FULL ARTICLE

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Physiological gait test: an effective method for analyzing balance, locomotion, and neuromuscular disorders in rats and a comparison to the elevated beam test

Teste de marcha fisiológica: um método eficaz para analisar distúrbios de equilíbrio, locomoção e neuromusculares em roedores

Lorena Pantaleon[1,2]; André Rinaldi Fukushima[1,2,3]; Natália Moreira[4]; Leonardo Ribeiro De-Paula[1]; Guilherme Ribeiro[1]; Juliana Weckx Peña-Muñoz[2]; Beatriz do Prado Pacca Faria[5]; Marcelo Mendonça[2]; Gabriel Ramos de Abreu[1]; Júlia Zacarelli-Magalhães[1]; Jan Carlo Bertassoni Delorenzi[6]; Paula A. Faria Waziry[7]; Helenice de Souza Spinosa[1]; Esther Lopes Ricci[1,3,6]

[1]Universidade de São Paulo, Faculdade Medicina Veterinária e Zootecnia, Departamenteo de Patologia, Programa de Pós-Graduação em Patologia Experimental e Comparada, São Paulo – SP, Brazil
[2]Centro Universitário das Américas, São Paulo – SP, Brazil
[3]Faculdade de Ciências da Saúde do IGESP, São Paulo – SP, Brazil
[4]Universidade Paulista, São Paulo – SP, Brazil
[5]Universidade Municipal de São Caetano do Sul, São Paulo – SP, Brazil
[6]Universidade Presbiteriana Mackenzie, Centro de Ciências Biológicas e da Saúde, São Paulo – SP, Brazil
[7]Western Atlantic University School of Medicine, Plantation – FL, USA

ABSTRACT

The evaluation of animal locomotor activity is a behavioral tool widely used to measure the mechanisms underlying a particular disease, disorder, or injury, as well as the effects of exposure to a xenobiotic. The elevated beam test is one of the most used tests in rodents to assess balance and motor coordination. Despite being inexpensive and utilizing a simple apparatus, the high beam test requires a long period of animal training and habituation. The development and characterization of an alternative test, namely the gait test, has the potential to circumvent the time and effort required for animal training, deeming it an effective, inexpensive, and fast method for the analysis of behaviors that are comparably assessed by the high beam test. Therefore, the present study focused on determining the effectiveness and feasibility of the gait test for assessing rodent locomotion and balance as a replacement for the elevated beam test. For this purpose, male rats were divided into three groups: one control group exposed to a saline solution (NaCl 0.9%) and two experimental groups exposed to a single dose of either 0.2 or 1.0 mg/kg of ivermectin intraperitoneally for induction of locomotor disturbance. The high beam and gait tests were performed 15 min and 24 h after drug administration. Results show that the experimental groups had difficulty performing the tasks of either test at both time points analyzed compared to the control groups. At the high beam, experimental animals had trouble maintaining balance and walking. At the gait test, experimental animals showed alterations in gait, which were quantitated by: (a) shortening of step length, (b) decrease of stride, (c) altered step symmetry, and (d) altered stride area. Such results are indicative of compensatory efforts and were comparable between both tests. Altogether, the data indicate that the gait test meets all requirements for assessing motor coordination in rodents. The gait test is therefore validated as a complement to the elevated beam test for the study and analysis of neurodegenerative impairment and other disorders involving neuromuscular disturbances.

Keywords: Locomotor activity. Motor coordination. Motor balance. Neurodegenerative diseases. Ivermectin.

RESUMO

A avaliação da atividade locomotora animal é uma ferramenta comportamental bastante utilizada para mensurar os mecanismos subjacentes a uma determinada doença, distúrbio ou lesão e efeitos da exposição a um xenobiótico. Um dos testes mais utilizados em roedores para avaliar o equilíbrio e coordenação motora é o teste da trave elevada que, apesar de ser um teste barato e que exige um aparato simples, é necessário um longo período de treino e habituação dos animais. O desenvolvimento e caracterização de um teste alternativo, chamado de teste da marcha, tem o potencial de contornar o tempo e o esforço necessários ao treino dos animais, considerando-o um método eficaz, barato e rápido para a análise de comportamentos avaliados comparativamente pelo alto teste de feixe. Portanto, o presente estudo concentrou-se em determinar a eficácia e viabilidade do teste de marcha para avaliação da locomoção e equilíbrio de roedores em substituição ao teste da trave elevada.

