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TOXOPLASMOSIS: MORPHOLOGICAL AND MORPHOMETRIC EVALUATION OF SPINAL CORD NEURONS FROM NONSYMPTOMATIC SEROPOSITIVE DOGS

TOXOPLASMOSE: AVALIAÇÃO MORFOLÓGICA E MORFOMÉTRICA DOS NEURÔNIOS DA MEDULA ESPINHAL DE CÃES SOROPOSITIVOS ASSINTOMÁTICOS

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Abstract
The aim of this work was to analyze the neuron morphology and morphometry of cervical, thoracic and lumbar areas of nonsymptomatic seropositive dogs' spinal cord for toxoplasmosis. Twenty indefinite-breed adult dogs were used; ten dogs were healthy, with negative serology for toxoplasmosis, and were used as the control group (group 1), and ten dogs were nonsymptomatic but seropositive for toxoplasmosis (group 2). After the microtomy, with interval of 100 micrometers (µm), the histological 5-µm-thick cuts were dyed by hematoxylin-eosin and Masson's trichrome techniques. The glass slides were analyzed under light microscope to examine the neuron morphology. The parameters considered for the morphometric analysis were area, perimeter, maximum diameter, minimum diameter and shape factor of cytoplasm and nucleus of neuron. The results were statistically analyzed by Student's t test at 5% probability level. The morphological characteristics between the two groups were similar and according to literature. The morphometric results showed that there were changes in neurons size and structure, and increase and loss of star shape were noticed in seropositive animals. The results suggest that the neurons of these dogs, yet nonsymptomatic, can have lost their conductor function.
Keywords: canine; central nervous system; histology; *Toxoplasma gondii*.

Resumo
Este trabalho objetivou analisar a morfologia e a morfometria dos neurônios das regiões cervical, torácica e lombar da medula espinhal de cães assintomáticos soropositivos para toxoplasmose. Utilizaram-se 20 cães sem raça definida, adultos, sendo dez cães hígidos, com sorologia negativa, utilizados como controle (grupo 1) e

dez cães assintomáticos mas soropositivos para toxoplasmose (grupo 2). Após microtomia semi-seriada, com intervalos de 100 micrômetros (µm), os cortes histológicos de medula espinhal, à espessura de 5 µm, foram corados pelas técnicas da hematoxilina-eosina e do tricrômico de Masson. As lâminas foram analisadas à microscopia de luz para verificar a morfologia dos neurônios. Para o estudo morfométrico, os parâmetros analisados foram: área, perímetro, diâmetro máximo, diâmetro mínimo e fator de forma do citoplasma e núcleo dos neurônios. Os resultados obtidos foram analisados estatisticamente, mediante o teste t de Student ao nível de 5% de probabilidade. As características morfológicas entre os dois grupos foram semelhantes e em conformidade com a literatura. Os resultados morfométricos demonstraram que há alteração no tamanho e estrutura dos neurônios, com aumento e perda do formato estrelado nos animais soropositivos. Os resultados sugerem que os neurônios destes cães, ainda que assintomáticos, possam ter perdido sua função condutora.

Palavras-chave: canino; histologia; sistema nervoso central; *Toxoplasma gondii*.

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Introduction

Inflammatory diseases of the central nervous system (CNS) are important causes of neurologic disorder in dogs[1]. Although neurologic canine diseases caused by protozoa are not very common[2], *Toxoplasma gondii (T. gondii)* and *Neospora caninum*[3] are the agents normally responsible for the cases of encephalitis caused by protozoa.

Canine toxoplasmosis has captured researchers' attention since it was described in 1910 by Mello in Italy[4] and by Carini[5] in Brazil, and the desease's life cycle and different modes of transmission have been extensively discussed in veterinary literature[6]. Dogs are considered very receptive animals for this zoonosis, probably, due to their carnivorous eating habit, which facilitates the ingestion of tissues contaminated by cysts and the contact with sporulated oocysts in contaminated soil[7].

