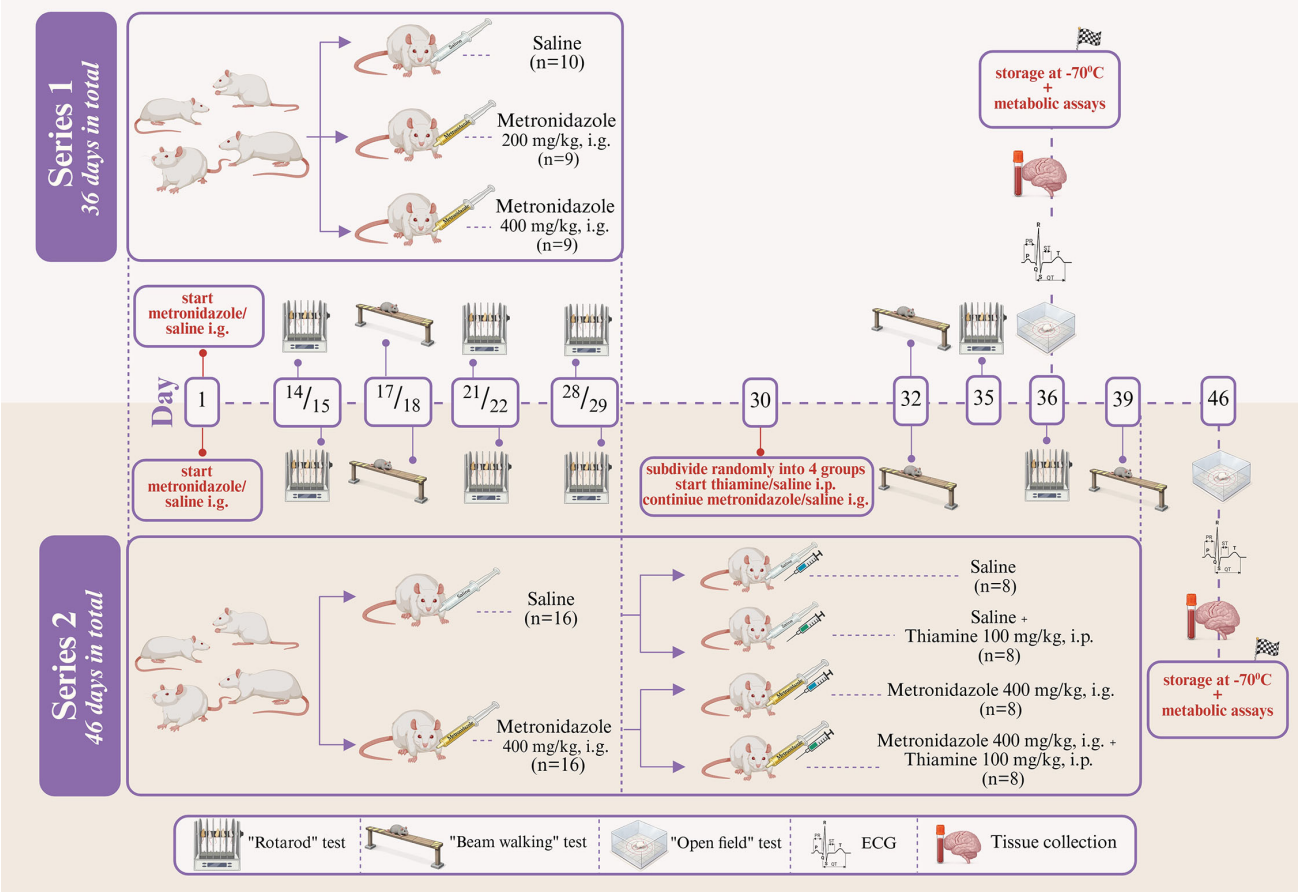

Fig. 1. Timeline of the experiment. The study included two independent experimental series. In series 1, rats received daily intragastric administration of metronidazole at 200 mg/kg (n = 9), 400 mg/kg (n = 9), or physiological saline (n = 10). In series 2, animals received daily intragastric administration of metronidazole at 400 mg/kg (n = 16) or physiological saline (n = 16); from day 30 to 39, half of the animals in each group (n = 8) additionally received intraperitoneal thiamine at 100 mg/kg or saline. Physiological function was assessed using the rotarod test (series 1—days 14, 21, 28, and 35; series 2—days 15, 22, 29, and 36) and the beam walking test (series 1—days 17 and 32; series 2—days 18, 32, and 39). Before the end of the experiment (day 36 in series 1; day 46 in series 2), electrocardiograms (ECG) were recorded, and the open field test was performed, after which blood and cerebral cortex samples were collected for further metabolic assays. Created in BioRender. Belousova, K. (2026) https://BioRender.com/ku6vwb9.

(pH 6.8–7.0, adjusted with NaOH) was injected intraperitoneally using insulin syringes, in accordance with recommendations for working with laboratory animals [34]. Experimental doses were selected based on interspecies dose conversion guidelines recommended by the U.S. Food and Drug Administration [35]. The metronidazole dose of 200 mg/kg in rats corresponds to a human equivalent dose of 2 g, which is within the standard therapeutic human dose range of 0.5–2 g per day for acute and recurrent infections [36]. The 400 mg/kg dose is consistent with previously published rodent models of prolonged metronidazole administration [26,32]. Thiamine was administered at a dosage of 100 mg/kg, similarly to other studies with rats [37,38], and equivalent to a human dose of 960 mg/day, which is comparable to 600–1500 mg/day doses used in patients with suspected Wernicke's encephalopathy [39,40,41].

Because the two experimental series had an identical protocol during the first 29 days, data from animals that did not receive thiamine (i.e., control and metronidazole 400 mg/kg groups from both series) were pooled whenever possible to assess the effects of metronidazole. The effects of thiamine were assessed using only series 2, in which thiamine or saline was administered from day 30 to 39. Accordingly, figures are organized to separately present the effects of metronidazole and the effects of thiamine, with consistent color coding and legends throughout to facilitate interpretation. Potential limitations arising from the differences in the experimental design between the two series are addressed in the "Limitations" section.

2.3 Behavioral Testing

To evaluate the behavioral impairment in experimental animals, the beam walking test, rotarod test, and open

field test were employed. In the beam walking test, the placement of each paw on the upper or lower board was recorded [42]. The number of paw placements on the lower board (errors), the number of limb slips from the upper board to the lower board (where the limb touched both boards), and the total number of steps taken from the starting line until the animal entered the dark compartment were counted separately for forelimbs and hindlimbs. The data from three trials were averaged. The severity of sensorimotor deficit was calculated as the ratio of the sum of the number of errors plus half the number of slips to the total number of steps, expressed as a percentage.

