REVISTA NEUROCIÊNCIAS

Original

Anxiolytic effects of *Leishmania braziliensis* proteins in a zebrafish animal model

Efeitos ansiolíticos das proteínas de Leishmania braziliensis em modelo animal de zebrafish

Efectos ansiolíticos de las proteínas de Leishmania braziliensis en modelo animal de zebrafish

Natália Sousa de Sousa[1], Rayssa Mirelle Campos Gurgel[2], Pedro de Freitas Santos Manzi de Souza[3], Dara da Silva Mesquita[4], Roberto Nicolete[5], José Eduardo Ribeiro Honório Junior[6]

1.Biomedicine. Centro Universitário Christus. Fortaleza-CE, Brazil. Orcid: https://orcid.org/0000-0003-1648-3126
2.Biomedicine. Centro Universitário Christus. Fortaleza-CE, Brazil. Orcid: https://orcid.org/0000-0003-4986-569X
3.Biomedicine. Centro Universitário Christus. Fortaleza-CE, Brazil. Orcid: https://orcid.org/0000-0003-1893-2992
4.Biomedicine. Centro Universitário Christus. Fortaleza-CE, Brazil. Orcid: https://orcid.org/0000-0002-8186-8375
5.PhD. Fundação Oswaldo Cruz (FioCruz). Fortaleza-CE, Brazil. Orcid: https://orcid.org/0000-0002-6101-1155
6.Biomedicine, PhD. Centro Universitário Christus. Fortaleza-CE, Brazil. Orcid: https://orcid.org/0000-0001-8320-8525

Resumo

Introdução. A leishmaniose está entre as 22 doenças mais negligenciadas em todo o mundo. Pouco se fala das evidências do acometimento neurológico que a leishmaniose pode acometer, devido às ulcerações formadas na pele em curto e em longo prazo. Todavia, alguns autores já citam que a *leishmania* pode causar inflamação no sistema nervoso central, podendo desencadear processos neuropsicomportamentais como a ansiedade. Objetivo. O presente estudo enfoca a avaliação dos efeitos das proteínas totais de *Leishmania* no sistema nervoso central no modelo de Zebrafish. **Método**. Foram realizados, inicialmente, o cultivo da *Leishmania* para serem extraídas as proteínas totais pelos métodos de Sonicação e Bradford. Após a extração das proteínas totais, foram inoculados no peixe-zebra para a observação da sintomatologia ocasionada pela carga de proteínas, e, após os 7 dias de inoculação, realizamos os testes comportamentais (Campo Aberto e Claro & Escuro). **Resultados**. Os peixes tratados com proteína de *Leishmania* apresentaram várias alterações cutâneas, bem como alterações comportamentais. Os testes comportamentais de campo aberto mostram um número de cruzamentos altos em relação ao grupo controle. No teste claro e escuro, o peixe-zebra teve uma permanência maior no campo claro, sendo observada uma ação ansiolítica nos animais que receberam a administração das proteínas. **Conclusão**. As proteínas totais de *Leishmania* podem, sim, ter uma ação ansiolítica no modelo de zebrafish.
Unitermos. Leishmania; Proteínas; Ansiedade; Sistema Nervoso Central

Abstract

Introduction. Leishmaniasis is among the 22 most neglected diseases worldwide. Few information is related about the evidence of neurological involvement that leishmaniasis can cause due to the ulcerations formed in the skin along short and long term. However, some authors have cited that leishmaniasis can cause inflammation in the central nervous system, which can trigger neuropsychomotor processes such as anxiety. **Objective.** The present study focuses on the evaluation of the effects of total Leishmania proteins on the central nervous system in the Zebrafish model. **Method.** Leishmania was initially cultured to extract total

proteins by Sonication and Bradford methods. After total protein extraction, the zebrafish were inoculated to observe the symptomatology caused by protein load, and after seven days of inoculation, we performed the behavioral tests (Open Field and Bright & Dark). **Results.** The fish treated with Leishmania protein showed several cutaneous alterations, as well as behavioral changes. The open field behavioral tests showed a high number of crosses compared to the control group. In the light and dark test, the zebra fish had a longer stay in the light field, and an anxiolytic action was observed in the animals that received the protein administration. **Conclusion**. The total Leishmania proteins may have an anxiolytic action in the zebrafish model.
Keywords. Leishmania; Proteins; Anxiety; Central Nervous System

Resumen

Introducción. La leishmaniosis está entre las 22 enfermedades negligenciadas en todo el mundo. Se habla poco de los indicios que la leishmaniosis puede provocar de forma neurológica, debido a las ulceraciones que se forman en la piel a corto y largo plazo. Pero algunos autores, ya citaron que la leishmaniosis, puede causar inflamación en el sistem nervioso central, pudiendo desencadenar procesos neuropsico comportamentales como la ansiedad. **Objetivo**. El presente estudio se enfoca en la evaluación de los efectos de las proteínas totales de la Leishmaniosis en el sistema nervioso central en el modelo del pez cebra (zebrafish). **Método**. Inicialmente se realizó un cultivo de Leishmaniosis para extraer las proteínas totales mediante los métodos de sonicación y Bradford. Después de la extracción de las proteínas totales estas fueron inoculadas en el pez cebra para observar los síntomas causados por la carga de proteínas, y tras 7 días de inoculación realizamos las pruebas de comportamiento (campo abierto, luz y oscuridad). **Resultados**. Los peces tratados con la proteína de Leishmania mostraron diversas alteraciones en la piel y en el comportamiento. Las pruebas de comportamiento en campo abierto muestran un elevado número de cruzamiento en comparación con el grupo de control, en la prueba de luz y oscuridad, los peces cebra tuvieron una mayor permanencia en el campo luminoso, y se observó una acción ansiolítica en los animales que recibieron la administración de proteínas. **Conclusión**. Las proteínas totales de Leishmania pueden tener una acción ansiolítica en el modelo del pez cebra.
Palabras clave. Leishmania; Proteínas; Ansiedad; Sistema Nervioso Central