The infection has been noticed in cats and dogs in many countries, demonstrating its cosmopolitan feature. Severe and fatal cases have been reported in spite of clinical manifestations being uncommon[3,6,8,9], and although many animals are serologically positive for toxoplasmosis, few develop clinical signs of the disease[10]. Ataxia, seizures, tremors, cranial nerve disorders, progressive paresis and paralysis are the most common clinical manifestations of CNS toxoplasmosis[11-15]. It is presumed that the disease manifestation occurs by local or systemic immune deficit of the host organism, so that immunosuppressed patients may have primary or recurrent infection[16].

Areas of discoloration, necrosis and cerebellar atrophy have been observed macroscopically in CNS of infected dogs. Lesions seen early in blood vessels consist of endothelial cell proliferation, necrosis, and perivascular cuffing. Neuronal necrosis, mild malacia, and some astrocytosis may be seen. Multifocal leptomeningeal infiltrates of macrophages, plasma cells, and some lymphocytes and neutrophils are found[10]. The most common histopathological alterations in the CNS are characterized by nonsuppurative meningoencephalomyelitis associated with vasculitis, necrosis, malacia and gliosis with possible involvement of peripheral nerves[10,17]. Histopathological evaluation of spinal cords of *T. gondii*-infected mice revealed high counts of infiltrated

inflammatory cells, presence of cysts mostly in the gray matter, neuronal degeneration and hemorrhage, with no obvious changes in the myelin staining intensity or axonal density[18].

The neurons possess a big nucleus with a proeminent nucleolus and distended chromosomes. Cytoplasm of nuclear body, or perikaryon, is rich in ribosome and they might be concentrated in small cytoplasmatic areas, and under optical microscope, we can see granular basophilic bodies, known as Nissl bodies[19,20]. The shape of the cellular body varies greatly[19], but also the size is extremely variable, and according to the type of neuron, it may vary from 3 to 150 μm[19,21]. According to Carvalho et al.[22], the size of cytoplasm and nucleus of the neurons from canine spinal cord is also very variable, depending on the location of neurons. Neurons placed in lumbar area are, statistically, bigger than those found in cervical and thoracic areas. They also mention that these lumbar neurons, statistically, are more rounded shaped than the neurons from other areas, this fact is proved by morphometric parameter analysis cellular shape factor.

It is known that important histopathological changes are observed in the CNS with clinical toxoplasmosis, so the objective of this research was to analyze the occurrence of early changes in the morphometry and morphology of neurons from spinal cord of apparently healthy dogs, but with high serological reactivity to toxoplasmosis, in order to better investigate and document the possible histological changes before clinical signs.

Material and Methods

For the proposed objectives we used twenty indefinite-breed adult dogs, weighing from seven to 15 kg, from the Zoonosis Control Center of Araraquara city, state of São Paulo, Brazil. The samples were collected in 2000 and the studied was concluded by 2007. The serology for *T. gondii* was performed by Enzyme-Linked Immunosorbent Assay (ELISA), with the technique described by Domingues et al.[23], at the Department of Veterinary Pathology of the School of Agrarian and Veterinary Sciences, São Paulo State University, Jaboticabal, Brazil. The reactivity of sera was analyzed in terms of ELISA levels (from 0 to 9) and the animals used in this study were those that presented reactivity 8 or 9. Ten dogs, with negative serology for *T. gondii*, were used as control group (group 1) and ten nonsymptomatic dogs, with levels of reactivity for *T. gondii* over 8, detected by ELISA, were used as reagent group (group 2).

After the formation of groups 1 and 2, fragments of spinal cord corresponding to cervical, thoracic and lumbar area were collected post-mortem, being removed from the medullary canal using a blunt-pointed forceps, and fixed in Bouin's solution during 24 hours and processed, as usual, to be included in paraffin.