In the rotarod test, the latency to fall from a rotating horizontal rod with gradually increasing speed and the total distance travelled before falling were recorded [43]. Prior to testing, animals were trained to stay on the rod at a constant rotation speed (5 rpm). During test sessions, the speed increased from 5 to 30 rpm over a period of 300 s.

In the Open Field test, the following physiological parameters were recorded over a period of 3 minutes: locomotor activity (number of grid lines crossed), number of center entries, total time spent in the center, number of rearings, total grooming time, number of grooming bouts, total freezing time, and number of defecation acts.

In series 1, the beam walking test was conducted on days 17 and 32, and the rotarod test on days 14, 21, 28, and 35 (Fig. 1). In series 2, the protocol was expanded: the beam walking test was performed on days 18, 32, and 39, and the rotarod test on days 15, 22, 29, and 36. Finally, on day 36 in series 1 and on day 46 in series 2, the Open Field test was recorded, followed by a non-invasive electrocardiogram (ECG) [44,45]. From the recorded ECG data, the mean R–R interval, its variation range (dX), and root mean square of the successive differences (RMSSD) reflecting parasympathetic activity as well as the stress index (SI) characterizing sympathetic regulation of heart rhythm were calculated [44,46].

2.4 Tissue Collection and Sample Preparation

After recording physiological parameters on day 36 or 46 (Fig. 1), animals were decapitated using a guillotine (OpenScience, Moscow, Russia) as previously described [37]. To eliminate blood odor and minimize stress in animals, the guillotine was washed with water and treated with ethanol after each use. The method complied with current guidelines [47] and was approved by the Bioethics Commission of Lomonosov Moscow State University (Protocol No. 139-a-2, May 19, 2022). Immediately after decapitation, the brain was removed, the cerebral cortices were separated from the brainstem, and within 60–90 seconds after decapitation the tissues were frozen in liquid nitrogen. Blood was collected during decapitation from the neck vessels into heparin-containing tubes. Tissue samples were stored at −70 °C.

2.5 Quantification of Thiamine and Its Derivatives

ThDP in whole blood was determined by an enzymatic method using the activation of yeast transketolase apo-enzyme, as described previously [31].

Metabolites from rat cerebral cortex were extracted using a methanol-acetic acid method described previously [31,48]. Frozen tissue samples were homogenized in eight volumes of ice-cold methanol, after which 1.5 volumes of 0.2% acetic acid were added and proteins were removed by centrifugation. To remove lipid-soluble fluorescent compounds, 225 µL of the methanol-acetic acid extract was treated with 21 µL of chloroform and centrifuged at 21,000 g for 3 min [49]. The tissue volume required for this method hardly allows the same analysis to be carried out in the cerebellum. Thiamine and its phosphorylated derivatives were determined in the upper phase by a fluorometric method based on the oxidation of thiamine and its phosphorylated derivatives by potassium hexacyanoferrate (III) in an alkaline medium, forming the corresponding thiochrome derivatives detected fluorometrically (360/430 nm) [50,51]. Separate determination of free thiamine and phosphorylated forms was achieved by selective extraction of free thiochrome with isobutanol [50,52]. Background fluorescence correction was made using a sample treated with toluene-sulfonyl chloride (TSC) similarly to a method with benzenesulfonyl chloride, described before [53]. Each 81 µL sample was mixed with 10 µL of 1.25 M NaOH and 10 µL of 10.5 mM $K_3[Fe(CN)_6]$; 12 µL of 11.4% TSC or free acetonitrile was added to the background or test sample aliquots, respectively, before the addition of $K_3[Fe(CN)_6]$, the reaction was performed at 0 °C with vortexing, followed by the addition of 280 µL isobutanol, vortexing and centrifugation of the mixture at 21,000 g for 3 min. Thiochrome fluorescence was measured in 200 µL of the upper isobutanol phase, and thiochrome phosphate fluorescence – in 40 µL of the lower aqueous phase upon a 5-fold dilution with water. Calibration used pooled samples spiked with thiamine (0.1–10 µM) or ThDP (0.3–100 µM).

2.6 Statistics

Statistical analysis was performed using Prism version 8.0 (GraphPad Software Inc., La Jolla, CA, USA). Based on our previous experiments, including the measurements of thiamine and its derivatives in the blood and cerebral cortex upon thiamine administration to rats and expected similar amplitudes of the effects and distributions of the parameters, a two-tailed *t*-test with 80% power and a significance level of 0.05, a minimum of eight animals per group was required [31,54]. The data from the saline- and 400 mg/kg metronidazole-treated groups before or without thiamine administration can be pooled for analysis of the results of the two experimental series together. The ROUT test for outliers was applied in GraphPad Prism v. 10.0 (GraphPad Software, LLC, San Diego, CA, USA). Data were analyzed

using one-way or two-way ANOVA with repeated measurements where necessary, followed by Sidak's post-hoc test.

3. Results

3.1 Physiological Consequences of Metronidazole Administration to Rats

To assess the effect of long-term metronidazole administration on physiological parameters, a panel of tests, namely the rotarod test, beam walking test, Open Field test, and ECG, was used.

The rotarod test was originally developed as a screening tool for neurological effects of pharmacological agents in rodents [55]. It is widely employed to evaluate coordination and detect motor deficits in models of traumatic brain injury and neurodegenerative diseases [56,57] and demonstrates high sensitivity to cerebellar pathology, in particular to motor impairments caused by Purkinje cell degeneration [58]. Nevertheless, in the present study the rotarod test showed the lowest sensitivity among the behavioral tests used: no statistically significant differences were detected in either experimental series (**Supplementary Fig. 1**).

3.1.1 Metronidazole Increases the Error Rate in the Beam Walking Test

The beam walking test was used to assess sensorimotor disturbances arising from dysfunction of the motor cortex and corticospinal tract [59]. This test is applied in models of traumatic brain injury and stroke [60] and is more sensitive than the rotarod test for detecting effects of pharmacological compounds [61].