Para isso, ratos machos foram divididos em 3 grupos, sendo 1 grupo controle exposto à solução salina (NaCl 0,9%) e 2 grupos experimentais expostos à dose única de 0,2 e 1,0 mg/kg de ivermectina por via intraperitoneal para indução da alteração locomotora. Os testes de trave elevada e marcha foram realizados 15 min e 24 h após a administração da droga. Os resultados mostram que os grupos experimentais tiveram dificuldade em realizar as tarefas de qualquer teste em ambos os momentos analisados em comparação com os grupos de controle. Na trave elevada, os animais experimentais tiveram dificuldade em manter o equilíbrio e andar. No teste de marcha, os animais experimentais apresentaram alterações na marcha, que foram quantificadas por: (a) encurtamento do comprimento da passada, (b) diminuição da passada, (c) alteração da simetria da passada e (d) alteração da área da passada. Tais resultados são indicativos de esforços compensatórios e foram comparáveis entre os dois testes. Em conjunto, os dados indicam que o teste de marcha atende a todos os requisitos para avaliação da coordenação motora em roedores. O teste de marcha é, portanto, validado como um complementar para o teste da trave elevada e para o estudo e análise de comprometimento neurodegenerativo e outros distúrbios envolvendo distúrbios neuromusculares.

Palavras-chave: Atividade locomotora. Coordenação motora. Equilíbrio motor. Doenças neurodegenerativas. Ivermectina.

Correspondence to:
André Rinaldi Fukushima
Universidade de São Paulo, Faculdade Medicina Veterinária e Zootecnia, Departamento de Patologia Experimental e Comparada
Av. Prof. Orlando Marques de Paiva, 87
CEP: 05508-270, São Paulo – SP, Brazil
e-mail: fukushima@alumni.usp.br

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Introduction

Evaluation of locomotor activity in an animal model

Normal locomotion in mammals involves an organized sequence of coordinated events comprising the four limbs, posture, balance, the precision of ballistic movement, positioning, and support, which are accompanied by a complex neurological and musculoskeletal communication system. Thus, deficits in any aspect of the motor pathways can produce abnormalities in movements and behaviors, such as walking, predation, exploration, reproduction, copulation, and fight and flight, among others (Dunnett & Brooks, 2018).

In primary and preclinical research studies, animal movement and balance assessment are behavioral tools often used to describe the phenotype of a particular disease, the consequence of injury, or exposure to a xenobiotic (Wertman et al., 2019). Due to legal and ethical reasons, testing that utilizes animal models, especially rodents, provides a practical and effective alternative to testing in humans and other animal species, allowing investigation, analysis, and possible elucidation of underlying mechanisms. Such research may provide possible causes and therapies or pinpoint specific neural circuits responsible for the behavioral deficits characteristic of the disorders (Allbutt & Henderson, 2007).

The development of animal models and protocols for evaluating the various aspects of animal behavior needs to undergo a validation process to guarantee the quality of the analysis and the result's reliability (Fukushima et al., 2018). According to Fukushima et al. (2018), validating a model or a protocol is one of the basic requirements for quality control to ensure that the method used is adequate for identifying and quantifying a specific behavioral parameter.

Some behavioral analysis protocols can also evaluate drug pharmacokinetics (Zaccarelli-Magalhães et al., 2018). An example is the open field test that was developed in 1934 by Calvin Hall (1934), which has been used for many years since it allows the simultaneous analysis of several behaviors, including motor activity, environmental exploration, emotionality, and anxiety-like behavior, in diversified animal models. Zaccarelli-Magalhães et al. (2018) have shown that behavioral tests are valuable tools for evaluating corresponding changes in blood serum concentrations of drugs that act directly on motor coordination and animal activity. A decrease in analyzed behavioral parameters evidences the correlation with drug plasma concentration reduction.

Furthermore, several studies require evaluating balance and coordination among the locomotor aspects that can be analyzed. In rodents, especially with rat models, the single most used method for assessing such parameters is the elevated beam test, which consists of a simple apparatus that estimates the rat's ability to balance itself and move across a narrow, raised platform (Allbutt & Henderson, 2007). Despite being a low-tech and cost-efficient test that provides

reliable results on motor coordination, the high beam method requires several animal training sessions. During training, the animals learn to walk on the apparatus and get habituated to positive reinforcement, usually a palatable food reward for completing the task. Although simplistic, the test poses a disadvantage: not all experimental animals are trainable and capable of reaching the learning curve threshold needed to cross the beam after the standard training period (Moreira et al., 2017).

Another parameter of measurable locomotor characteristics is the physiological gait, which is directly related to balance and can be quickly evaluated. Adequate execution of gait depends on neurological integrity (central and peripheral proprioceptive systems, peripheral nerves and roots, extrapyramidal system, cerebellar afferent and efferent neurons, pyramidal system, including upper and lower motor neurons) and orthopedic structures (ligaments, tendons, joints, and skeletal striated muscle) (Pinto et al., 2015).