After the microtomy, with interval of 100 micrometers (μm), the histological 5-μm-thick cuts were dyed by hematoxylin-eosin (HE) and Masson's trichrome (MT) techniques. The glass slides were analyzed under light microscope using a Olympus BX50 photo microscope.

The morphometric analysis was performed in cytoplasm and nucleus of neurons. The following parameters were analyzed, in micrometers (μm): area (μm²), perimeter (μm), maximum diameter (μm), minimum diameter (μm), and shape factor. The measurement, in μm, of cellular parameters was done using the image analysis software, Image Pro-plus, joined to a binocular microscope, both by Carl Zeiss.

The shape factor was expressed by the mathematic formula $\frac{(\text{perimeter})^2}{(4.\pi.area)}$, which was programmed into the image analysis software. This factor was calculated, indirectly, from the circle perimeter, whose equation is $2.\pi.R$, and also from the circle area, whose equation is $\pi.R^2$. Substituting the perimeter² and the area we obtained:

$$\text{Shape factor} = \frac{(2.\pi.R)^2}{4.\pi.(\pi.R^2)} = \frac{(4.\pi^2.R^2)}{(4.\pi.\pi.R^2)} = \frac{(4.\pi^2.R^2)}{(4.\pi^2.R^2)} = 1$$

The smallest value of this factor was equal one, meaning that the shape of the cytoplasm and/or nucleus was similar to the shape of a circle. When this factor is bigger than one, we understood that the shape of the structure was irregular.

For each one of the parameters analyzed, 30 neurons in each area per animal were measured, totalizing 300 neurons per region of spinal cord in each experimental group. The results were statistically analyzed by Student's t test at 5% probability level ($p<0.05$), using Statistic Analysis Software SAS.

Results and Discussion

The neuron morphological characteristics of cervical, thoracic and lumbar areas from the spinal cord of dogs showed similarities between groups 1 and 2 were compared. The average value of area, maximum diameter, minimum diameter, perimeter, and shape factor of cytoplasm and nucleus of neurons in cervical, thoracic and lumbar areas from spinal cord of dogs are respectively listed in Tables 1 to 5.

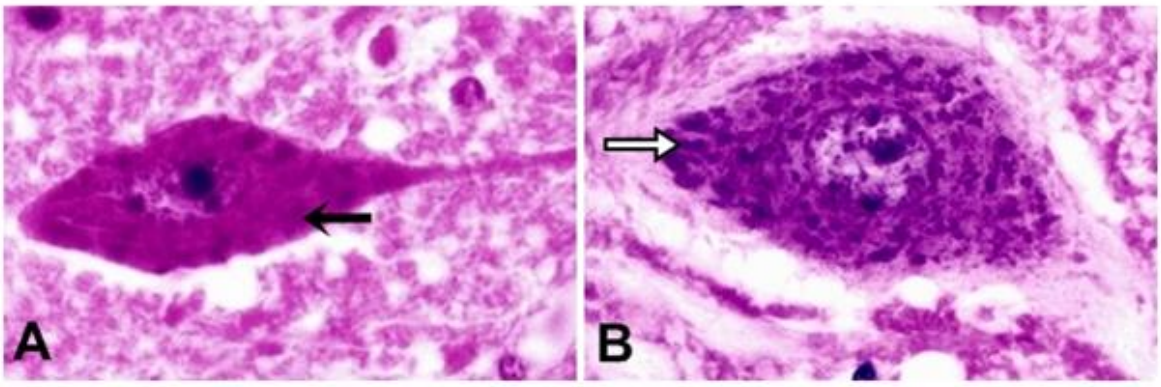

Figure 1: Photomicrographies of neurons from cervical area of spinal cord of control dogs (**A**, HE, 100x) with acidophilic cytoplasm (→); and toxoplasmosis seropositive dogs (**B**, MT, 100x) showing Nissl corpuscles (⇨) in neuron's cytoplasm.