The beam walking test was performed on days 17/18 and 32 in both experimental series, and also on day 39 in series 2 (Fig. 1). Combined results obtained on days 17/18 and 32 in the two experimental series are presented in Fig. 2. Animals receiving metronidazole (400 mg/kg) exhibited an increased error rate in limb placement already at days 17/18 (ANOVA factor metronidazole, $p < 0.05$), particularly in the forelimbs – the error rate increased 1.4–fold ($p = 0.03$) compared to the control group (Fig. 2A). The effect of metronidazole increased in a dose-dependent manner at both time points, achieving significance ($p = 0.03$) in case of hindlimb and high (400 mg/kg) dosage (**Supplementary Fig. 2A,B**). However, the effect of metronidazole on motor impairments was less pronounced on day 32 (ANOVA factor "metronidazole", $p = 0.21$), as observed from the experimental data from both series (Fig. 2B). The "limb" factor was significant both on days 17/18 ($p < 0.001$) and day 32 ($p < 0.0001$). It should be noted that differences in the study design between the two experimental series emerged from day 29 onwards and affected the day 32 tests. Specifically, in series 2 animals continued to receive metronidazole whereas in series 1 the assessment of sensorimotor functions was performed 3 days after discontinuation of the drug (Fig. 1). These differences could have influenced the magnitude of the observed effect. No effect of metronidazole in the beam walking test was observed on day 39, evaluated in experimental series 2 only (Fig. 1). Thus, the test results indicate that repeated administration of metronidazole induces a sensorimotor deficit in rats within 17/18 days; the deficit is better observed in forelimbs, and its severity slowly diminishes with time.

3.1.2 Thiamine Administration Reduces the Error Rate in the Beam Walking Test

To correct the developed motor impairments, thiamine was administered at a dose of 100 mg/kg from day 30 to 39 (10 days in total) to half of the animals from the metronidazole-treated and control groups (Fig. 1, experimental series 2). Thiamine substantially reduced the severity of sensorimotor deficit in the beam walking test already after three injections (Fig. 3). As above, forelimbs were more sensitive to thiamine treatment than hindlimbs (**Supplementary Fig. 3**). Specifically, the effect was significant both in control rats (Fig. 3A,B) and in the ones receiving metronidazole (Fig. 3C), with the strongest reduction in slip proportion observed in forelimbs on day 39 after 10 injections in total (Fig. 3A,C and **Supplementary Fig. 3A**). That is, the sensorimotor deficit was reduced 51% in the "metronidazole + thiamine" group, compared to metronidazole alone ($p < 0.01$, Fig. 3C). In control animals, thiamine caused 43% reduction in slip proportion, compared to the controls receiving saline ($p = 0.04$, Fig. 3A). Thus, thiamine administration caused a reduction in sensorimotor deficit, the effect which is opposite to the one induced by metronidazole neurotoxicity.

3.1.3 Results of the Open Field Test and ECG Analysis

The open field test is widely used to assess general locomotor and exploratory activity as well as anxiety levels. ECG recording allows evaluation of the functional state of the cardiovascular system and parameters of autonomic regulation. The open field test and ECG recording were performed on the final day of the experiment (Fig. 1). Since animals in the two experimental series received metronidazole for different periods of time (29 and 39 days in series 1 and 2, respectively), this also made it possible to evaluate the influence of metronidazole treatment duration on the studied parameters (Fig. 4A–C).

As shown in Fig. 4A, metronidazole administration causes a decrease in heart rate variability manifested as a reduced RMSSD parameter ($p = 0.017$), which indicates an imbalance in the autonomic nervous system and increased stress. No other significant effects of metronidazole on ECG parameters have been revealed (Fig. 4B and **Supplementary Fig. 4**). Additionally, 400 mg/kg metronidazole increased the number of defecation acts ($p = 0.03$), which may reflect its antibiotic action on the gut microbiota as well as serve as an indication of stress induced by metronidazole neurotoxicity (Fig. 4C).

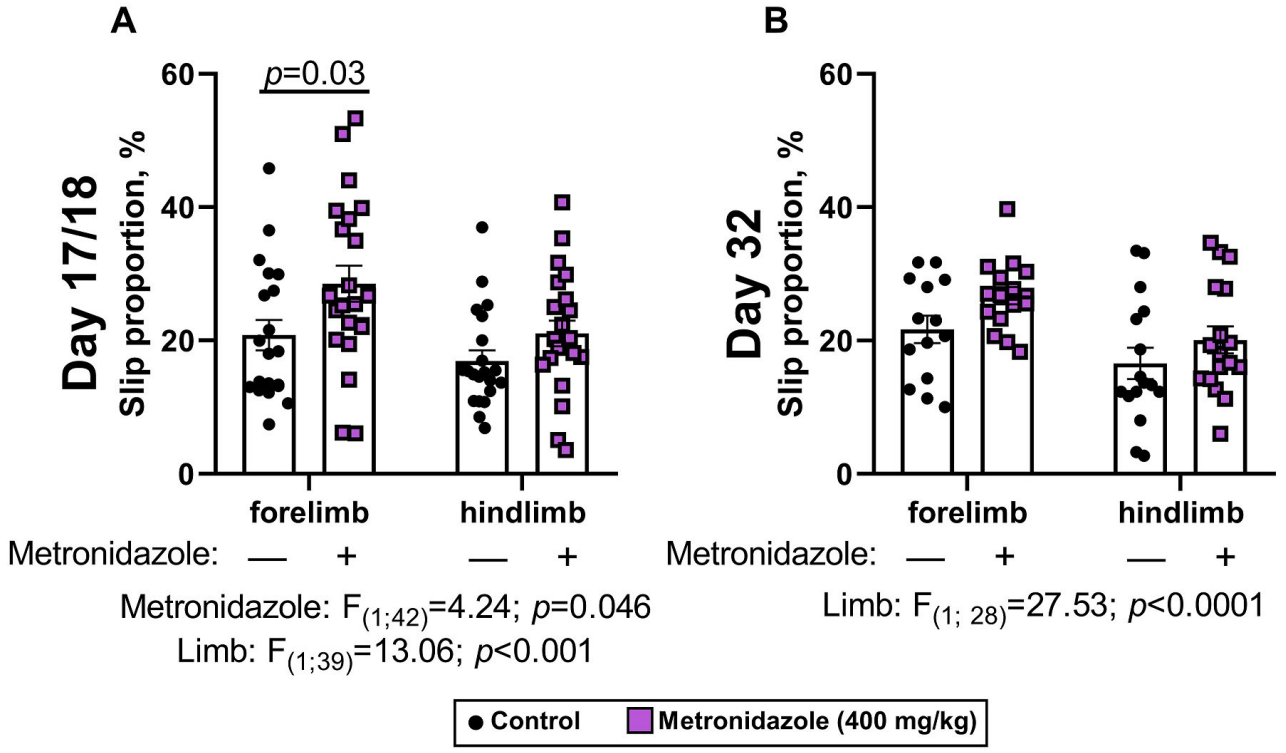

Fig. 2. Effect of metronidazole on sensorimotor deficit in the beam walking test. The graphs combine results from both experimental series for the forelimb and hindlimb on days 17/18 (A) and 32 (B) of the experiment. Error rate is shown as the proportion of slips. Data from the control rats (n = 18) and those receiving 400 mg/kg metronidazole (n = 17) are shown according to the figure legend; each point represents an individual animal. Data are presented as mean ± standard error of the mean (SEM). Statistically significant ($p < 0.05$) results of RM–ANOVA are shown below the graphs as *p*-values and F-statistics for each factor. Sidak's post-hoc test was used for pairwise comparisons between the groups, with statistically significant differences indicated as *p*-values in the graphs.