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Corresponding address: José Eduardo R Honório Junior. R. Princesa Isabel 1920. Farias Brito. Fortaleza–CE, Brazil. CEP 60015-061. Phone: 3022-0709. E-mail: eduribiologo@yahoo.com.br

INTRODUCTION

Leishmaniasis is among the 22 neglected diseases, in which cutaneous leishmaniasis is the primacy worldwide[1]. According to the World Health Organization 2017 (WHO), leishmaniasis intimidates about 350 million men, women, and children worldwide, leading to about 1 to 2 million new cases each year[2].

Leishmania presents several symptoms described in the literature, among them, one can mention alterations in the

central nervous system (CNS). It has been observed that dogs infected by this disease presented clinical and neurological signs, and in further analysis of their cerebrospinal fluids, they presented antigens for *Leishmania*[3].

The immune response to an infection can produce various pro-inflammatory cytokines that can cross the blood-brain barrier[4,5]. *Leishmania* is a protozoan that can produce immunomodulatory mechanisms that can regulate or even modify the inflammatory response in the central and peripheral nervous system by causing the regulation of pro- and/or anti-inflammatory cytokines[6,7]. Cytokines are activated due to the activation of the Th1 immunity in contrast to the Th2 immune response that monitors the Th1 functions, observing the activation of macrophages in benefit of disease progression[8,9]. This immune response generated by cutaneous *Leishmania* could lead to neuroinflammation[7].

The zebrafish comes as an innovation for the scientific community, and its use has increased significantly in recent decades. It is a vertebrate species with high physiological and genetic homology with humans, easy to manipulate, and morphological similarity to the central nervous system[10]. Thus, this study aims to evaluate the effects of total *Leishmania* proteins (PrLeish) in the promastigote form on the CNS in the zebrafish animal model.

METHOD

Obtaining the *Leishmania*

The virulence of *Leishmania (Viannia) braziliensis* strain (MCAN/BR/98/R619 - provided by Fiocruz Rio de Janeiro) was maintained by a regular passage in cultures at 22.4°C in N.N. N (Neal, Novy, Nicolle) medium containing Schneider supplemented with 20% inactivated fetal bovine serum (SBF), 2% sterile human urine, 5 ml of blood agar medium. Promastigotes in the stationary phase with up to three culture passages were used. *Leishmania* aliquots were placed in an Eppendorf tube and frozen to maintain sample stability and use at various stages of testing was possible. After *Leishmania* culture, it was centrifuged at 3,000 rpm for 10 minutes at 4°C to obtain the sediment formed in the falcon tube. The sediment was taken to the oven at a temperature of 22.4°C for seven days for replication of the *Leishmania*. After this period, the viability was observed on a slide.

Acquisition and determination of *Leishmania* proteins

The *Leishmania* pool was suspended in PBS buffer solution for homogenization for 15 minutes, moving, gently, until the complete suspension was achieved. Soon after, the samples were inserted into an ice bath. To disrupt the cells, the suspension was sonicated in three cycles with the amplitude of 100% for 1 min, thus centrifuging the lysate for 15min at 3500 RPM at 4°C. Finally, the supernatant was separated from the cell pellet carefully. The supernatant is the total cell lysate. After filtration of the supernatant, a

clarified fluid of the soluble cell protein was obtained. After separation of the clarified fluid from the soluble *Leishmania* proteins, which were assayed by the modified Bradford method for ELISA[8]. The Bradford assay was performed in a 96-well ELISA plate, consisting of a final volume of 200ul for each well of the plate. From the total concentration of 71.95 mg/mL PrLeish protein, three doses were determined at concentrations of 1/2, 1/4, and 1/8 from the total dose, plus controls and blank. The samples were read at an absorbance of 595nm.

Zebrafish animal model

The study was approved by the Unichristus Animal Use Ethics Committee, number 029/19. The animals were divided into groups of 10 according to the experimental conditions to identify statistically significant and biologically relevant differences between baseline performance and treatment effects. This value of animals per group was obtained using a statistical program for experimental samples called Piface by Russell V. Lenth version 1.76 from June 29, 2011[10,11]. We used 60 adult *Danio rerio* animals of undetermined sex and age from local pet shops, kept for at least one week in groups of 10 in 3.5-liter aquariums (Autonomous Aquatic System/ZEBTEC) - before the experiment started, with daily commercial feed-ration (Tetra, Germany).

Acclimatization

Zebrafish (ZFas) were kept in glass aquaria (5ZFa/L), at a temperature of 24 to 28°C, in light-dark cycles of 14/10 or 12/12 hours. Tap water (pH 7-8), previously treated with anti-chlorine (ProtecPlus®; 2 drops/L), and air pumps with submerged filters can be used. Change the water partially or totally every 24h or, at the most, 48h of the experiments. In the partial exchange, you can reach up to 70% renewal of the aquarium water. After acclimation, the ZFas can be fed with feed (Spirulina®) *ad libitum*.