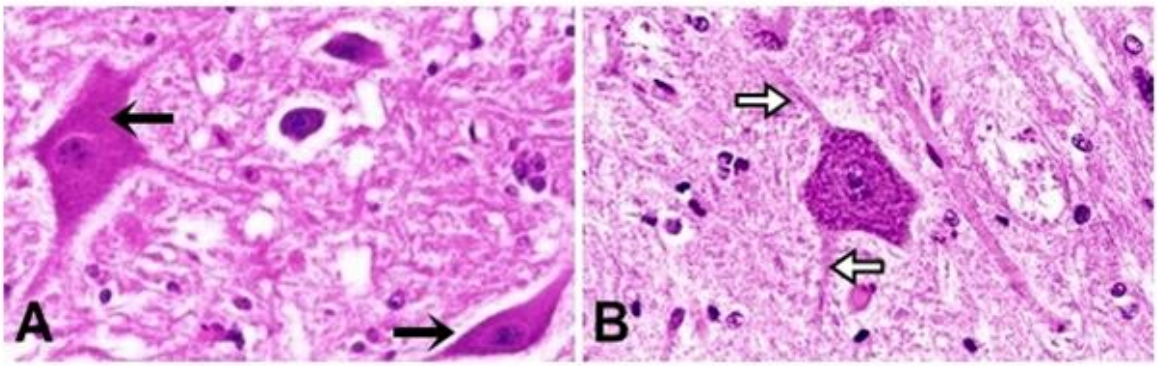

Figure 2: Photomicrographies from thoracic area of spinal cord of control dogs (**A**, HE, 40x) showing neurons with different sizes and shapes (➔); and toxoplasmosis seropositive dogs (**B**, HE, 40x) with neuron projections (⇨).

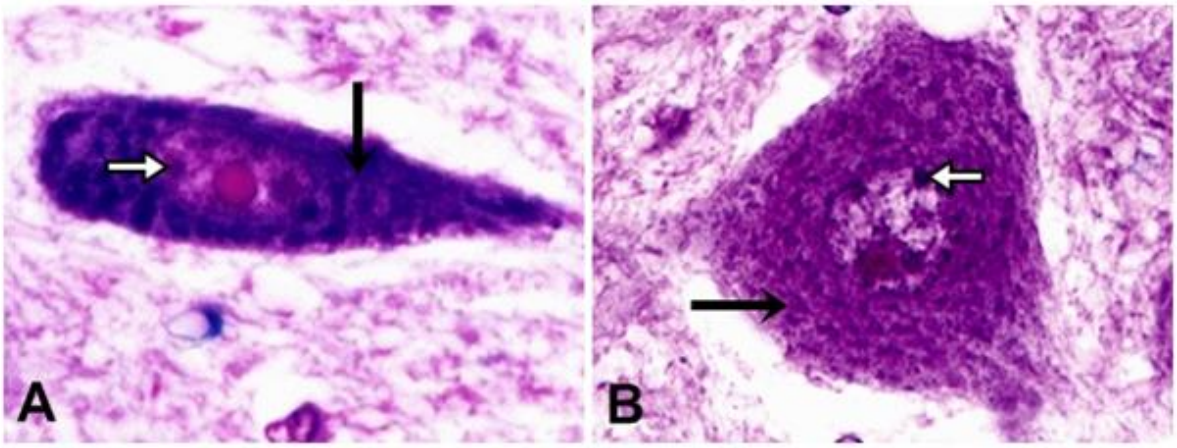

Figure 3: Photomicrographies from lumbar area of spinal cord of control dogs (**A**, MT, 100x) and toxoplasmosis seropositive dogs (**B**, MT, 100x) showing neurons with basophilic cytoplasm (➔) and nucleus with visible nucleolus (⇨).