Changes in gait patterns are related to morphological and functional changes in the cerebellum, including those induced by damage to the nigrostriatal dopaminergic system (Yamamoto et al., 2019). Motor deficits are the main consequence of striatal dysfunction (Dunnett & Brooks, 2018). The basal ganglia (striatum: caudate nucleus and putamen; globus pallidus, subthalamic nucleus, and substantia nigra), broadly speaking, are also involved in gait control, mainly through their interaction with cortical motor areas, which are considered supplementary motor areas (Fernagut et al., 2002).

Gait analysis bestows quantifiable behavioral data correlated to a particular disease, injury, or drug exposure that can affect movement (Wertman et al., 2019). The study suggested evaluating neurodegenerative diseases with significant motor components (Pinto et al., 2015). Additionally, this evaluation is inexpensive, does not require animal training, can be completed in one day, and can easily be replicated.

Objectives

To demonstrate the effectiveness of the gait test for the evaluation of the locomotion and balance of rodents, as well as to establish the gait test as a complementary alternative for the elevated beam test.

Materials and Methods

Animals

Twenty-seven adult Wistar male rats, approximately 60 days old, were obtained from the Central Laboratory Animal Facility of the Institute of Biomedical Sciences of the University of São Paulo (ICB-USP) and used for the experiments.

The animals were housed in polypropylene cages (41 × 34 × 18 cm – 3 animal/box) with autoclaved shavings as bedding and kept in a room with a relative humidity of 55% ± 10, controlled temperature (22 °C ± 2 °C) and light/dark cycles of 12 h (light on at 6:00 am and off at 6:00 pm). The animals received filtered water and autoclaved food (Nuvilab®) *ad libitum* throughout the experimental procedure.

This investigation was approved by the Ethics Committee on the Use of Animals (CEUA) of the Faculty of Veterinary and Animal Science of the University of São Paulo (FMVZ-USP) under protocol number 6095071020.

Experimental design

The 27 adult Wistar male rats were divided into three groups: 1 control group (Cont), which received saline solution (NaCl 0.9%) intraperitoneally (IP), and two experimental groups (IV 0. 2 and IV 1. 0 – n=9 animals/group), which received a single dose of 0.2 or 1.0 mg/kg of commercially available ivermectin solution (Ivomec® injectable) via IP. The ivermectin dose of 0.2 mg/kg is most used in treatments. In comparison, the amount of 2.0 mg/kg was chosen based on experimental results obtained by Bernardi et al. (2011), which was shown to cause impairment in rodent behavior 15 min after administration. According to Chiu et al. (1990), the half-life of ivermectin in rats is 24 to 48 hours. Thus, behavioral evaluations were performed 15 min and 24 h after pharmacological administration.

The injectable Ivomec® (1% ivermectin – Merial Saúde Animal Ltda., Paulínia/SP) was prepared following the protocol of Moreira et al. (2017). One drop of Tween 80 was added for every 5 ml of 0.9% NaCl, which was gradually mixed with the drug to obtain the final concentrations of 0.2 and 1.0 mg/ml of ivermectin.

Methods

Elevated beam test

This test was performed on a raised wooden beam (Figure 1) 2 m long and 18 mm wide, with a platform of 10 cm^2 at each end painted white (platforms A and E); the beam was supported at 3 points that were 20 cm from the surface, two under each platform (A and E) and one at the center (point C). 0.5m from each platform – towards the center of the beam – two marks delineated a region of 1 m in length (between points B and D), where the performance of each animal was evaluated in the test (Moreira et al., 2017; Rodrigues-Alves, 2007).

To evaluate motor coordination at the elevated beam test, the protocol described by Moreira et al. (2017) was

implemented. Initially, the animals underwent conditioning training to learn to walk on the raised beam. Each animal was placed individually for 5 min on the beam for 10 consecutive days, and this period enabled the rats to get acquainted with the apparatus before the test day. Training included palatable food for positive reinforcement (cooked corn kernels) placed at each platform. On the first training day, each rat was placed on platform A, and a corn kernel was placed on the opposite platform (E). On the second training day, the rat was placed on marked position B on the beam, facing platform A, and a cork kernel was placed on platform A. This procedure was repeated for 5 min. During the following days, the rat was placed on the beam at incremental distances from the platform with positive reinforcement until it could cross the raised beam and reach each opposing platform (E). Once the animal learned to cross from platform A to platform E, the same procedure was repeated so that it would learn to perform the reverse path and become enabled to travel from one platform to another within 5 minutes.