Treatment protocols

The animals were divided into six groups as follows:

GROUPS	***TREATMENT***
GROUP I	Control animals without any treatment
GROUP II	Diazepam 1 mg/Kg, intraperitoneal
GROUP III	PBS solution - orally
GROUP IV	PrLeish 1/2 of the total dose, orally
GROUP V	PrLeish 1/4 of the total dose, orally
GROUP VI	PrLeish 1/8 of the total dose, orally

ANXIETY ASSESSMENT PROTOCOL

ZFas from the treatment and control groups were placed in individual beakers (300mL) containing 150mL of heated water. The animals were anesthetized in cold water (12ºC-15ºC) until they were immobile. The ZFa were then transferred to a moist sponge for application of PrLeish, Diazepam, and PBS then returned to the original beakers and allowed to recover for 30 min. The PrLeish was carefully administered orally (v.o) using a pipette (5 µl). After the last

anxiety test, the animals were euthanized (placed in cold water at 5°C for 20 min)[12,13].

Locomotor activity: open field test

This test aims to evaluate the locomotor activity of the treated animals. They were treated (orally; *v.o*) with 5 µL of PrLeish (1/2; 1/4; 1/8 mg/ml) or vehicle (Control; PBS 3%). After 30 minutes of the treatments, the ZFa were submitted to the Open Field Test for the evaluation of locomotor activity (No. of Crossing Lines, NCL), in glass Petri dishes (100 x 15 mm). The locomotor impairment exerted by the protein pool was evaluated in relation to the crossing count of the ZFas in the quadrants of the plates, for 5 minutes. An untreated group (Naive) was included and used as a baseline (100%). Thus, calculate the percentage of NCL according to: $\%NCL=[NCL_{(Treatment)}/NCL_{(Naive)}] \times 100\%$[14].

Anxiolytic activity: light & dark test

This practice aims to evaluate the anxiolytic effect of PrLeish in ZFas. Male and female animals were treated, orally, with PrLeish (1/2; 1/4; 1/8mg/ml; 5µL) or vehicle (Control; PBS 5µL) or Diazepam (DZP; 1mg/ml; 5µL). A Naive group was also included after 30 min, ZFas were added to the light zone of a glass aquarium (30x15x20cm), divided into a light and dark zone, with drug-free water (3cm). The anxiolytic activity was characterized by animals remaining in the light zone for most of the analysis time (5min),

characteristic of animals treated with anxiolytic drugs (Diazepam)[13].

Prleish inoculation protocol

The groups were separated after three days of environmentalization, both with 10 fish in each tank, the control groups were treated separately. The first group without any kind of treatment (Naive) received only feed and daily cleaning for seven days. Groups II and III received daily amounts of 5 µl of Diazepam and PBS for seven days, in addition to daily feed and cleaning. Groups IV, V, and VI received for seven days, daily applications of 5 µl of PrLeish with the dosages for each group separately. On the eighth day, behavioral tests were performed in all six groups.

Statistical analysis

Graph Pad Prism 6.0 software was used for the statistical analysis of the data. The results were analyzed by *one-way* ANOVA followed by *Tukey*'s *post hoc* test. The results were expressed as mean±error and the significance level was considered $p<0.05$. We use alphabetical letters to indicate the significance level.

RESULTS

Quantification of the *Leishmania* proteins

According to the Bradford method, the amount of total protein obtained was 71.95 mg/mL. Each PrLeish treatment group had the following total protein concentrations: at dose

1/2 35.96 mg/mL, 1/4 a total of 17.98 mg/mL, and the last concentration of 1/8 a total of 8.99 mg/mL.

