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FUNCTIONAL EVALUATION OF GAIT IN WISTAR RATS AFTER DEMYELINIZATION WITH ETHIDIUM BROMIDE IN SUPPLEMENTATION OF VITAMIN D

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Abstract:

Introduction: *In the world, the glycotoxic model has been used through the use of ethidium bromide (BE) which involves demyelination with the process of remyelination in the central nervous system (CNS). This experimental method helps to detail the events involved in responding to the finding of remyelination in diseases such as Multiple Sclerosis.*

Objective: *This study aims to assess whether Vitamin D has the potential to alter the gait movement of demyelinated rats with ethidium bromide (BE) through experimental glycotoxic models with the use of ethidium bromide (BE) in order to conjugate knowledge about nerve regeneration and the development of strategies that promote the recovery of nervous tissue.*

Results: *The treatment group with 100.000 IU/week demonstrated a significant difference in gait on hind legs compared to the positive control group in specificities in CPMO and APMA.*

Conclusion: *It was possible to concluded in this study that the treatment with Vitamin D supplementation in rats with lesions in the central nervous system showed a faster functional performance and with a greater grip evidenced in gait in high doses of Vitamin D supplementation, in this case, equivalent to 50.000 IU/week to 100.000 IU/week.*

Keywords: *Ethidium bromide, Multiple Sclerosis, demyelination, Vitamin D, gait.*

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INTRODUCTION:

Constantly the improvement in human knowledge in the technology inherent to the treatment of injuries in the Central Nervous System (SNC) [16], encourages the search of thousands of researchers to answer in an illuminating way related to the new perspectives regarding the satisfactory recovery of the patient in primary demyelinating diseases such as sclerosis multiple [16, 28], These responses are being brought up by researchers through the remyelination process and involve the generation of a new myelin sheath. For example, the use of ethidium bromide in the Central Nervous System is used in some studies in order to carry out the process of demyelination by chemical actions [28], where as remyelination can occurs through a new generation of cells known as oligodendrocytes that are generated due to demyelination with ethidium bromide which simulates primary demyelinating diseases such as multiple sclerosis and this process occurs from multipotent parents known as OLG progenitor cells (OPCs) that will act by actions in response to the ethidium bromide demyelination process in order to divide, migrate and differentiate mature oligodendrocytes [16,28]. Therefore, several studies have used the glycotoxic model with the use of ethidium bromide (BE) [28]. These studies involved pathophysiology in the process of demyelination and remyelination, mainly of the Central Nervous System (CNS) in rats, and so using this method helps in the detailed understanding of the cellular events involved as well as in the response to the finding of remyelination in primary demyelinating diseases such as Sclerosis Multiple (EM) in humans [28].

Ethidium bromide (3.8 diamino-5-ethyl-6-phenyl-phenanthridine) when injected into the Central Nervous System is able to induce primary demyelination and cause local loss of oligodendrocytes and also determines the focal disappearance of astrocytes [28]. The disappearance of astrocytes allows invasion of pial cells and lymphocytes to the neuropile as well as Schwann cells from the Peripheral Nervous System and, thus, has the ability to express its remyelinating potential in the Central Nervous System [28].

The prohormone known as Vitamin D plays an important role not only in bone development, but also in other systems of the human body [06, 10,24]. Currently, Vitamin D deficits have been common in patients with primary demyelinating diseases such as multiple sclerosis [22, 23, 28]. With neuroimmune illness and skin aging, the ability to synthesize Vitamin D decreases significantly. Maclaughlin and Holick (2011) described that the skin's ability to synthesize Vitamin D is reduced by more than 50% [20].

However, neuroimmune disease effects intestinal absorption from the mouth to the anus. In addition, Vitamin D supplementation is used to complement the treatment of Multiple Sclerosis [20, 22], who has beneficial effects on the progression of autoimmune diseases [06]. The use of Vitamin D in the form of weekly supplementation seeks to contribute to reducing the inflammation of outbreaks caused by the disease and prevents the worsening of autoimmune diseases, who has a regulatory effect on innate immunity and is generally associated with the multifaceted regulation of acquired immunity [02, 23]. However, hydroxylation at position C-25 in the liver is not affected by the cellular process in the illness and the hydroxylation capacity at position C-1 is reduced by the functional limitation mainly related to the aging of the kidneys, and therefore presents a lesser response to stimulation of CYP27B1 parathyroid hormone [07, 23].

It is due to this hydroxylation process, that 25 (OH) D is considered normal with a blood dose of 30 ng/dl in patients with Multiple Sclerosis [20-23]. Due to the worsening of the disease, a longitudinal decline in the levels of 25 (OH) D occurs [20]. As a result of such

evidence presented, Vitamin D has been the target of numerous and growing studies in the last decade, which includes its interaction in the neuroimmune system, because the existence of Vitamin D receptor expression in a wide variety of tissues in the body, mainly the brain and spinal cord [20,22, 25]. These studies have performed the relationship between Vitamin D deficit and several autoimmune diseases [20,23]. This group includes insulin-dependent diabetes mellitus (IDDM), multiple sclerosis (MS) and systemic lupus erythematosus (LES) and rheumatoid arthritis (RA) [20-23].

Thus, it is suggested that Vitamin D is an extrinsic factor capable of affecting the prevalence of autoimmune diseases [08], since Vitamin D is able to interact with the immune system through actions on the control in the production of cells such as lymphocytes, macrophages and natural killer cells (NK) and also interferes with the production of cytokines in vivo. This interaction in the immune system causes Vitamin D to exert its biological action on nervous tissue through connections to its receptors known as VDR [18, 21,23].

Thus, several cells of the immune system and mainly the nervous one have Vitamin D receptors (VDR) [18,23]. Thus, when stimulated, this receptor will generate a chemical signal that is able to influence the expression of the gene within the cell and, therefore, it is able to cause the cell to end up acting through the division and supplementation of Vitamin D [25], being fundamental to improve the general clinical condition mainly in improving the gait of patients with Multiple Sclerosis [13, 16]. In addition to bringing benefits in neurological terms [21].