The nucleus was basophilic and the cytoplasm was acidophilic when stained with HE (Figures 1A, 2A and 2B), whereas the nucleus was acidophilic and the cytoplasm was basophilic when stained with MT (Figure 3). Photomicrography taken from these cells showed, in the different areas, cytoplasm with accumulation of deeply basophilic granules distributed in perikaryon, known as Nissl corpuscles, which correspond to rough endoplasmic reticulum, an organelle rich in polyribosomes (Figure 1B). The perikaryon also presented irregular projections of cytoplasm, providing great variation in shape and size of these cells was observed in both groups (Figure 2).

We observed the nucleus of neurons was generally big, spherical or egg shaped, little stained, with loose chromatin and one or more apparent nucleolus, placed in the center of the cellular body (Figure 3A).

The neuron morphological characteristics from cervical, thoracic and lumbar areas from the spinal cord of dogs belonging to group 2 were preserved, and were similar to the neuron characteristics of dogs in group 1. The results of this study concerning the shape of the cytoplasm, quantity of

projections of cytoplasm, number of visible nucleolus, and the chromatin pattern of these cells, were included, altogether, in the reports of Junqueira and Carneiro[19] and Machado[21].

As stated by Junqueira and Carneiro[19], Ross and Pawlina[20], Machado[21], and Carvalho et al.[24], cytoplasm with accumulation of intensively basophilic granules were noticed, distributed in perikaryon and dendrites, but absent in axon and in its axon hillock.

As for the cytoplasm and neuron nucleus shape, the discoveries from this research agree with Junqueira and Carneiro[19], Machado[21], and Carvalho et al.[24], when they reveal a cellular body with variable shape and a nucleus that is big, spherical, little stained and with loose chromatin and one or more apparent nucleolus.

The cytoplasmic projections, dendrites and axons, visible in this study, occurred in bigger neurons, confirming the findings by Machado[21], who reported that routine histological techniques show only the neuronal body and, in bigger neurons, the initial portions of their projections.

The morphological results indicate that there was no visible morphological change, under light microscope, in neurons of animals belonging to group 2 when they were compared to the animals in group 1, in that occasion, what allow us to conclude that the morphological characteristics observed agree with those described in classic literature.

Table 1: Comparison of the average values (μm²) of the variable cytoplasm area and nucleus of neurons, in cervical, thoracic and lumbar areas from spinal cord of negative (group 1) and seropositive (group 2) animals

	CYTOPLASM		
	Cervical	**Thoracic**	**Lumbar**
Group 1	288.15±55.81 a	262.69±38.76 a	641.01±212.94 a
Group 2	317.97±122.56 a	512.03±187.71 b	982.35±160.94 b
	NUCLEUS		
	Cervical	**Thoracic**	**Lumbar**
Group 1	79.62±6.66 a	73.90±9.56 a	106.25±14.43 a
Group 2	70.31±23.21 a	94.70±34.21 b	174.08±38.83 b

Same letters in same column do not differ by Student's t test ($p>0.05$).

When the cervical area was analyzed no significant difference was observed ($p>0.05$) for cytoplasm and nucleus of neuron, in both groups; however, the average values of thoracic and lumbar areas showed significant difference ($p<0.05$) in both groups, and group 2 presented the highest values for this parameter in these areas (Table 1).

Significant difference ($p<0.05$) was observed in maximum diameter of the nucleus of cervical area and cytoplasm in thoracic area, and the highest average values were found in group 2. There were no significant differences ($p>0.05$) between groups for the average values of maximum diameter of cytoplasm of cervical area, of nucleus of thoracic area, and of both cytoplasm and nucleus of lumbar area (Table 2).

Significant difference ($p<0.05$) was observed for minimum diameter between thoracic and lumbar areas, for both cytoplasm nucleus of neurons. The highest values were found in group 2. There were no significant differences ($p>0.05$) between the groups for minimum diameter of cytoplasm and nucleus of cervical area (Table 3).

No significant difference was observed ($p>0.05$) for perimeter of cytoplasm of cervical area and of nucleus of neuron from thoracic area forboth groups; however, significant difference ($p<0.05$)

occurred between the groups for the perimeter of nucleus of cervical area, of cytoplasm of thoracic area, and for both cytoplasm and nucleus of lumbar area (Table 4).