The animals were considered trained when, in seven days, they could make two complete crossings (A ↔ E) without stopping within 5 min; the others were excluded from the experiment. The rats continued to be trained between the 7th and 10th day, and on the 11th day, the test was performed (Moreira et al., 2017).

The test consisted of evaluating the performance of the animals at the central part of the beam from the attribution of scores, according to Table 1. Thus, when the rat walked in the central area of the beam (between points B to D), a score was assigned for each step given with the pelvic limb facing the observer. At the end of the test, the scores obtained by each animal were added for two completed crossings (Moreira et al., 2017).

Gait test

The gait test was performed to evaluate the motor coordination and balance of the animal, through the direct recording of its gait (Figure 2), from the footprints left by the paws of the pelvic limbs, as proposed by Pinto et al. (2015) and Dunnett & Brooks (2018). The procedure utilized a flat horizontal platform that was 60 cm long and 10 cm wide, with walls of 15 cm on each side. The platform floor was lined with white filter paper, as illustrated in Figure 3.

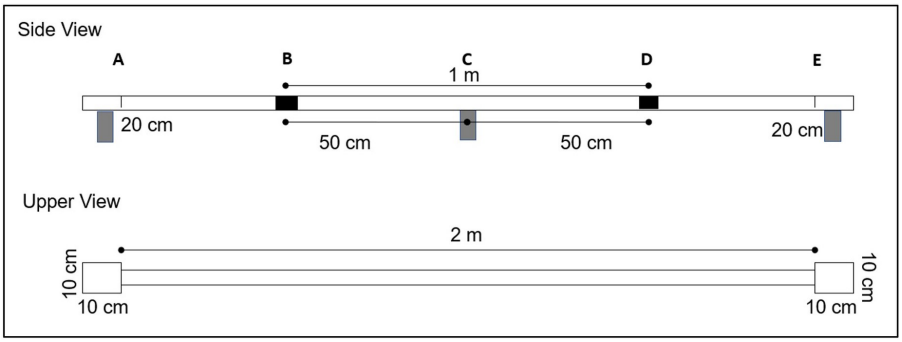

Figure 1 – Schematic drawing of the raised beam. Source: Adapted from Udo (2021).

Figure 2 – Direct detection of rats' footprints obtained by the gait test.

Table 1 – Scores attributed to mouse behavior in the central part of the high beam

Score	Behavior
0	The animal keeps the hind paw fully resting on the beam surface.
1	The animal supports part of the paw on the surface of the beam so as not to exceed the lower edge.
2	The animal slips, and the paw overtakes the lower edge of the beam.

Source: Rodrigues-Alves (2007).

For gait analysis, each animal had the plantar pads of the pelvic limbs stamped with black paint; then, the animal was placed to walk in the apparatus. Based on the footprints left by the plantar pads on the apparatus floor, we have obtained the parameters illustrated in Figure 4, which were evaluated and described in Table 2, according to Jacobs et al. (2014) and Yamamoto et al. (2019). The parameters were quantified with the AutoCAD® software for better standardization and accuracy of the measurements.

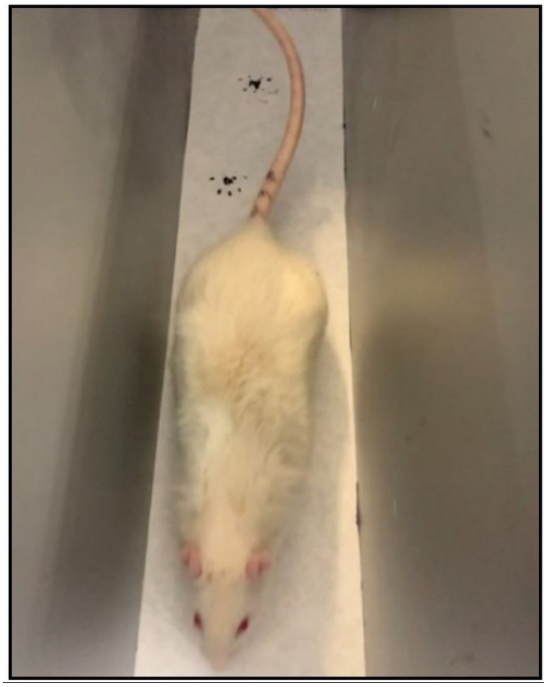

Figure 3 – Picture of a rat inside the gait test apparatus. Notice the footprints stamped on the platform. Source: Pantaleon (2021).

Statistical analyses

Statistical analysis was performed utilizing GraphPad Prism 6® *software* (GraphPad Software, Inc., San Diego, CA, USA). The Bartlett test was used to verify the homoscedasticity of the data. Parametric data were utilized as one-way ANOVA, followed by Dunnett's post-test. Results were considered significant when $p<0.05$.