In addition, the genetic influence on Multiple Sclerosis is related to Vitamin D, in addition, previous experimental studies have shown that when the immune system cell is exposed to Vitamin D, there is a decrease in the inflammatory capacity of these cells [10-13]. In view of the above, this manuscript aims to assess whether Vitamin D has the potential to alter the gait movement of demyelinated rats with ethidium bromide (BE) using glycotoxic experimental models with the use of ethidium bromide (BE) in order to combine knowledge about nerve regeneration and the development of strategies that promote the recovery of nervous tissue.

METHODOLOGY:

Male Wistar lineage rats were used, weighing approximately 290 - 358 g with eight weeks of life from the Central Animal Hospital at the Federal University in Mato Grosso do Sul in the area of Biological Health Sciences, Campo Grande, MS. The animals were kept in a closed room with controlled temperature. Artificial lighting with 12-hour photo period and with Nuvilab feed intake. All experimental procedure were carried out in line with ethical principles in animal experimentation. This research was submitted and approved by the Ethics Committee on Animal Experimentation - CEUA / UFMS, under protocol number 993/2018.

The sample used was composed of 70 adult Wistar rats. The animals were divided into seven groups: (A) negative control group (n = 10), (B) positive control group (n = 10), (C) Vitamin D supplementation group with 10.000 IU/week (n = 09), (D) Vitamin D supplementation group with 20.000 IU/week (n = 09), (E) Vitamin D supplementation group with 40.000 IU/week (n = 10), (F) Vitamin D supplementation group with 50.000 IU/week (n = 10), (G) Vitamin D supplementation group 100.000 IU/week (n = 12). Groups A, C, D, E, F and G were demyelinated with ethidium bromide in the brain stem according to the method used by Dominguita et al (2011), with injection of 10 µl of 0.1% ethidium bromide in the cistern baseline of adult Wistar rats. Group A and B animals were treated with vehicle only (0.2 ml saline). Vitamin doses were administered orally weekly. The animals were kept in the conditions mentioned above. Animals affected in gait were separated after evaluation on the seventh day of those not affected. They were submitted to gait analysis during the treatment period with Vitamin D, in this case, fourteen days, twenty-one, forty-two, sixty-nine and eighty-three days after the demyelination procedure with injected ethidium bromide. The animals were encouraged to go through an elaborate space 80 cm long and 0.10 m wide and high. The parameters used in this study to characterize gait were: stride length of the forelimb (CPMA), stride height with forelimb (APMA) [16], stride length of the posterior limb (CPMP) and stride height of the posterior limb (APMP) [16]. The test to perform the functional gait assessment was performed weekly on each rat. From the seventh day of post-demyelination with ethidium bromide through the functional gait index (MFI), where this method was used in order to more accurately assess quantitative information when using a formula which takes measurements into account made on the impressions of the rats used in the study and when walking in the wooden corridor with squared paper.

A paper tape with the same size as the corridor used for the rat to walk was used and this tape favored the

rats to keep the paper in situ during the period of analysis of the rats' gait and thus avoided any movement or folding that might change the footprint measurement [21]. The rats were held by the trunk and by the tail and thus, a second researcher painted the back legs with the use of a soft brush, with a mixture of Indian ink and two measures of non-toxic school glue, which allowed the ink to have a higher density and adherence in the plantar region of the rat. The time determined to stimulate the rats to go through the elaborated space was the total time to perform the test 5 minutes [16].

Table 01: Distribution of animals according to the Vitamin D supplementation dose.

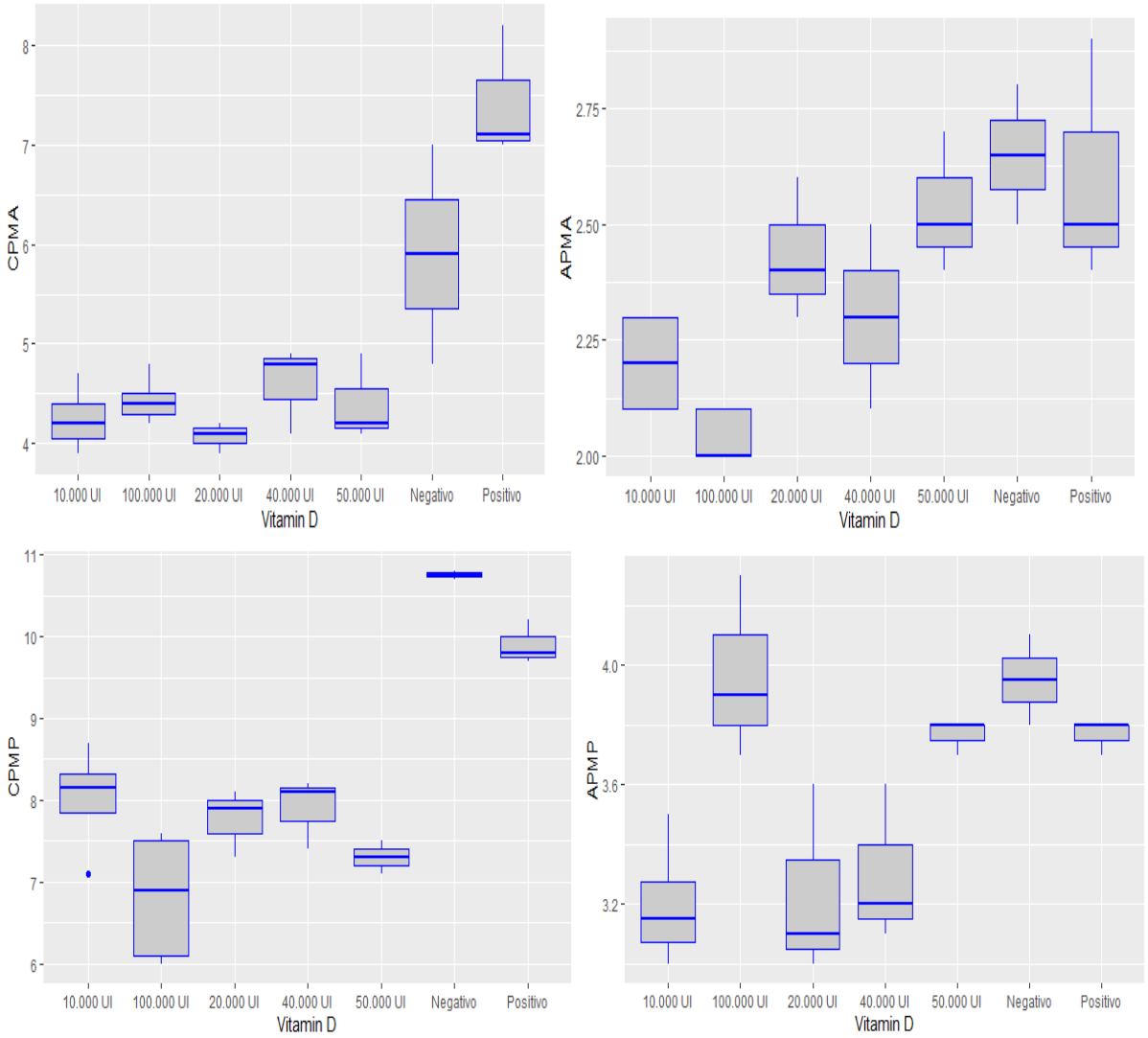
ANIMALS DISTRIBUTION				
Grups	**n -total animals used in the study (n = 70)**	**DOSE**	**Affected**	**Not Affected**
Negative Control	10	**Negative Control**	2	08
Positive Control	10	**Positive Control**	3	07
Grup C	09	**10.000 UI**	4	05
Grup D	09	**20.000 UI**	3	06
Grup E	10	**40.000 UI**	3	07
Grup F	10	**50.000 UI**	3	07
Grup G	12	**100.000 UI**	5	07
TOTAL ANIMALS			**23**	**47**