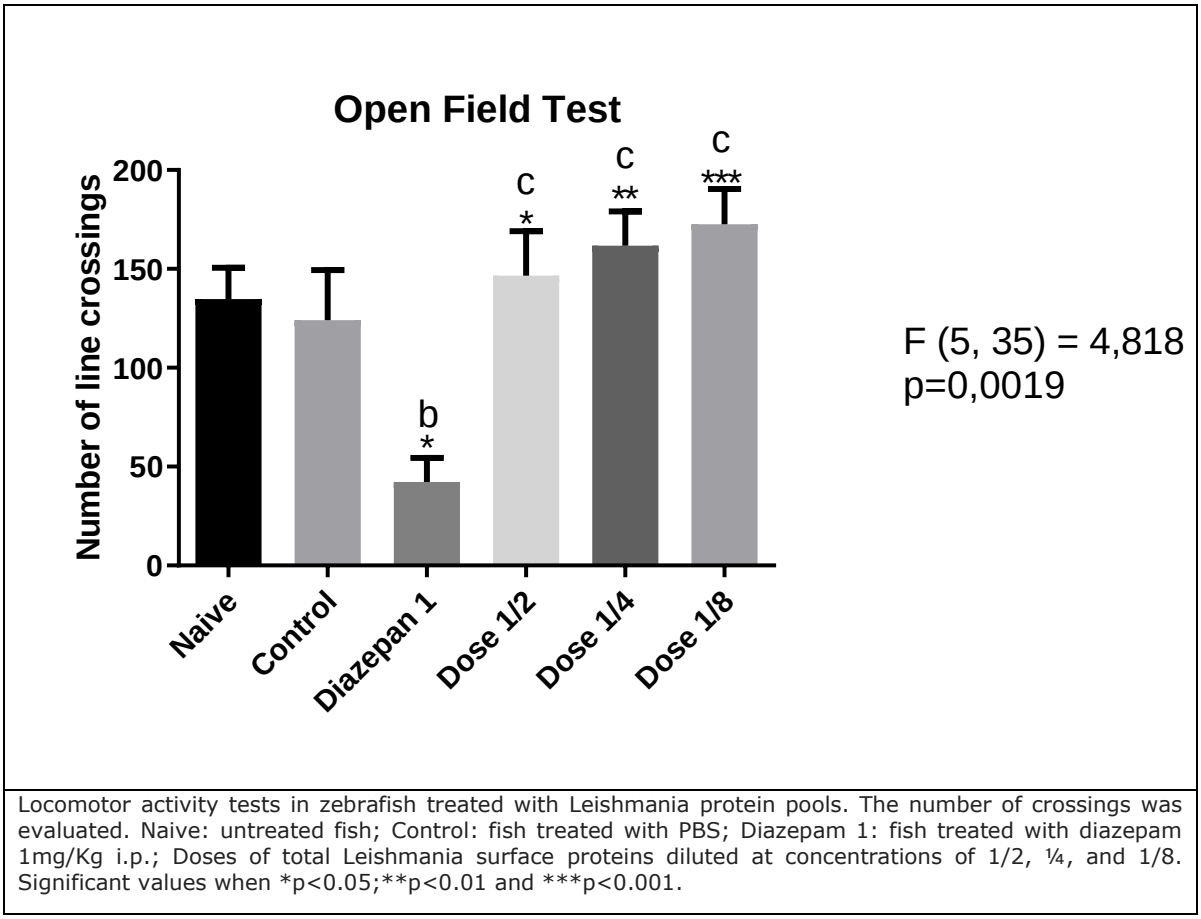
Locomotor activity of zebrafish

According to the literature, Magalhães[15] proposed a methodology in which the increase in the number of crossings of the ZFa in the open field in relation to the control can be interpreted as anxiogenic activity. Animals that did not receive treatments were considered naive (100% locomotor activity) and calculated locomotor activity (Figure 1). In the open field test, it could be observed that animals treated with Leishmania surface proteins in the three doses had increased locomotor activity compared to diazepam [Dose 1/2 (146.6±22.5); Dose 1/4 (161.8±17.8), Dose 1/8 (172.5±17.9 and Diazepam (42.2±12.2)]. Diazepam showed a decrease against the control (124.0±25.4) too, but not against the naive (134.7±15.9). These results show that all three doses had an increase in locomotor activity in the Zebrafish model on chronic treatment.

Anxiolytic activity in zebrafish

The parameters evaluated in this test were the permanence in the bright field, the latency time, from which it leaves the bright field and goes to the dark field for the first time and the number of crossings in a time of five minutes[16].

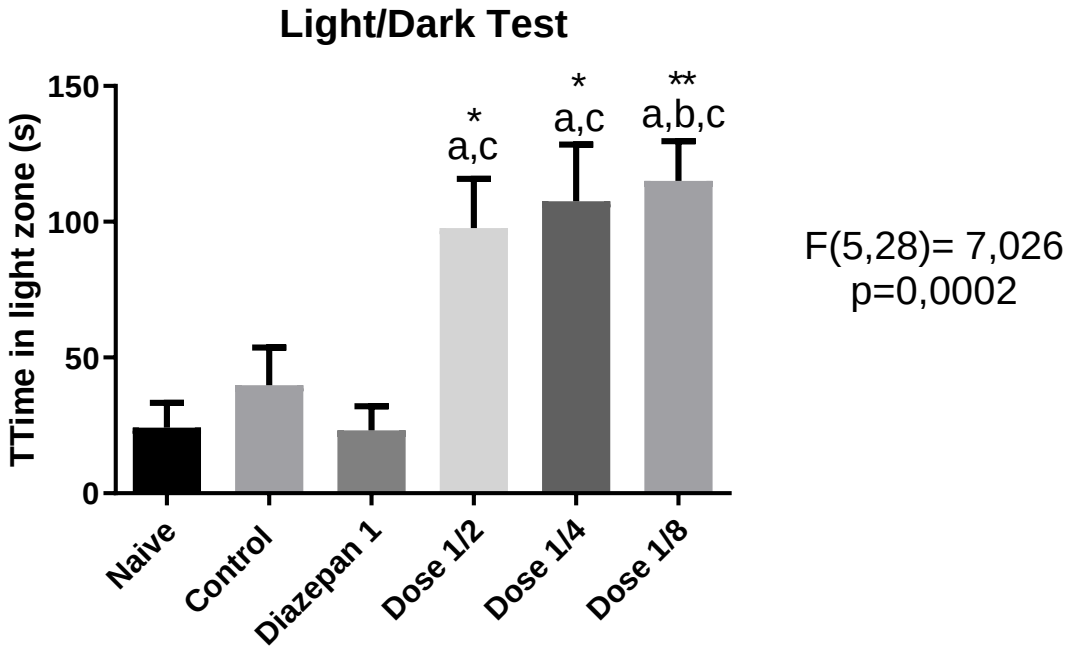
Figure 1: Locomotor Activity Ratio Zebrafish Model.



Locomotor activity tests in zebrafish treated with Leishmania protein pools. The number of crossings was evaluated. Naive: untreated fish; Control: fish treated with PBS; Diazepam 1: fish treated with diazepam 1mg/Kg i.p.; Doses of total Leishmania surface proteins diluted at concentrations of 1/2, ¼, and 1/8. Significant values when *p<0.05;**p<0.01 and ***p<0.001.

In the Light & Dark test, the ZFa treated with PrLeish showed in all three doses an increase compared to diazepam, and naïve [Dose 1/2 (97.6±18.2), Dose 1/4 (107.6±20.8), Dose 1/8 (115.0±14.6), Diazepam (23.2±8.8) and Naive (24.2±9.1)], but it was not significant compared to the control (39.8±13.9). This result shows that PrLeish has an anxiogenic effect within the Zebrafish animal model (Figure 2).