Thiamine administration had no effect on heart rate variability (Fig. 4D), resulting in a metronidazole effect ($p = 0.023$) similar to the one observed without thiamine (Fig. 4A,D). However, a moderate thiamine-induced increase in the dX parameter was observed ($p = 0.044$), which mostly affected healthy rats (Fig. 4E). The aforementioned effect of metronidazole on defecation did not reach significance when involving the thiamine-treated and control animals (Fig. 4F). No other significant effect of thiamine or metronidazole on other parameters of the ECG and Open Field tests was observed (**Supplementary Fig. 4**).

Thus, combined data of ECG and Open Field tests indicate a metronidazole-induced autonomic imbalance, which is not fully reversed by 10-day thiamine administration.

3.2 Metronidazole Administration Regulates the Thiamine Pool in Rat Blood and Cerebral Cortex

In view of the previously identified physiological metronidazole-induced deficits, the presumed involvement of thiamine-dependent mechanisms in their development, and the protective effects of thiamine opposite to metronidazole in behavioral tests, we have evaluated the thiamine status of the experimental animals. The blood and cerebral cortex were used as tissues corresponding, respectively, to alterations in thiamine content of the body and the cerebral cortex.

It was found that after 29 ($p = 0.0001$) and 39 ($p < 0.0001$) days of 400 mg/kg metronidazole treatment, followed by a week of recovery (Fig. 1), the whole blood ThDP concentration was 21–27% higher compared to the corresponding control groups (Fig. 5A). In contrast, the opposite pattern was observed in the cerebral cortex. The concentration of phosphorylated derivatives decreased by 17–21% ($p < 0.01$), while free thiamine content did not change (Fig. 5B,C). The trend for a reduction in thiamine derivatives (mostly ThDP and thiamine monophosphate (ThMP)) content in animals receiving 400 mg/kg metronidazole was revealed at 29 days ($p = 0.084$). The effect was dose-dependent, reaching statistical significance ($p = 0.016$) when analyzing the data of series 1 alone (**Supplementary Fig. 5**).

Thiamine administration caused a slight decrease in the significance of the metronidazole effect on the blood ThDP level, reflected by a significant interaction ($p = 0.033$) between the metronidazole and thiamine factors of

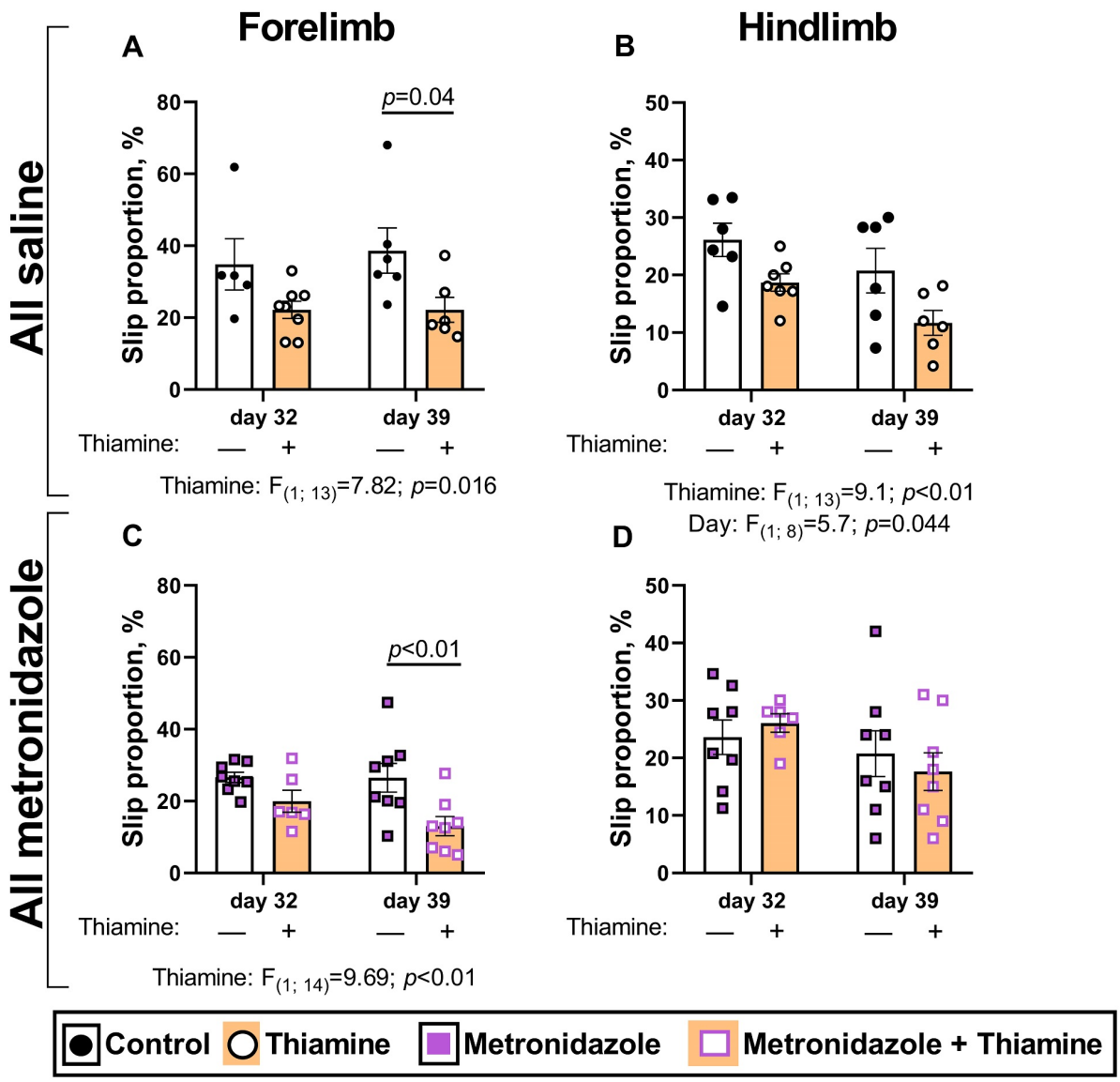

Fig. 3. Effect of thiamine on sensorimotor deficit in the beam walking test. The data from the forelimb (A,C) and hind limb (B,D) error rates from the beam walking test on days 32 and 39 are shown. Animals of experimental series 2 received either saline (n = 8) or saline + thiamine 100 mg/kg (n = 7) (A,B), or metronidazole 400 mg/kg (n = 8) or metronidazole 400 mg/kg + thiamine 100 mg/kg (n = 8), as described in the bottom panel of Fig. 1. Each point represents an individual animal. Data are presented as mean ± SEM. Outliers were excluded as described in Section 2.6. Statistically significant ($p < 0.05$) results of RM-ANOVA are shown below the graphs as *p*-values and F-statistics for each factor. Sidak's post-hoc test was used for pairwise comparisons between groups, with statistically significant differences indicated as *p*-values in the graphs.