Statistical analysis:

The collected data were demonstrated through descriptive statistics and still, the analysis of variance (ANOVA) with post-hoc was used through the Fisher´s test [21]. All statistical analysis was performed with the R-Studio for Windows program to determine the functional gait index (MFI) with 07, 14, 21, 42, 69 and 83 in all calculations, a critical level of 5% ($p < 0.05$).

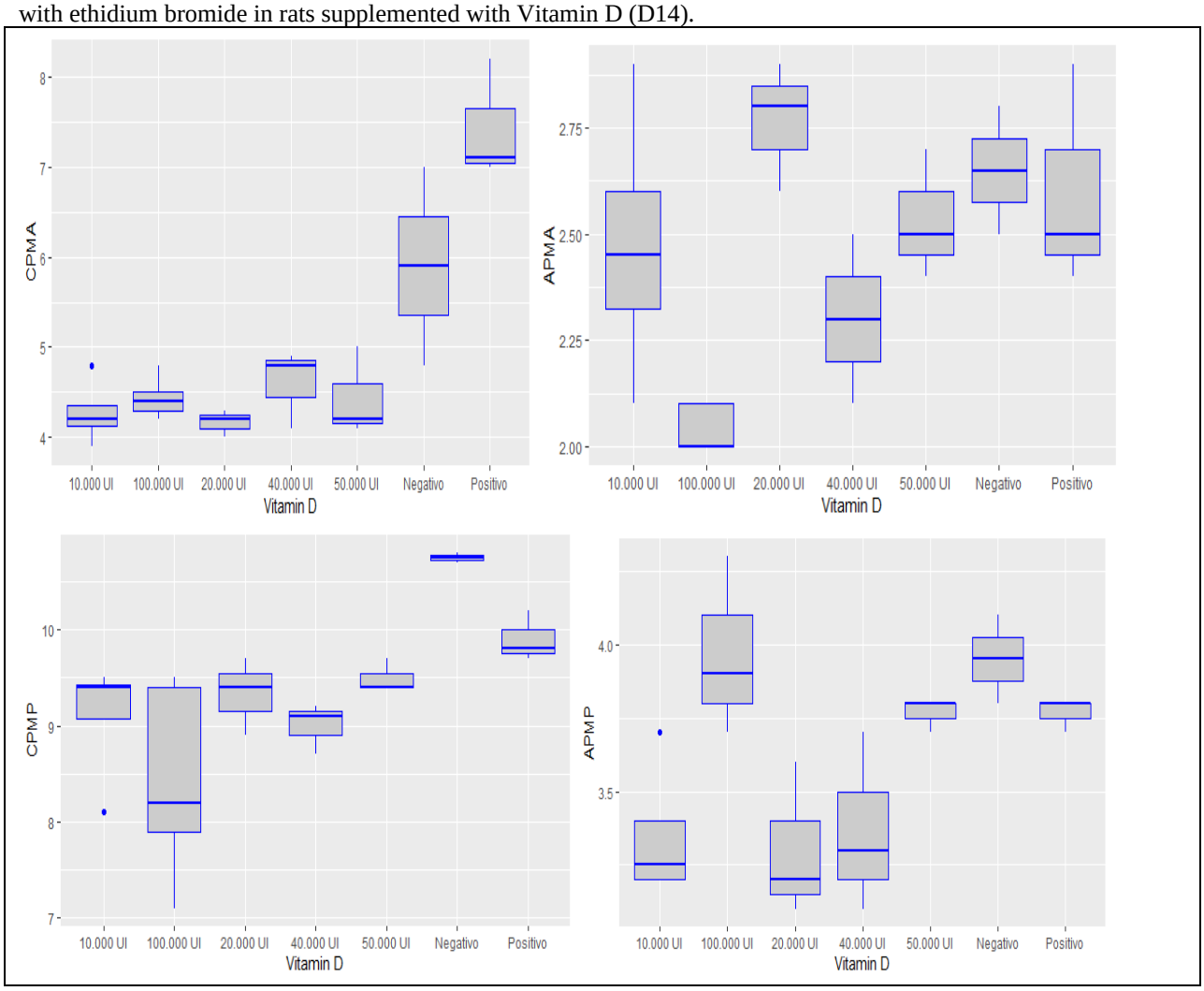
RESULTS:

Figure 01: Box-Plot of the indicator in relation to the Functional March Index (MFI) after seven days of demyelination with ethidium bromide in rats supplemented with Vitamin D (D07).