Figure 2. Light/Dark Test Ratio Zebrafish Model.



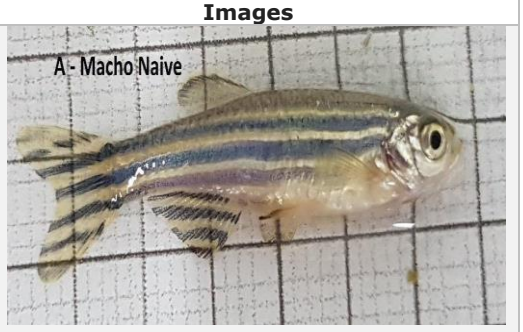
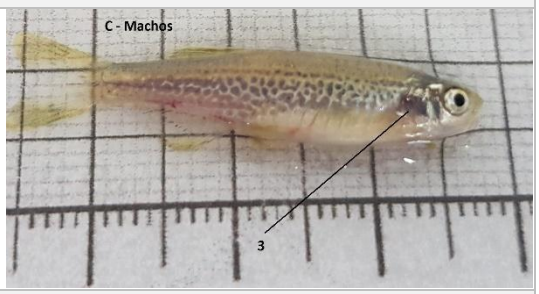
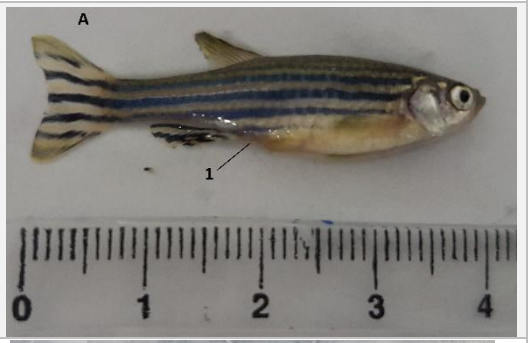
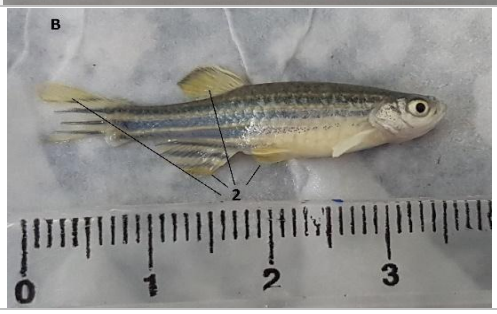
Dark light tests in zebrafish treated with the *Leishmania* protein pool. Time in the zebrafish in the light environment in seconds was evaluated. Naive: untreated fish; Control: fish treated with PBS; Diazepam 1: fish treated with diazepam 1mg/Kg i.p.; Doses of total *Leishmania* proteins diluted at concentrations of 1/2, 1/4, and 1/8. Significant values when *p<0.05 and **p<0.01.

Morphological changes in zebrafish

The animals treated for 7 days with PrLeish showed dark spots concentrated on the abdomen of the fish with a reddish yellow color, a situation that varied a lot among the infected fish and according to the doses, but was not found in the control, naive, and diazepam animals. Other animals had yellowish spots on the ends of the dorsal, caudal, and anal fins. The operculum of several animals had red spots on both males and females. In addition, the males showed more

intense discoloration than the females altering the blue striped coloring pattern of the zebrafish.

Some social behaviors of the zebrafish were altered after treatment with PrLeih. The treated animals showed a decrease in foraging, as well as an apparent progressive loss of their locomotor and visual senses. Some changes can be seen in the table below:

Groups	Morphological Changes	Images
GROUP I- without any treatment.	No changes, normal diameter, coloration, and feeding	
GROUP II- Diazepam	They did not show any type of alteration. Normal diameter, coloration, and feeding.	
GROUP III-PBS Solution	They did not show any type of alteration. Diameter, coloration, and feeding were normal.	
GROUP IV- PrLeish 1/2	They presented yellowish coloration, blackened spots on the skin, reddish spots on the skin and eyes, traditional reddish gills presented purplish color, loss of appetite, and loss of locomotion.	
GROUP V- PrLeish 1/4	Yellowish color, reddish spots on the skin and eyes, tiny fins, gills of a traditional reddish color, loss of appetite, and loss of locomotion.	
GROUP VI- PrLeish 1/8	Yellowish color, blackened spots on the skin, reddish spots on the skin and eyes, shortened tail and fins, discoloration of the skin, loss of appetite, and loss of locomotion.	

DISCUSSION

Our results showed that animals treated with *Leishmania* Totals Proteins (PrLeish) showed altered skin coloration and the development of inflammatory processes. In addition, the treated animals had the development of anxiogenic behavior compared to the control.

In PrLeish we can find a group of proteins called leishmanolysins. The leishmanolysins help in the infection of macrophages in the human host by the promastigote forms[17]. In a study, were able to extract from the promastigote forms around 352 proteins, which were involved in the infection process and pathogenesis of *Leishmania*. Some authors worked with leishmania proteins and concluded that more studies are needed to understand the pathways that the proteins use[18,19]. For example, it can be cited, the leishmanolysin GP63 as it is known is the main infection protein in the promastigote form. In addition, we can also find LIT1 (*Leishmania* Iron Transporter 1) and LFR1 (*Leishmania* Ferric Reductase 1), which are transmembrane proteins that interact by reducing Fe 3+ to Fe 2+, the soluble form of iron, and thus help the parasite to remain inside the cell[20,21].