two-way ANOVA. The amount of thiamine phosphoderivatives in the rat cerebral cortex was reduced by metronidazole ($p < 0.001$) independently of thiamine administration (Fig. 5E). Cerebral thiamine was not significantly changed after thiamine administration (Fig. 5F).

Thus, metronidazole-induced neurotoxicity is not associated with vitamin B1 deficiency in the whole body but may be caused by alterations in thiamine metabolism primarily in the nervous tissue. Importantly, the protocol used for thiamine administration cannot reverse the stable metronidazole-induced reduction in the cerebral levels of thiamine phosphoderivatives.

4. Discussion

4.1 Metronidazole and Its Analogues: Features of Neurotoxicity and Potential for Recovery

The mechanisms underlying metronidazole-induced neurotoxicity have not been definitively established. Replacing the drug with other 5-nitroimidazole derivatives

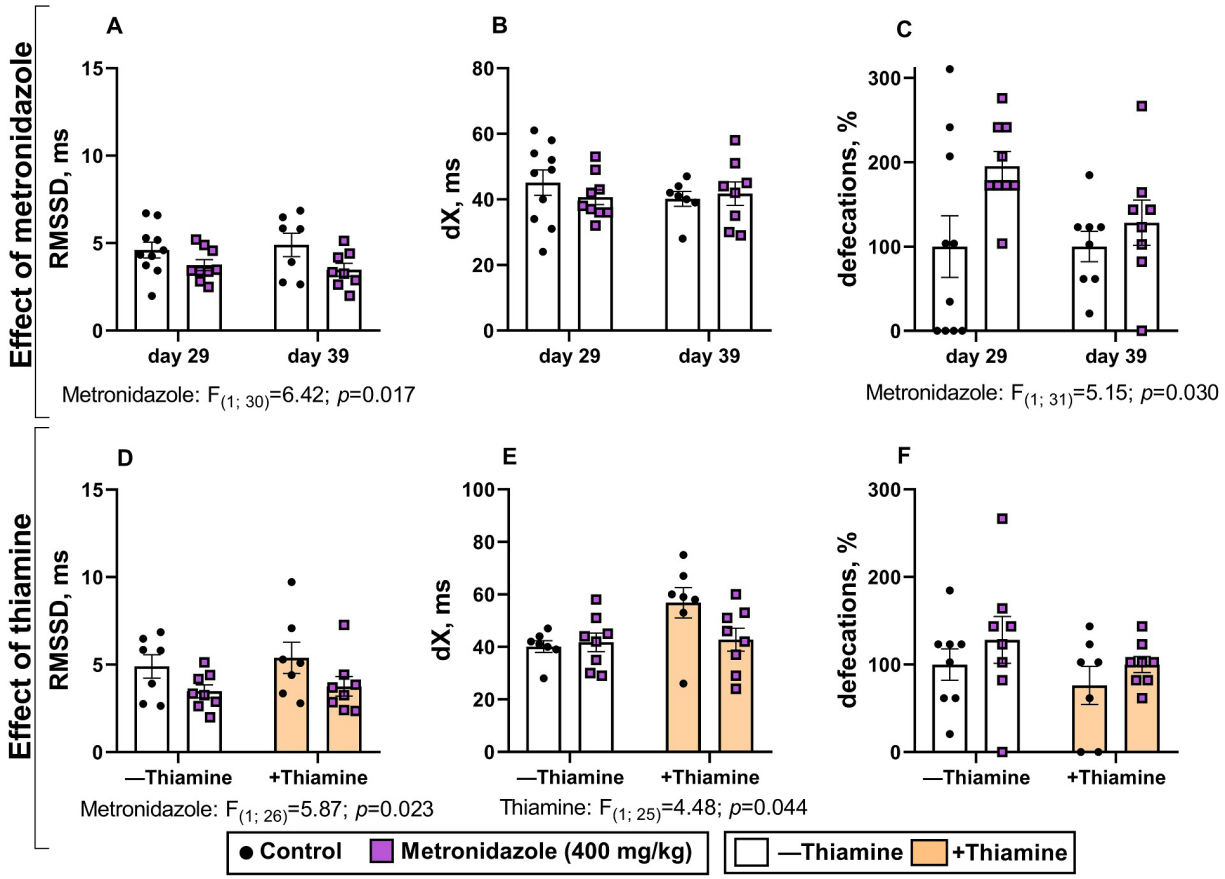

Fig. 4. Effects of metronidazole (A–C) and thiamine (D–F) on parameters of ECG and the Open Field test. ECG heart rate variability parameters RMSSD – parasympathetic index, relaxation index (A,D); dX – variation range, total rhythm variability (B,E) and the number of defecation acts in the Open Field test (C,F) expressed as a percentage of the control group values are shown; other parameters are provided in **Supplementary Fig. 4**. Graphs (A–C) present the data obtained after 29 and 39 days of metronidazole administration in the experimental series 1 and 2, respectively; graphs (D–F) show the effect of thiamine on the studied parameters in animals from the series 2. Each point represents an individual animal. Data are presented as mean ± SEM. Statistically significant ($p < 0.05$) results of two-way ANOVA are shown below the graphs as p-values and F-statistics for each factor.

(ornidazole, tinidazole, secnidazole) does not guarantee a reduced risk of neurological complications [4]. All these drugs have a huge structural similarity to each other and a thiamine fragment, and potentially can participate in the thiaminase-catalyzed reaction described for metronidazole [12,14,62]. The four 5-nitroimidazole derivatives differ mainly in their pharmacokinetic properties [63]. In addition to metronidazole toxicity, cases of neurotoxicity have also been described upon prolonged use of ornidazole and tinidazole, accompanied by typical clinical manifestations and MRI changes that regress after discontinuation of the drugs [62,64]. No such cases have been identified for secnidazole, which is likely due to its use predominantly in short or single-dose courses [65]. Thus, these common features suggest that neurotoxicity is a class effect, characteristic of most 5-nitroimidazole drugs and underscores the need to study the mechanisms of its development in order to find preventive approaches.