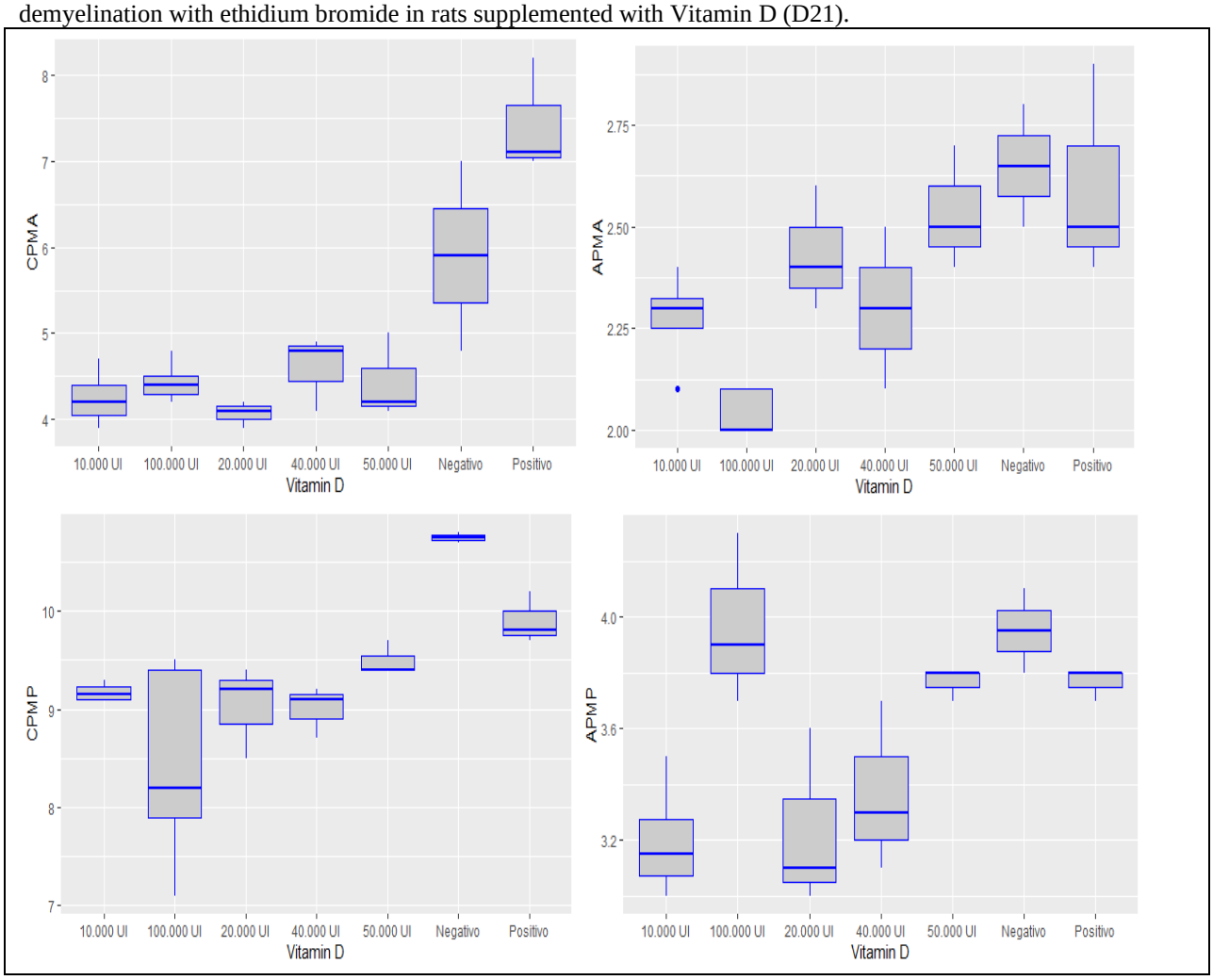
Analyzing the graph in figure 01, it is possible to notice that the supplementation of Vitamin D after seven days of demyelination with ethidium bromide, it is possible to verify changes in the length of the legs in the posterior paws when printing the area and, consequently, a decrease in the stride length, and the support base in the gait of Wistar rats. The animals in the groups received average levels of different doses administered during the treatment period. Higher dose rats showed decreasing responses to CPMA, APMA, CPMP and APMP indicators.

Figure 02: Box-Plot of the indicator in relation to the Gait Functional Index (MFI) after fourteen days of demyelination with ethidium bromide in rats supplemented with Vitamin D (D14).