Results

Figure 5 shows the scores obtained on the raised beam, performed 15 min and 24 h after ivermectin administration. One-Way ANOVA showed an increase in the sum of scores in both experimental groups about the control group for both times of measurements, 15 min ($F(2.17)=8.474$, $^{*}p<0.05$, $^{**}p<0.01$) and 24 h ($F(2.16)=8.543$, $^{*}p<0.05$, $^{**}p<0.01$) after exposure.

Figure 6 shows the results of the parameters analyzed from the gait test performed 15 min after the administration of ivermectin. One-way ANOVA showed a decrease in step length ($F(2.13)=6.452$, $^{*}p<0.05$) and symmetry ($F(2.12)=8.801$, $^{*}p<0.05$, $^{**}p<0.01$) in both experimental groups and an increase in the stride area ($F(2.12)=3.822$, $^{*}p<0.05$) for the group exposed to a dose of 0.2 mg/kg about the control group. One-Way ANOVA showed no significant difference between the experimental groups and control for the stride length ($F(2.13)=1.070$), for the pitch angle ($F(2.13)=0.4701$) and the distance between the two feet ($F(2.13)=0.3897$) ($p>0.05$).

Figure 7 shows the results of the parameters analyzed from the gait test performed 24 h after ivermectin administration. One-way ANOVA showed a decrease in

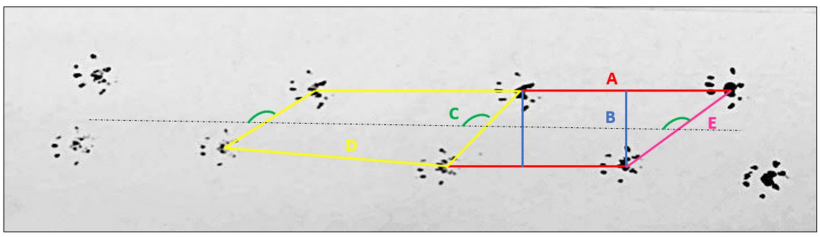

Figure 4 – Gait test parameters evaluated in rats: (A) stride length (red line); (B) step length (blue line); (C) pitch angle (yellow ram); (D) area of the stride (quad of red and blue lines); (E) distance between feet (magenta line). Source: Pantaleon (2021).

Table 2 – Description of the parameters analyzed in the gait test

Parameter	Description
Stride length	Distance between two footprints of the same paw (Figure 5A; in red)
Step length	Width between right and left limbs (Figure 5B; in blue)
Symmetry	Step length (Figure 5B) divided by stride length (Figure 5A). A symmetrical step should be equal to 0.5
Pitch angle	The angle of a footprint relative to the center of the step (Figure 5C; in green)
Stride area	The trapezoid area formed every two steps (Figure 5D; in yellow)
Distance between feet	Distance between the two feet in the same step (Figure 5E; in magenta)

Source: Pantaleon (2021).

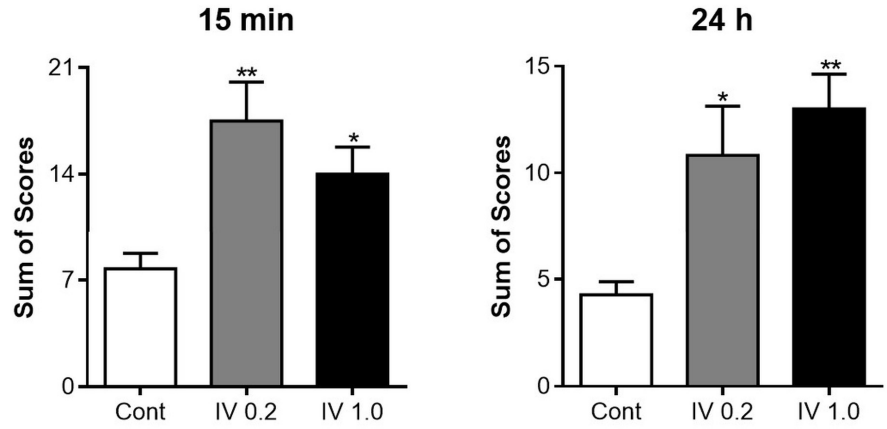

Figure 5 – Sum of scores obtained on the elevated beam test performed in adult male rats 15 min and 24 h after administration of ivermectin (IV - 0.2 or 1.0 mg/kg) or saline solution (Control - Cont) intraperitoneally. The means and their standard errors are displayed. N=6-8 animals per group. One-Way Anova.