Motor disturbances are among the major manifestations of metronidazole-induced neurotoxicity [66,67,68]. This is consistent with the present study, in which long-term metronidazole administration to rats induced a sensorimotor deficit that was detected in the beam walking test (Fig. 2, **Supplementary Fig. 2**). However, no impairment was observed in the rotarod test, which is also designed to assess motor coordination (**Supplementary Fig. 1**). Similar results were obtained in a model of benzodiazepine-induced ataxia, where the rotarod test likewise failed to reveal motor deficits in mice despite a pronounced clinical effect in humans [61]. This may further demonstrate the higher sensitivity of the beam walking test or indicate that repeated metronidazole administration predominantly disrupts fine motor coordination without causing more severe motor disorders detectable by the rotarod.

The sensorimotor deficit was the most pronounced at days 17/18 of drug administration (Fig. 2), was only partially detected at day 32 (**Supplementary Fig. 2B**), and

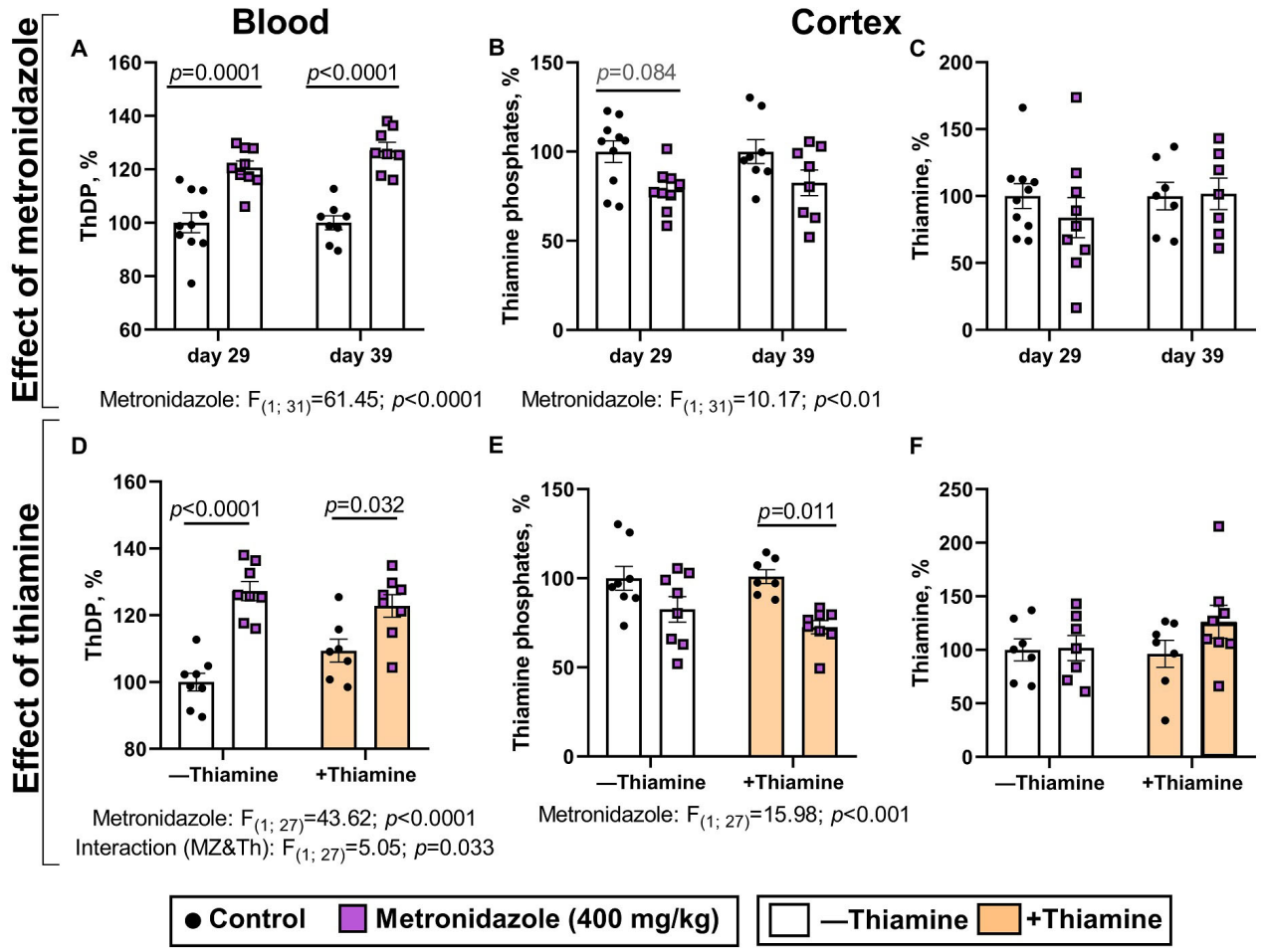

Fig. 5. Effects of metronidazole (A–C) and thiamine (D–F) on ThDP levels in blood (A,D) as well as on thiamine phosphoderivatives (B,E) and thiamine (C,F) in cerebral cortex. The 100% level for each parameter represents the following mean concentrations (both series combined): blood ThDP—0.46 ± 0.02 nmol/mL; cortical thiamine phosphoderivatives—8.13 ± 0.58 nmol/g fresh weight (FW); cortical thiamine—0.78 ± 0.07 nmol/g FW. Graphs (A–C) present the data obtained after 29 and 39 days of metronidazole administration in experimental series 1 and 2, respectively; graphs (D–F) show the effect of thiamine on the studied parameters in animals from series 2. Each point represents an individual animal. Data are presented as mean ± SEM. Statistically significant ($p < 0.05$) results of two-way ANOVA are shown below the graphs as factors and their interactions with *p*-values and F-statistics. Sidak's post-hoc test was used for pairwise comparisons between groups with significant differences ($p < 0.05$) and trends ($0.05 < p < 0.1$) indicated as *p*-values in the graphs.

was not detected thereafter on day 39 (Fig. 3A,B *vs* Fig. 3C,D). The gradual disappearance of symptoms on subsequent days may indicate an adaptation of the animals to long-term metronidazole administration, although several aspects of experimental design could contribute to the effect, namely the three-day withdrawal of the drug before the tests on day 32 in experimental series 1, and a smaller number of animals tested on day 39 (experimental series 2 only). The latter assumption is consistent with clinical observations, according to which the neurological symptoms gradually regress after metronidazole discontinuation [4]. Of note, the 17/18 day-long time course over which the sensorimotor disturbances were developed in rats is comparable to the range of treatment duration (14–52.5 days) required for the symptoms' onset, reported in the literature for metronidazole-induced neurotoxicity in human patients [4] and only slightly exceeds the duration of a standard therapeutic course of 7–14 days [36], highlighting the medical relevance of the obtained results.