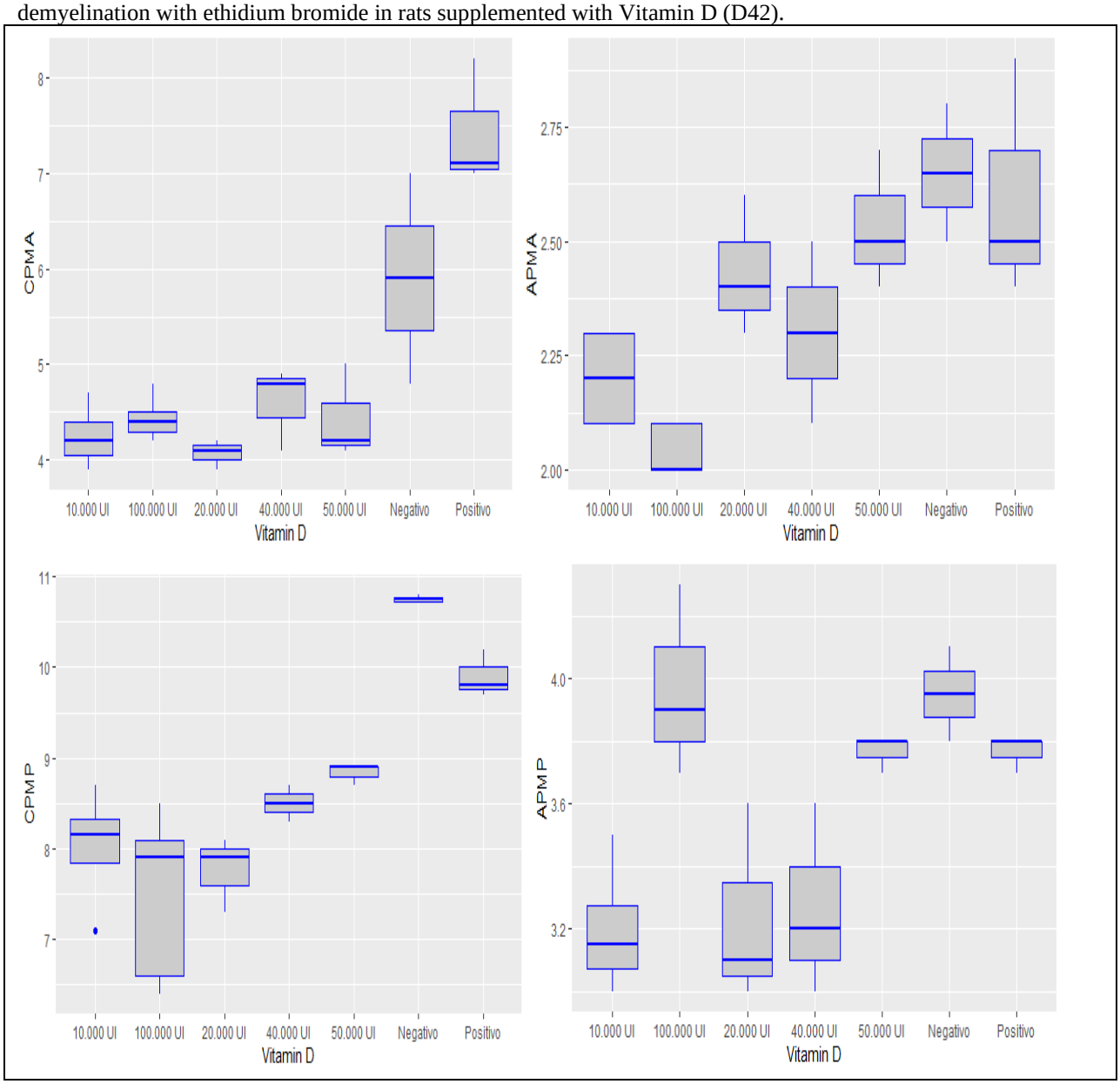
In figure 02, we provide a series of Box-Plot with indicator for functional gait indexes (MFI) after fourteen days of demyelination with ethidium bromide in Wistar rats that supplemented Vitamin D with variable doses. These graphs showed the relative use of Vitamin D doses in experimental groups with rats. What is remarkable about Fig. 02 is the complexity of the response in relation to the variability of doses used in treatment groups, particularly those focused exclusively on higher doses compared to those using lower doses. Therefore, the result suggests that the experiment in higher doses brings positive responses on all topics related to the change in the Functional Gait Index (MFI) after fourteen days of Vitamin D supplementation.

Figure 03: Box-Plot of the indicator in relation to the Gait Functional Index (MFI) after twenty-one days of demyelination with ethidium bromide in rats supplemented with Vitamin D (D21).



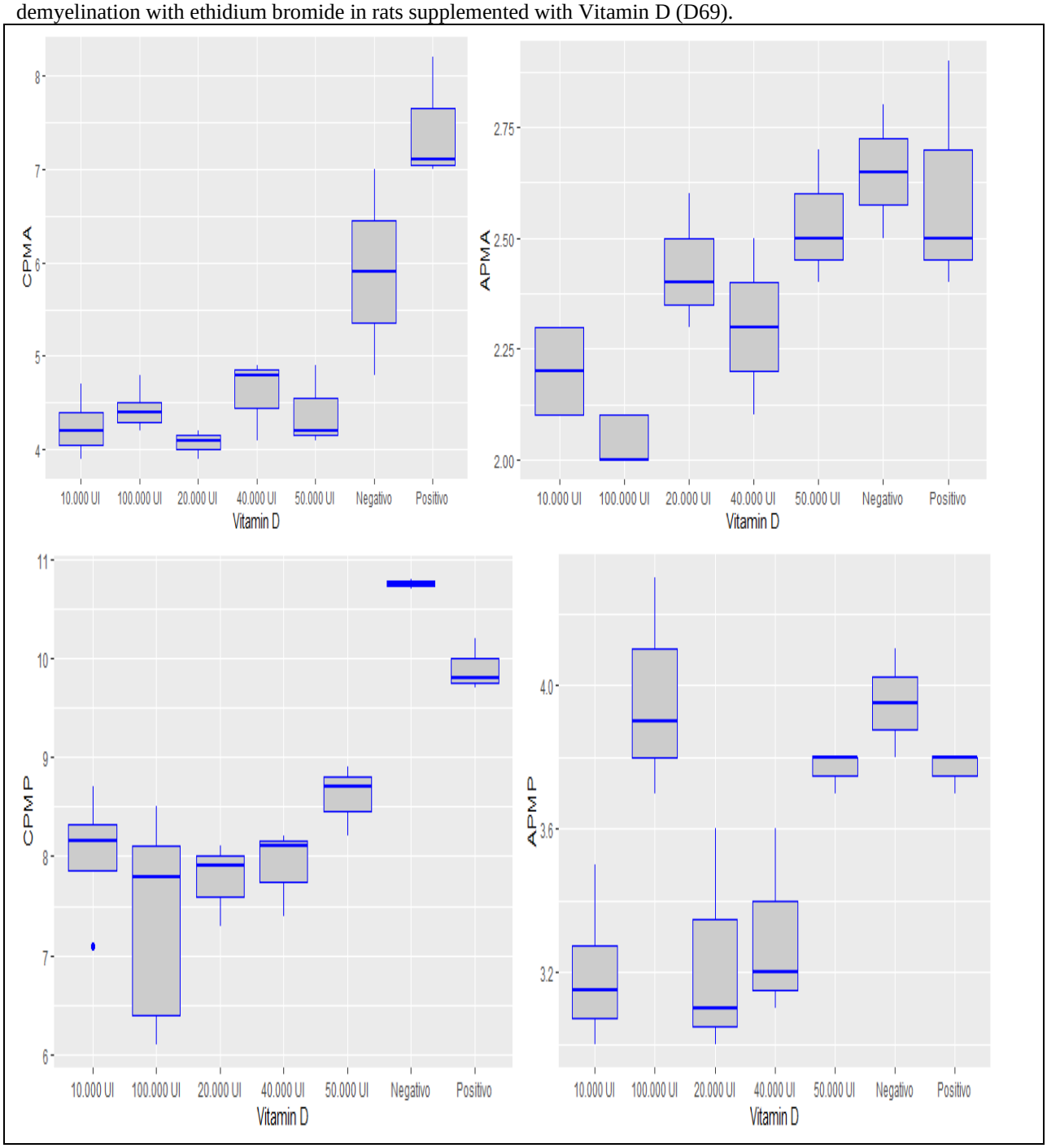
In figure 03, the Functional Gait Index (MFI) demonstrated that supplementing Vitamin D with higher doses with a time of twenty days is associated with the recovery process of the Functional Gait Index (MFI) ($p = 0.001406$), mainly APMP and CPMP indicator.