The *Leishmania* proteins when orally administered to the zebrafish, lead to various morphological changes from whitening of the skin to lesion-like spots, as in the operculum of these animals. These morphological alterations were well evidenced from the second day of application and only increased as the days went by. During the applications and

after the experimentation, their morphology was observed, which presented blackish spots, yellowish coloration, red spots that may resemble the ulcerations that are present in the human form during infection with *Leishmania braziliensis,* as well as progressive loss of appetite. These changes may suggest the formation of a chronic inflammatory process.

The early manifestations of leishmaniasis can cause the formation of lesions, which may have evolved into ulcers with elevated and well-defined borders. In some circumstances, secondary infections may arise due to moistened lesions and without any kind of treatment, they may evolve into a more aggressive lesion cutaneous leishmaniasis[22]. There is preliminary evidence confirming the association between the presence of mental disorders, such as anxiety, in patients with leishmaniasis, in addition to demonstrating the importance of correlated studies on different manifestations in types of leishmaniasis[23].

The zebrafish has a cellular defense system similar to that of man[24]. This similarity lies in the immune cell pathway, which takes the same cell differentiation pathway found in mammals[25]. The main human lineage cells that were evidenced in the zebrafish are macrophages, neutrophils, B-lymphocytes, and T-cells[26], so we can hypothesize that the most activating cells were the macrophages, like man, leading to the development of chronic inflammation in the zebrafish. Thus, the zebrafish model is an innovative model for research, because, besides its low cost and great genetic

homology, it presents anxiety-like behavior similar to humans, enabling low-cost research for new therapeutic targets[27-30].

Sickness behavior is associated with the inflammatory response, neuroinflammation and pain conditions in animals, due to the elevated and prolonged production of pro-inflammatory cytokines that may not lead to the development of serious behaviors such as anxiety[31,32]. The open field is a well-employed test in routine research, as it provides data on locomotor activity and anxiolytic effects[33]. Most pesticides tend to increase locomotion activity in Zebrafish, our open field test study showed that the group treated with diazepam decreased their locomotor activity and the group treated with PrLeish showed an increase in their locomotor activity[34].

These data lead us to the perception that PrLeish causes an anxiogenic effect on the central nervous system in zebrafish. In a study whose objective was to show the anxiolytic effects of chalcones using diazepam as a standard, the results showed that all doses caused motor impairment in relation to diazepam, showing a decrease in the locomotor activity of zebrafish, suggesting that chalcones and diazepam are compounds that decrease locomotor activity in zebrafish. Thus, proving that the open field directs toward more specific analyses for anxiety tests[34,35].

Male BALB/c mice that were infected with the stationary-phase promastigotes form of *L. amazonensis* had a significant reduction in locomotor activity compared to the

control, which differs from the results obtained in Zebrafish, but both the Leishmania species and the method of infection and the animal used differ from our study, highlighting the importance of further correlated studies[36].

The light/dark tests are determined by the light stimulus which shows the zebrafish's preference for the dark environment because it feels more protected[37,38]. The behavioral responses produced by the zebrafish are like the responses produced by rodents in other behavioral tests[20]. This test evidence that anxiety is a normal adaptive mechanism employed by humans, as by fish, to cope with potential danger[37]. Several pieces of research show the use of the Light & Dark Test to evaluate the possible anxiolytic effect of drugs on adult zebrafish (*Danio rerio*)[39-42].

A study showed a reduction in results with the use of the benzodiazepine Clonazepam, where there was differentiation and which shows us the natural behavior of zebrafish in dark places, whose fish treated with clonazepam showed a greater preference for the dark side of the aquarium, this behavior is natural for zebrafish and is linked to the fish's strategy of escaping from natural predators, or the area where it feels safer[43]. Our tests showed that PrLeish, in the light & dark test, shows an anxiogenic effect in the treated animals.

CONCLUSION

Finally, we conclude that although few studies have reported the effects of leishmaniasis on the central nervous

system of humans or other animals, our study showed that PrLeish caused morphological changes similar to inflammatory lesions in skin tissue, increased locomotor activity, and anxiogenic effect in zebrafish animal models, leading the animals to an infection-like state that could cause anxiety. To confirm and further develop these results we need to perform new tests on this zebrafish model.

REFERENCES

1.Molyneux DH, Savioli L, Engels D. Neglected tropical diseases: progress towards addressing the chronic pandemic. Lancet 2017;389:312-25. https://doi.org/10.1016/S0140-6736(16)30171-4

2.Global Health Observatory. Leishmaniasis. Geneva: World Health Organization; 2020 (accessed May 2021). Available in: https://www.who.int/gho/neglected_diseases/leishmaniasis/en/

3.Oliveira VdC, Boechat VC, Mendes Junior AAV, Madeira MdF, Ferreira LC, Figueiredo FB, *et al*. Occurrence of Leishmania infantum in the central nervous system of naturally infected dogs: Parasite load, viability, co-infections and histological alterations. PLoS ONE 2017;12:e0175588. https://doi.org/10.1371/journal. pone.0175588

4.Portes A, Giestal-de-Araujo E, Fagundes A, Pandolfo P, Geraldo AS, Lira MLF, *et al*. Leishmania amazonensis infection induces behavioral alterations and modulates cytokine and neurotrophin production in the murine cerebral cortex. J Neuroimmunol 2016;301:65-73. https://doi.org/10.1016/j.jneuroim.2016.11.003