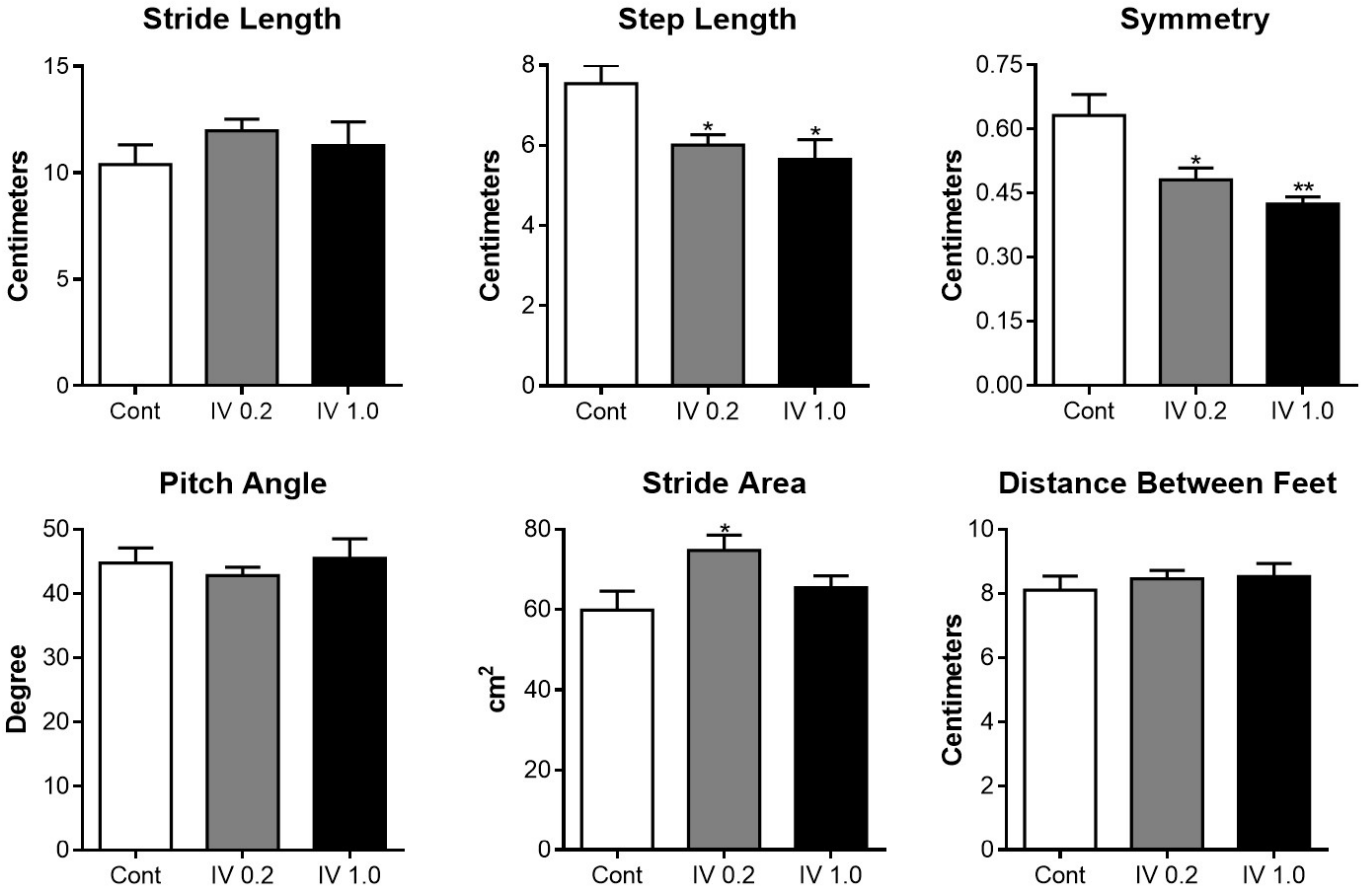

Figure 6 – Gait test parameters performed in adult male rats 15 min after administration of ivermectin (IV – 0.2 or 1.0 mg/kg) or saline solution (Control – Cont) intraperitoneal. The means and their standard errors are displayed. N=4-7 animals per group. One-Way Anova.

stride length (F(2.21)=10.98, ***p<0.001) and symmetry (F(2.19)=6.977, **p<0.01) in the group exposed to the dose 1.0 mg/kg of ivermectin and a decrease in the stride area (F(2.21)=7.407, *p<0.05, **p<0.01) in both experimental groups concerning the control group. One-Way ANOVA showed no significant difference between the experimental groups and control for the length of the step (F(2.22)=0.3824), the pitch angle (F(2.21)=1.578), or the distance between the two feet (F(2.22)=1,800) (p>0.05).