Figure 04: Box-Plot of the indicator in relation to the Functional March Index (MFI) after forty-two days of demyelination with ethidium bromide in rats supplemented with Vitamin D (D42).



In figure 04, the Box-Plot in relation to the functional gait index (MFI) after forty-two days of demyelination with ethidium bromide in rats with Vitamin D supplementation, there was a significant correlation between the CPMA, APMA, CPMP and APMP indicators ($p= 0.008857$) and the dose used in the treatment period of the experimental groups.

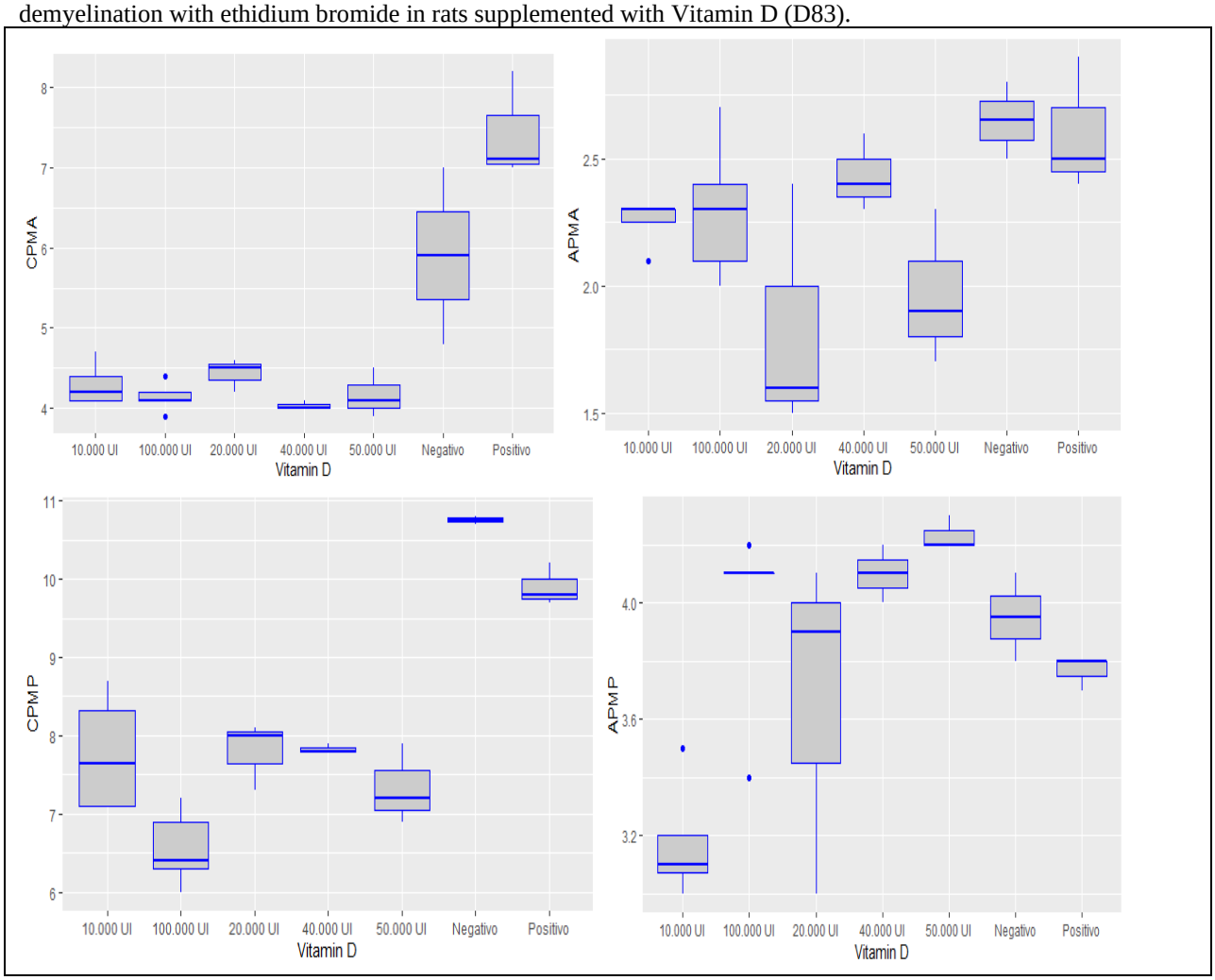
Figure 05: Box-Plot of the indicator in relation to the Gait Functional Index (MFI) after sixty-nine days of demyelination with ethidium bromide in rats supplemented with Vitamin D (D69).