5.Brundin L, Erhardt S, Bryleva EY, Achtyes ED, Postolache TT. The role of inflammation in suicidal behaviour. Acta Psychiatr Scand 2015;132:192-203. https://doi.org/10.1111/acps.12458

6.Debnath M, Berk M. Th17 pathway-mediated immunopathogenesis of schizophrenia: mechanisms and implications. Schizophr Bull 2014;40:1412-21. https://doi.org/10.1093/schbul/sbu049

7.Notarangelo FM, Wilson EH, Horning KJ, Thomas MA, Harris TH, Fang Q, *et al*. Evaluation of kynurenine pathway metabolism in Toxoplasma gondii-infected mice: implications for schizophrenia. Schizophr Res 2014;152:261-7. https://doi.org/10.1016/j.schres.2013.11.011

8.Ramos PK, Brito MV, Silveira FT, Salgado CG, De Souza W, Picanço-Diniz CW, *et al*. In vitro cytokines profile and ultrastructural changes of microglia and macrophages following interaction with Leishmania. Parasitology 2014;141:1052-63. https://doi.org/10.1017/S0031182014000274

9.Honório Jr JER, Vasconcelos GS, Rodrigues FT, Sena Filho JG, Barbosa-Filho JM, Aguiar CC, *et al*. Monocrotaline: histological damage and oxidant activity in brain areas of mice. Oxid Med Cell Longev

2012;2012:697541. https://doi.org/10.1155/2012/697541
10.Gerlai R, Lahav M, Guo S, Rosenthal A. Drinks like a fish: zebra fish (Danio rerio) as a behavior genetic model to study alcohol effects. Pharmacol Biochem Behav 2000;67:773-82. https://doi.org/10.1016/s0091-3057(00)00422-6
11.Lenth RV. Java applests for power and sample size. [Computer Software]. 2006; (accessed in 01/13/2020). Avaiable in: https://www.stat.uiowa.edu/~rlenth/Power
12.Almeida RS, Klotzle MC, Figueiredo Pinto AC. Composição do conselho de Administração no setor de energia elétrica do Brasil. R Admin UNIMEP 2013;11:156-80. http://www.raunimep.com.br/ojs/index.php/rau/article/view/509
13.Magalhães FEA, Batista FLA, Lima LMG, Abrante IA, Batista FLA, Abrante IA, *et al*. Adult Zebrafish (Danio rerio) As a Model for the Study of Corneal Antinociceptive Compounds. Zebrafish 2018;15:566-74. https://doi.org/10.1089/zeb.2018.1633
14.Tatem KS, Quinn JL, Phadke A, Yu Q, Gordish-Dressman H, Nagaraju K. Behavioral and locomotor measurements using an open field activity monitoring system for skeletal muscle diseases. J Vis Exp 2014;91:51785. https://doi.org/10.3791/51785
15.Magalhães FEA, de Sousa CÁPB, Santos SAAR, Menezes RB, Batista FLA, Abreu ÂO, *et al*. Adult Zebrafish (Danio rerio): An Alternative Behavioral Model of Formalin-Induced Nociception. Zebrafish 2017;14:422-9. https://doi.org/10.1089/zeb.2017.1436
16.Maximino C. Serotonin in the nervous system of vertebrates. In: Serotonin and Anxiety. New York: Springer; 2012; pp15-36. https://link.springer.com/chapter/10.1007/978-1-4614-4048-2_2
17.Arango Duque G, Descoteaux A. Leishmania survival in the macrophage: where the ends justify the means. Curr Opin Microbiol 2015;26:32-40. https://doi.org/10.1016/j.mib.2015.04.007
18.Gouveia JR A, Zampieri RA, Ramos LA, Silva EF, Mattioli R, Morato S. Preference of goldfish (Carassius auratus) for dark places. Rev Etol 2005;7:63-6.
http://pepsic.bvsalud.org/scielo.php?script=sci_arttext&pid=S1517-28052005000200002&lng=pt&nrm=iso
19.Amiri-Dashatan N, Rezaei-Tavirani M, Ahmadi N. A quantitative proteomic and bioinformatics analysis of proteins in metacyclogenesis of Leishmania tropica. Acta Trop 2020;202:105227. https://doi.org/10.1016/j.actatropica.2019.105227
20.Castro Neto AL, Brito ANALM, Rezende AM, Magalhães FB, de Melo Neto OP. *In silico* characterization of multiple genes encoding the GP63 virulence protein from *Leishmania braziliensis*: identification of sources of variation and putative roles in immune evasion. BMC Genomics 2019;20:118. https://doi.org/10.1186/s12864-019-5465-z
21.Laranjeira-Silva MF, Wang W, Samuel TK, Maeda FY, Michailowsky V, Hamza I, *et al*. A MFS-like plasma membrane transporter required for *Leishmania* virulence protects the parasites from iron toxicity. PLoS Pathog 2018;14:e1007140. https://doi.org/10.1371/journal.ppat.1007140