Discussion

The present work evaluated the feasibility of the physiological gait test for partial and complementary substitution of the elevated beam test. The high beam test

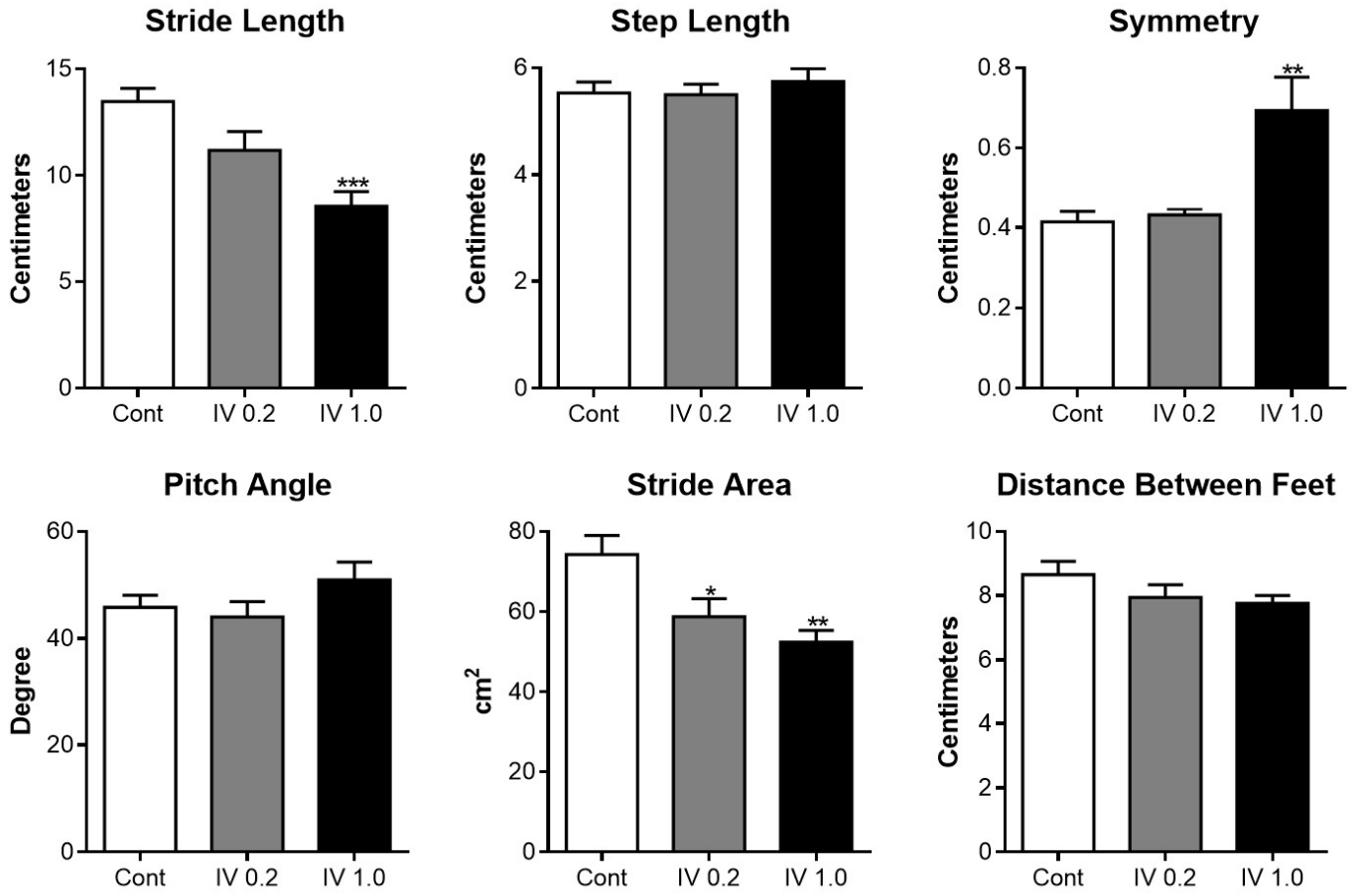

Figure 7 – Gait test parameters performed in adult male rats 24 h after administration of ivermectin (IV - 0.2 or 1.0 mg/kg) or saline solution (Control - Cont) intraperitoneal. The means and their standard errors are displayed. N=6-9 animals per group. One-Way Anova.

has been a prototype for many years to determine proper locomotion and balance in rodents. However, it requires animal training; not all animals can perform this test. On the other hand, the gait test allowed direct observation of the behavior exhibited by the animals via measurements of spontaneous locomotion that did not require training, proving its usefulness as a practical, effective, and reproducible test.