In figure 05, the effect of Vitamin D administration with higher doses on the treatment time showed decreasing results, as responses to the APMA and CPMP indicators. For these parameters, the groups with higher doses showed a better result compared to the positive control group ($p = 0.0003767$). Regarding the Functional March Index (MFI), the groups with 100.000 IU/week, 50.000 IU/week and 40.000 IU/week showed results with a greater statistical difference in relation to the treatment group with doses equal to 10.000 IU/week and 20.000 IU/week. Week after one-way

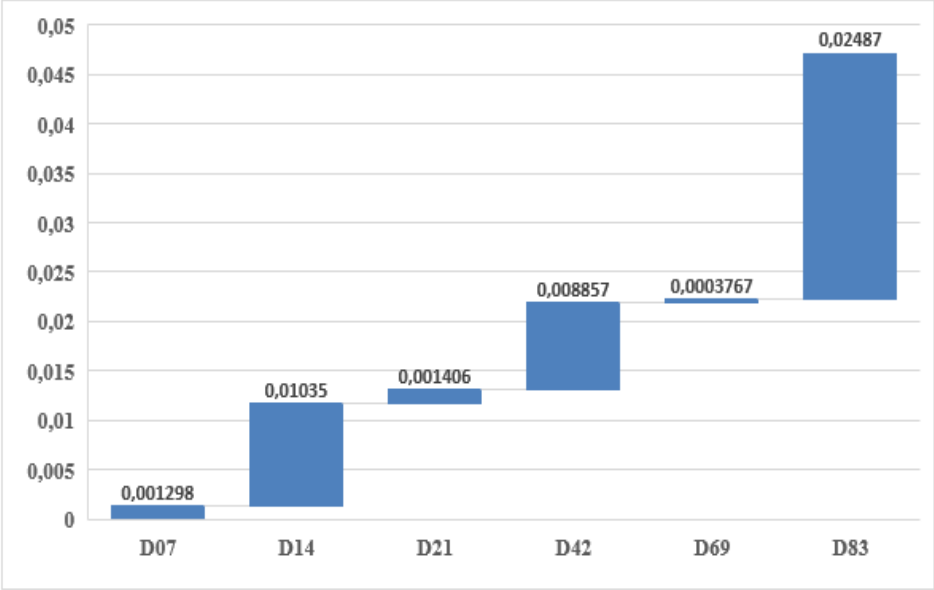
ANOVA tests after Fisher's test and supplemented with Vitamin D (p = 0.0003767), as the rats in the groups with the highest dose showed changes in the footprint and support base, that is, it is significant (p <0.05).

Figure 06: Box-Plot of the indicator in relation to the Gait Functional Index (MFI) after eighty-three days of demyelination with ethidium bromide in rats supplemented with Vitamin D (D83).



In figure 06, analyzes with ANOVA test of variable pathways in the doses of Vitamin D revealed the effect of time (days of treatment) on the Functional Gait Index (MFI) with (p = 0.02487) on measures of alteration of the Functional Index (MFI). It is possible to verify that the 100.000 IU/week group showed a significant difference in relation to the positive control group in CPMP and APMA (p = 0.02487).

Graph 01: p-Value for Functional Gait Index (MFI) over 83 days of experimentation with Rats Wistar.



The evaluation with ANOVA test indicated a significant effect of Vitamin D supplementation on gait behavior specifically in the Functional Gait Index (MFI), in this case, in D42, D69 and D83 ($p < 0.05$). Tests between the effect of Vitamin D supplementation indicated that the group with doses from 100.000 IU/week to 40.000 IU/week exhibited better response to gait indicators in CPMP and APMP ($p < 0.05$). Thus, there was a difference in the results compared in D07 and D83 and over 83 days of treatment there was a significant difference ($p < 0.05$).

DISCUSSION:

Since the Functional Gait Index (MFI) is derived from printed footprints of rats, its value is finalized mainly due to the intrinsic muscles of the paw [21]. The results of the gait analysis in this study indicated a significant functional improvement in the treatment groups with a high dose of Vitamin D supplementation with a value of 40.000 IU/week to 100.000 IU/week in the period of eighty-three days [21]. At 21 days, an improvement in gait was observed in both treatment groups, especially animals with supplementation of 50.000 IU/week and 100.000 IU/week in the CPMA and CPMP indicators, which was significant ($p = 0.001406$) [07-10]. This result corroborates the results of other researches such as the studies by Lacopetta et al (2018), which proves the action of Vitamin D, for example, in the normal neural function that depends on the absorption and activation of Vitamin D, which portrays a potent antioxidant activity, for reducing lipid peroxidation and capable of increasing enzymes and protecting against oxidation, as oxidative stress generally plays a cellular role capable of causing brain damage that can contribute to cognitive and motor dysfunction in neurodegenerative diseases such as primary demyelinating ones, in this case, Multiple Sclerosis in humans [10, 11]. This is because Vitamin D imposes brain functions on the physiological effects in promoting neurotransmission, neurogenesis, synaptogenesis, amyloid clearance and the prevention of neural death that has an important motor cognitive performance [21, 22, 23]. At twenty-one days, the Functional Gait Index (MFI) was significantly lower for the CPMA and APMP indicators and this indicates that the neural injury with ethidium bromide administration is under the most severe functional point of view in the first seven to fourteen days [04, 22, 23]. This is due to Vitamin D deficiency, which has been related to the decrease in muscle mass strength, which causes impairment in balance, such as increased falls in patients with Multiple Sclerosis and the elderly