22.Scorza BM, Carvalho EM, Wilson ME. Cutaneous Manifestations of Human and Murine Leishmaniasis. Int J Mol Sci 2017;18:1296. https://doi.org/10.3390/ijms18061296
23.Pires M, Wright B, Kaye PM, da Conceição V, Churchill RC. The impact of leishmaniasis on mental health and psychosocial well-being: A systematic review. PLOS ONE 2019;14:e0223313. https://doi.org/10.1371/journal.pone.0223313
24.Paul CD, Devine A, Bishop K, Xu Q, Wulftange WJ, Burr H, *et al*. Human macrophages survive and adopt activated genotypes in living zebrafish. Sci Rep 2019;9:1759. https://doi.org/10.1038/s41598-018-38186-y
25.Ellett F, Lieschke GJ. Zebrafish as a model for vertebrate hematopoiesis. Curr Opin Pharmacol 2010;10:563-70. https://doi.org/10.1016/j.coph.2010.05.004
26.Oosterhof N, Boddeke E, van Ham TJ. Immune cell dynamics in the CNS: Learning from the zebrafish. Glia 2015;63:719-35. https://doi.org/10.1002/glia.22780
27.Kalueff AV, Echevarria DJ, Stewart AM. Gaining translational momentum: More zebrafish models for neuroscience research. Progr NeuroPsychopharmacol Biol Psychiatr 2014;55:1-6. https://doi.org/10.1016/j.pnpbp.2014.01.022
28.Stewart AM, Ullmann JF, Norton WH, Parker MO, Brennan CH, Gerlai R, *et al*. Molecular psychiatry of zebrafish. Mol Psychiatry 2015;20:2-17. https://doi.org/10.1038/mp.2014.128
29.Khan KM, Collier AD, Meshalkina DA, Kysil EV, Khatsko SL, Kolesnikova T, *et al*. Zebrafish models in neuropsychopharmacology and CNS drug discovery. Br J Pharmacol 2017;174:1925-44. https://doi.org/10.1111/bph.13754
30.Carvalho TS, Cardoso PB, Santos-Silva M, Lima-Basto S, Luz WL, Assad H, *et al*. Oxidative Stress Mediates Anxiety-Like Behavior Induced by High Caffeine Intake in Zebrafish: Protective Effect of Alpha-Tocopherol. Oxid med cell longev 2019;2019:9. https://doi.org/10.1155/2019/8419810
31.Dantzer R. Cytokine, sickness behavior, and depression. Immunol Allergy Clin North Am 2009;29:247-64. https://doi.org/10.1016/j.iac.2009.02.002
32.Stelow E. Behavior as Illness Indicator. Vet Clin North Am Small Anim Pract 2018;48:9-10. https://doi.org/10.1016/j.cvsm.2017.12.002
33.Travi B, Rey-Ladino J, Saravia NG. Behavior of Leishmania braziliensis s.l. in golden hamsters: evolution of the infection under different experimental conditions. J Parasitol 1988;74:1059-62. https://doi.org/10.2307/3282237
34.Hussain A, Audira G, Malhotra N, Uapipatanakul B, Chen JR, Lai YH, *et al*. Multiple Screening of Pesticides Toxicity in Zebrafish and Daphnia Based on Locomotor Activity Alterations. Biomolecules 2020;10:1224. https://doi.org/10.3390/biom10091224
35.Ferreira MKA, da Silva AW, Silva FCO, Vieira Neto AE, Campos AR, Santos SAAR, *et al*. Anxiolytic-like effect of chalcone N-{4'[(2E)-3-(3-

nitrophenyl)-1-(phenyl)prop-2-en-1-one]} acetamide on adult zebrafish (Danio rerio): Involvement of the 5-HT system. Biochem Biophys Res Commun 2020;526:505-11. https://doi.org/10.1016/j.bbrc.2020.03.129.
36.Gugliandolo E, Palma E, Peritore AF, Siracusa R, D'Amico R, Fusco R, *et al*. Effect of Artesunate on *Leishmania Amazonesis* Induced Neuroinflammation and Nociceptive Behavior in Male Balb/C Mice. Animals (Basel) 2020;10:557. https://doi.org/10.3390/ani10040557
37.Rosemberg DB, Rico EP, Mussulini BHM, Piato ÂL, Calcagnotto ME, Bonan CD, *et al*. Differences in Spatio-Temporal Behavior of Zebrafish in the Open Tank Paradigm after a Short-Period Confinement into Dark and Bright Environments. PLoS ONE 2011;6:e19397. https://doi.org/10.1371/journal.pone.0019397
38.Serra EL, Medalha CC, Mattioli R. Natural preference of zebrafish (Danio rerio) for a dark environment. Braz J Med Biol Res 1999;32: 1551-3. https://doi.org/10.1590/S0100-879X1999001200016
39.Osorio Y, Rodriguez LD, Bonilla DL, Peniche AG, Henao H, Saldarriaga O, *et al*. Congenital transmission of experimental leishmaniasis in a hamster model. Am J Trop Med Hyg 2012;86:812-20. https://doi.org/10.4269/ajtmh.2012.11-0458
40.Brito TM. Validação da preferência claro/escuro como modelo comportamental de ansiedade no Carassius auratus (peixe dourado) (Dissertação). Ribeirão Preto: Universidade de São Paulo; 2011. https://doi.org/10.11606/D.59.2011.tde-29022012-092339
41.Magno LD, Fontes A, Gonçalves BM, Gouveia A Jr. Pharmacological study of the light/dark preference test in zebrafish (Danio rerio): Waterborne administration. Pharmacol Biochem Behav 2015;135:169-76. https://doi.org/10.1016/j.pbb.2015.05.014
42.Dahlén A, Wagle M, Zarei M, Guo S. Heritable natural variation of light/dark preference in an outbred zebrafish population. J Neurogenet 2019;33:199-208. https://doi.org/10.1080/01677063.2019.1663846
43.Gebauer DL, Pagnussat N, Piato ÂL, Schaefer IC, Bonan CD, Lara DR. Effects of anxiolytics in zebrafish: Similarities and differences between benzodiazepines, buspirone and ethanol. Pharmacol Biochem Behav 2011;9:480-6. https://doi.org/10.1016/j.pbb.2011.04.021