4.2 Effect of Thiamine on Sensorimotor Deficit. Pathologies and Mechanisms

A positive effect of thiamine in the beam walking test was observed on all testing days (32 and 39) both in animals receiving metronidazole and in the control group, with no significant interaction of ANOVA factors (Fig. 3), indicating a general neuroprotective action of the vitamin [69]. Notably, the beneficial effect of thiamine was not revealed

in the rotarod test (**Supplementary Fig. 1**). That is, both metronidazole-induced neurotoxicity and thiamine's beneficial effects can be detected in the beam walking test, but not in the rotarod test, which is an indication of similar mechanisms involved in the effects of the two treatments.

The neuroprotective effect of thiamine has been and continues to be studied extensively. It has been confirmed in other experimental models of nervous system injury. For example, in spinal cord injury, a single administration of thiamine 15–20 hours after the injury improved motor function recovery score by 18% compared with untreated animals [70]. The effect was accompanied by increased levels of deacylase sirtuin 5 and acetylation of glutamate dehydrogenase K503 residue, and a reduction in acetylated p53, although the thiamine coenzyme targets, namely the activities of ThDP-dependent OGDHC and PDHC, were not affected by thiamine in the spinal cord and brain of injured animals [70]. In a model of traumatic brain injury, in contrast, thiamine administration 1 h before the trauma restored OGDHC activity, which dropped significantly upon the trauma. Additionally, thiamine reduced the expression of the proinflammatory cytokine receptor TNF-R1. These data point to a major role of OGDHC in neuroinflammation, with thiamine being among the neuroprotective drugs possessing specific molecular targets [71]. Together, these data indicate that the neuroprotective effect of thiamine involves both the classic ThDP-dependent enzyme targets such as OGDHC, and is realized also through various non-coenzyme mechanisms involving thiamine's role in regulation of protein acylation [54,72], phosphorylation [31], NAD-dependent deacylases [72,73] and direct regulation of p53 by ThDP and NAD [74].

In the context of metronidazole-induced neurotoxicity, the antioxidant properties of vitamin B1 are of great importance [69]. The mechanism of the antioxidant action of thiamine definitely involves, but is not limited to, the maintenance of the pentose phosphate pathway, which is necessary for the functioning of the cellular NADPH-dependent redox system. Its nonoxidative phase includes a rate-limiting ThDP-dependent enzyme, transketolase, which possesses a nanomolar apparent Km for ThDP [75,76]. An additional mechanism of antioxidant thiamine activity includes its capacity to scavenge a variety of radical species directly [77,78,79]. As a result, thiamine and ThDP reduced the formation of reactive oxygen species *in vitro* [77,80] and *in vivo* [81], promoting its application in multiple diseases involving redox stress. In clinical studies, high doses of thiamine have been proven to be a promising tool for the treatment of Parkinson's disease [82,83,84], and a few other neurological conditions including multiple sclerosis, Friedreich ataxia, fibromyalgia and chronic cluster headache [85,86,87,88]. Moreover, independent studies have revealed thiamine as a good protectant against inflammatory bowel disease [89,90]. A synthetic thiamine derivative, benfotiamine, reduced the levels of pro-inflammatory mediators (COX-2, Hsp70, TNF-α, IL-6) in a cell model of lipopolysaccharide-stimulated BV-2 microglia [91]. Benfotiamine has also shown microvascular protective effects and a reduction in oxidative stress markers in the serum of patients with type 2 diabetes [92]. In contrast, thiamine deficiency facilitated oxidative stress and pathological manifestations in animal models of neurodegenerative diseases such as Alzheimer's disease [93].

Thus, the neuroprotective effect of thiamine observed in the present study is most likely not limited to the ThDP coenzyme role in energy metabolism. Metronidazole can generate reactive oxygen species, while thiamine, possessing direct and indirect antioxidant actions, eliminates them [16,17,77]. Therefore, the ability of thiamine to limit the development of oxidative stress induced by metronidazole could significantly contribute to its neuroprotective effect, besides the expected restoration of the impaired cerebral ThDP pool. These mechanisms certainly deserve further investigation.

4.3 Metronidazole Disrupts Thiamine Metabolism

Among the mechanisms of the thiamine role in metronidazole-induced pathology, one very interesting observation deserved particular attention. That is, analysis of thiamine and its phosphorylated derivatives, or ThDP, in cerebral cortex and blood revealed oppositely directed changes induced by metronidazole (Fig. 5). Specifically, the concentration of thiamine phosphates in the cortex decreased with metronidazole administration. The main component of the thiamine pool is ThDP, accounting for 71% or 92% in the cerebral cortex of humans and rats, respectively [94], as well as about 84% of the thiamine pool in human whole blood [95]. Thus, the data on metronidazole-induced reduction in cerebral thiamine phosphoderivatives (Fig. 5B,E) imply a reduction in cerebral ThDP. However, the whole blood ThDP was significantly increased in animals receiving metronidazole. This clearly indicates the absence of systemic vitamin B1 deficiency during prolonged metronidazole treatment, and points to local disturbances of thiamine metabolism in the central nervous system. These disturbances in cerebral thiamine phosphoderivatives/ThDP content should play a significant part in the sensorimotor deficit caused by metronidazole.

It is worth noting that our data on the effect of metronidazole on the content of thiamine and derivatives indirectly contradict the results of a previous study [26], which demonstrated decreased serum thiamine levels in rats after 60 days of 400 mg/kg metronidazole administration. Although a direct comparison of the studies is impossible due to different durations of the studies, different types of biological samples, and different forms of vitamin B1 analyzed, the thiamine concentrations determined in that work by ELISA were highly suspicious (158.2 ± 4.3 mg/dL in the control group, or 1.582 g/L thiamine, which is about 4.7 mmol/L) [26]. The latter enormously exceeds the generally

accepted 11–17 nmol/L thiamine concentrations for serum/-plasma of humans [95,96] or even 103 nmol/L concentration for the whole blood of rats [97]. In contrast, levels of blood ThDP determined in this work (460 ± 20 nmol/L, Fig. 5) are in agreement with the reference values of 345 ± 25 nmol/L for blood ThDP [97]. With that in mind, the previously reported reduction in serum thiamine induced by metronidazole is questionable, although it cannot be excluded, as only ThDP was measured in the rat blood in this study. The latter is increased by metronidazole (Fig. 5A and **Supplementary Fig. 5A**), and serum thiamine could be one of the sources for ThDP synthesis in red blood cells, which are the main ThDP-comprising compartment of the blood [95].