[04, 11, 13]. This was verified in studies that evaluated the kinetics of muscle contraction and found a significant prolongation in the muscle relaxation phase of rats with Vitamin D deficit, thus, the study by Pedrosa et al. (2005) corroborated with data related to the deficit of Vitamin D that causes a reduction in the active transport of calcium into the sarcoplasmic reticulum through a fundamental process for muscle relaxation [23, 25]. The improvement in gait amplitude means a greater number of functional axons, which directly represents the number of regenerated motor nerve fibers [24, 25]. The mean amplitude of the functional gait index after twenty days of the ethidium bromide demyelination process was 4.6 cm at sixty-nine days was 4.3 cm for groups treated with 100.000 IU/week [21]. However, values close to those of the 50.000 IU/week group were found after forty-two days of the ethidium bromide demyelination process. This study evaluated the extent of recovery in the Functional Gait Index in the period of eighty-three days in Vitamin D supplementation with potential action on the central nervous system of rats submitted to neurochemical injury with ethidium bromide from seven days to eighty-three days [20-25]. The general amplitude values in the indicators in the Functional Gait Index showed a recovery in partial gait of 85% at 69 days and normal return at eighty-three days [14, 22]. Measurement was possible at forty to two days, at which time significant values were observed in the control groups and dose greater than 50.000 IU/week. This study corroborates the study by Gumieiro et al (2015) in which it proved a latent increase after the injury induced by ethidium bromide in the central nervous system [25]. Probably the treatment groups with supplementation doses of Vitamin D with values of 50.000 IU/week to 100.000 IU/week showed positive response in progress mainly in the CPMA and CPMP indicators [11, 16, 18]. Motor improvement can be considered a greater remyelination than that of the control group in the treatment period from fourteen days to eighty-three days [16]. This may have occurred, as each axon with injury may emit two to three axial extensions during the regenerative process that advance towards the injured tissue to reestablish the connection and function [16, 23]. This study was carried out with the choice of a suitable, applicable and low-cost and standardized method that simulates neurological injuries caused in gait in human beings with Multiple Sclerosis [16, 22, 24]. The choice for the Wistar rat is mainly due to the spinal vascularity similar to the human. The Gait Functional Index (MFI) evaluation model is more used in studies with central nervous system injury induction similar to that found in humans and analyzes the motor functionality of rats with the ability to verify neurological recovery after spinal cord injury in rats [24, 25]. These data evidenced in epidemiological studies have indicated that Vitamin D supplementation is associated with beneficial adverse brain results in humans and rodents [13]. In previous studies with mice, Vitamin D deficit during adulthood affected significant brain functions [13, 18, 19]. Thus, it must be considered a biologically plausible risk factor for the development of neuropsychiatric and neurodegenerative diseases. Following this support for Vitamin D supplementation in the proliferation, differentiation and apoptosis of neuronal cells, a previous study developed in BALB/c mice, which tested the effects of Vitamin D deficit in adults on hippocampal neurogenesis, proving that Vitamin D deficit leads to increased proliferation and decreased survival of neurons from the hippocampus born in adults [13,18,19]. Thus, exposure to Vitamin D deficit during adulthood in mice was sufficient to impair cognition and alter brain function, because affected the pathways involved in neuroprotection and neurotransmission instead of direct regulation of proliferation, differentiation and apoptosis of neuronal cells [14, 15, 16]. Therefore, this method was chosen for the study, because it was easy to apply and allowed to assign scores in an objective manner with a slight inter-observer variation and thus made it possible to assess the rat for neurological deficit at the time of observation, but mainly to monitor its daily clinical evolution [3-8]. It was a comprehensive method that verified several criteria such as the following: amplitude, frequency, coordination and consistency of gait related to the main joints of the lower limb, gait stability and tail support for balance in gait steps [8]. The results obtained with the statistical analysis showed that the treatment groups that received Vitamin D supplementation for more than twelve weeks after the neural injury with ethidium bromide and doses greater than 40.000 IU/week showed better neurological recovery over twenty-two years [4-7]. Observation days and obtained a better evaluation from the seventh day of the ethidium bromide demyelination procedure ($p < 0.05$). Supplementing Vitamin D, promotes the proliferation and differentiation of neural stem cells in the subventricular zone and generally the migration of these cells to the site of the injury to the corpus callosum produces basic myelin proteins [8, 10, 14]. The present study reinforces the importance of treatment with Vitamin D supplementation with doses of 40.000 to 100.000 IU/week [14-16]. This approach can ease the comorbidities caused by diseases such as Multiple Sclerosis, with similar changes suffered by the rats in the study, such as impaired walking, restricted movement, emotional changes and neurological deficit [18, 22, 25]. This study

corroborates that Vitamin D supplementation in patients with primary demyelinating diseases such as Multiple Sclerosis when presenting a direct correlation between high levels of supplementation and the functional performance evidenced by the slower and lesser grip of the moment when supplementation of Vitamin D occurs with doses less than 20.000 IU/week [14, 15, 16]. The strength of this study is that the sampling is highly selected and allows obtaining laboratory data and functional performance in ideal condition, without the effect that comorbidities or drugs in primary demyelinating diseases such as Multiple Sclerosis can have on motor performance [04, 13, 23, 26]. This study reinforces the importance of advancing the diagnosis and interventions in Vitamin D supplementation in motor functionality, especially considering the evidence that states that high doses of Vitamin D (50.000 IU/week to 100.000 IU/week) allow for better physical performance, strength and balance in addition to reducing the incidence and aggravation, such as falls and fractures, which are determinant indicators for preventing disability in patients with primary demyelinating diseases such as Multiple Sclerosis [22, 23, 25].

CONCLUSION:

The results present in this study allow us to establish the following conclusion: The damage caused by the direct administration of ethidium bromide in the central nervous system region in this study characterized a serious injury that can be compared to the development of Multiple Sclerosis in humans. The use of Vitamin D supplementation treatment in rats with lesions in the central nervous system showed a faster functional performance and greater grip, evidenced in gait in high doses of Vitamin D supplementation, in this case, equivalent to 50.000 IU/week to 100.000 UI/week. Thus, Vitamin D supplementation may ease the comorbidities caused by primary demyelination as in Multiple Sclerosis equivalent to those suffered by rats in this study.

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