Measurements of behavior comparisons were determined by disrupting regular coordination with the use of ivermectin (0.2 and 1.0 mg/kg) at two time points after administration: (1) 15 min and (2) 24 h. Both the elevated beam test and the gait test verified that the administration of ivermectin impaired the motor coordination of the animals. On the high beam, this effect was observed with both doses of ivermectin after 15 min of administration and remaining until 24 h.

In a similar investigation employing disruption of motor coordination by Rodrigues-Alves et al. (2009), the group used moxidectin, another anthelmintic drug of the milbemycin class with a mechanism of action that is similar to ivermectin. Moxidectin treatment at doses of 0.2, 2.0, and 20 mg/kg impaired the motor coordination of rats after 24 and 72 h. The authors indicated that this effect was due to the drug's action in the central GABAergic system, mainly in $GABA_B$ receptors, corroborating the appropriateness of utilizing GABA agonists to attenuate rodent motor coordination.

In the present investigation, a high sum of scores obtained by an animal demonstrated difficulty in maintaining and walking on the beam and a more significant impairment in its balance. This deficit was observed and significant at 15 min of administration of ivermectin and remained until 24 h post-administration.

In the gait test, after 15 min of administration, it was possible to observe a decrease in step length and symmetry in both experimental groups and an increase in the stride area of the group exposed to a dose of 0.2 mg/kg. After 24 h, gait changes were still evidenced by decreased stride length and symmetry in the group exposed to a dose of 1.0 mg/kg of ivermectin and decreased stride area in both experimental groups.

A decrease or increase of the parameters analyzed in gait demonstrates an imbalance of the animal and compensatory walking behavior. Motor deficits are characteristic consequences of striatal dysfunction (Dunnett & Brooks,

2018); changes in gait patterns are related to morphological and functional changes in the cerebellum, including those induced by damage to the nigrostriatal dopaminergic system (Wu & Hallett, 2013).

The striated body is part of the core at the base of the brain and participates in the control of voluntary movements (Hikosaka et al., 2000), such as gait, mainly through its interaction with cortical motor areas, i.e., supplementary motor area. Interruption of such a system may result in gait disorders, such as Parkinson's disease, Striated, subcortical, and frontal cortex lesions, or degenerative processes (Elble et al., 1996; Hanakawa et al., 1999; Hausdorff et al., 1998).

Another neurotransmitter, dopamine, controls motor activity (Bear et al., 2008). Dopamine is a catecholamine involved in reward mechanisms, emotions, and cognitive and endocrine functions (Bear et al., 2008. Dopaminergic neurons are found mainly in the substantia *nigra* of the mesencephalon that protrudes into the striatum, the so-called nigrostriatal pathway, which is essential for the control of involuntary motor zones (Machado, 2007; Schultz, 2007). The degeneration of dopaminergic neurons in the *substantia nigra* is the leading cause of the development of neurodegenerative motor diseases, such as Parkinson's, whose underlying mechanisms have been described in detail by Pantaleon et al. (2021). Based on this knowledge, several authors have evidenced changes in gait patterns with changes in striatal neurochemistry, especially in the dopaminergic system. Fernagut et al. (2002) state that slowness, the so-called hypokinesia of gait, evidenced by a reduction in the stride length of the posterior limb, is a characteristic of diseases that affect the basal ganglia and this reduction is correlated with the magnitude of the loss of cells of the striated body. Therefore, the stride length is a simple method to obtain an index of motor disorders due to rodent dysfunction of the basal ganglia.

Conclusions

Altogether, the data obtained in the present study demonstrated that the parameters analyzed by the gait test provided information regarding rodents' balance and motor coordination. Moreover, due to its low cost, easiness of execution, fast results of performance, replicability, and the fact that it does not require habituation or previous training of animals, the gait test proved to be a viable complementary option for replacement of the high elevated test. Thus, in this study, we can conclude that it is a valid test for analyzing models with specific neurodegenerative impairment and other motor disorders.

Conflict of Interest

We, the authors of this article, declare that there are no conflicts of interest regarding the research conducted and the content pre.sented herein. We affirm our commitment to upholding the highest standards of scientific integrity and transparency.

Ethics Statement

We declare that the work entitled " Physiological gait test: an effective method for analyzing balance, locomotion, and neuromuscular disorders in rats and a comparison to the elevated beam test" has been submitted to the Animal Ethics Committee and has been approved under the CEUA protocol number 6095071020 (ID 008679).

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