Owing to the characteristics of thiamine transport and metabolism [12], the decrease in total thiamine phosphates in the cerebral cortex may be caused by enhanced dephosphorylation of ThDP and ThMP with subsequent efflux of thiamine from the brain, since the concentration of thiamine itself does not increase substantially (Fig. 5). Alternatively, the same effect could be achieved by a downregulation of ATP-dependent thiamine phosphorylation by thiamine diphosphokinase, which, according to data obtained in neuroblastoma cells, is a key mechanism required for thiamine transport into cells [98]. Indeed, metronidazole derivatives generated in the thiaminase reaction have been shown to inhibit thiamine diphosphokinase [12,13,14]. These data favor the mechanism of observed metronidazole-induced reduction in cerebral cortex thiamine phosphate levels to be linked to the proposed inhibition of thiamine diphosphokinase in the brain. It should be taken into account that, in this case, metronidazole derivatives would have to penetrate the blood-brain barrier (BBB), which has not been demonstrated directly. However, metronidazole itself can cross the BBB very well [99,100], and so can thiamine [101,102]. Importantly, according to clinical observations, neurological symptoms in patients with metronidazole-induced encephalopathy regress after parenteral thiamine administration, despite normal serum thiamine concentrations [30]. Moreover, in patients with suspected Wernicke's encephalopathy, normal blood levels of both thiamine [103,104] and ThDP [105] are also frequently recorded.

Thus, the data obtained indicate that metronidazole-induced neurotoxicity is associated with a disturbance of cerebral thiamine-dependent metabolism resulting in a drop in phosphorylated thiamine derivatives in the cerebral cortex, and an increase in the blood ThDP, revealing no signs of systemic vitamin B1 deficiency.

5. Limitations

Several aspects of the present study warrant consideration when interpreting the findings. The first concerns the behavioral assessment at day 32, as the two experimental series followed different protocols starting from day 29. In series 1, metronidazole had been discontinued, whereas in series 2 the drug was still being given. This difference arose because the two series addressed different questions: series 1 assessed the dose-dependent effects after a fixed 29-day treatment, while series 2 examined whether thiamine could reverse an already established deficit. Although clinical experience indicates that neurological symptoms regress gradually after drug withdrawal and recovery may require several weeks, this difference in timing could still have influenced the magnitude of the observed effect. Statistical evaluation supports pooling of the data from the two series, which also reduces the number of animals required. Nevertheless, this design difference should be kept in mind when interpreting the day-32 results.

Additionally, the analysis was limited to whole blood and the cerebral cortex, and an important difference was revealed between the two tissues. Whole blood was chosen because it is the standard clinical material for assessing thiamine status and reflects responses from the whole body. Among brain regions, the cortex was selected because it provided sufficient tissue volume, cortical involvement in metronidazole-induced encephalopathy has been reported clinically, and this region allowed direct comparison with our previous work on thiamine metabolism. Nevertheless, other brain regions and peripheral tissues were not examined, and the conclusion that systemic vitamin B1 deficiency was absent therefore rests on blood data alone. Thus, the observed metronidazole-induced disturbances in the thiamine pool appear to be specific to the cerebral cortex representing the brain in this study and are not detected in whole blood.

It should also be acknowledged that the 400 mg/kg dose of metronidazole in rats exceeds the clinically equivalent human dose estimated from the standard conversion formula by approximately two-fold. This dose was chosen to model a more pronounced and potentially longer-lasting neurotoxic effect, in line with other studies in rats, but direct extrapolation to clinical dosing requires caution. In addition, the behavioral data showed considerable inter-individual variability, which may have reduced the ability to detect subtle differences despite the use of a broad panel of tests and repeated-measures ANOVA. The beam walking test nevertheless proved to be the most sensitive measure and provided the clearest evidence of the sensorimotor deficit. Finally, histological confirmation of neurotoxicity was not performed, and the study was restricted to male Wistar rats.

6. Conclusions

Taken together, our findings demonstrate for the first time that metronidazole-induced sensorimotor dysfunction is associated with cerebral cortex-related thiamine dysmetabolism, and that high-dose thiamine offers a partially effective countermeasure. These results encourage a further exploration of thiamine-based interventions for drug-

induced encephalopathies, and highlight the importance of extensive studies of thiamine and its derivatives levels in the blood and other organs. The example of metronidazole shows that drugs can induce tissue-specific reductions in physiologically critical ThDP, specifically the cerebral ThDP, with no such effect or even an opposite action on the whole blood ThDP levels, thereby complicating proper diagnosis and treatment of neurological disorders.

Abbreviations

BBB, blood-brain barrier; ECG, electrocardiogram; OGDHC, 2-oxoglutarate dehydrogenase complex; MZ, metronidazole; PDHC, pyruvate dehydrogenase complex; RMSSD, root mean square of the successive differences in R-R intervals; ThDP, thiamine diphosphate; ThMP, thiamine monophosphate; TSC, toluene-sulfonyl chloride.

Availability of Data and Materials

The data presented in this study are available in this article and the **Supplementary Material**. The datasets used and analyzed during the current study are available from the corresponding author on reasonable request.

Author Contributions

VA and AG designed the research study. KB, AI, AL, OS, MM, AM, AG, AA, and VA performed the research. KB, AG, AA and VA analyzed the data. KB wrote the draft and prepared figures. VA prepared the final text. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

Ethics Approval and Consent to Participate

The study was carried out in accordance with the ARRIVE guidelines. The research protocol was approved by the Bioethics Commission of Lomonosov Moscow State University (Protocols No. 139-a-2, May 19, 2022 and No. 139-3-a, December 4, 2025). Animal experiments were conducted in accordance with the Guide for the Care and Use of Laboratory Animals and the relevant regulations of the European Union Directives 86/609/EEC and 2010/63/EU.

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Conflicts of Interest

The authors declare no conflicts of interest.

Declaration of AI and AI-Assisted Technologies in the Writing Process

During the preparation of this work, the authors used BioRender (for the timeline of the experiment and the graphical abstract only), including its built-in tools. DeepSeek was used to check spelling and grammar. After using this tool, the authors reviewed and edited the content and take full responsibility for the content of the publication.

Supplementary Material

Supplementary file includes **Supplementary Figs. 1–5**. Supplementary material associated with this article can be found, in the online version, at https://doi.org/10.31083/FBL56591.

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