Table 2: Comparison of the average values (μm) of the variable maximum diameter of cytoplasm and nucleus of neurons, in cervical, thoracic and lumbar areas from spinal cord of negative (group 1) and seropositive (group 2) animals

	CYTOPLASM		
	Cervical	**Thoracic**	**Lumbar**
Group 1	46.52±4.81 a	40.25±4.76 a	56.75±6.99 a
Group 2	41.54±4.95 a	48.17±5.69 b	59.53±5.60 a
	NUCLEUS		
	Cervical	**Thoracic**	**Lumbar**
Group 1	15.19 ±1.15 a	14.22±1.43 a	16.25±1.07 a
Group 2	12.85±1.23 b	14.55±1.03 a	17.46±1.32 a

Same letters in same column do not differ by Student's t test (p>0.05).

Table 3: Comparison of the average values (μm) of the variable minimum diameter of cytoplasm and nucleus of neurons, in cervical, thoracic and lumbar areas from spinal cord of negative (Group 1) and seropositive (Group 2) animals

	CYTOPLASM		
	Cervical	**Thoracic**	**Lumbar**
Group 1	11.83±1.25 a	11.62±0.95 a	19.32±3.82 a
Group 2	12.82±2.93 a	17.19±4.54 b	27.08±4.14 b
	NUCLEUS		
	Cervical	**Thoracic**	**Lumbar**
Group 1	6.05±0.54 a	5.84±0.38 a	7.59±0.74 a
Group 2	6.19±1.69 a	7.57±2.31 b	11.86±1.94 b

Same letters in same column do not differ by Student's t test (p>0.05).

Table 4: Comparison of the average values (μm) of the variable perimeter of cytoplasm and nucleus of neurons, in cervical, thoracic and lumbar areas from spinal cord of negative (Group 1) and seropositive (Group 2) animals

	CYTOPLASM		
	Cervical	**Thoracic**	**Lumbar**
Group 1	133.92±12.92 a	113.18±12.04 a	160.36±21.36 a
Group 2	120.92±16.82 a	138.74±22.33 b	180.31±20.73 b
	NUCLEUS		
	Cervical	**Thoracic**	**Lumbar**
Group 1	36.66±1.61 a	34.51±3.00 a	40.48±2.79 a
Group 2	32.16±4.09 b	36.96±4.76 a	48.54±5.21 b

Same letters in same column do not differ by Student's t test (p>0.05).

Table 5: Comparison of the average values of the variable shape factor of cytoplasm and nucleus of neurons, in cervical, thoracic and lumbar areas from spinal cord of negative (Group 1) and seropositive (Group 2) animals

	CYTOPLASM		
	Cervical	**Thoracic**	**Lumbar**
Group 1	5.55±1.27 a	4.34±0.55 a	3.54±0.62 a
Group 2	4.23±0.83 b	3.36±0.43 b	2.82±0.62 b
	NUCLEUS		
	Cervical	**Thoracic**	**Lumbar**
Group 1	1.40±0.12 a	1.35±0.08 a	1.27±0.04 a
Group 2	1.27±0.09 b	1.25±0.13 a	1.13±0.06 b

Same letters in same column do not differ by Student's t test ($p>0.05$).

Significant difference ($p<0.05$) was noticed in shape factor in the three areas for both cytoplasm and nucleus of neurons, in most of comparisons, except shape factor of nucleus of thoracic area, where there was no statistic difference ($p>0.05$) between the groups. The lowest average values were found in group 2, indicating that the neurons of these animals are more rounded shaped than the neurons of animals in group 1 (Table 5).

Concerning the morphometric analysis, we could observe that the neurons of animals belonging to group 2 showed statistically significant differences when compared to the neurons of dogs belonging to group 1. They had higher average values, suggesting that these neurons might be suffering process of edema, making the cell bigger, due to the presence of the parasite inside the neuron, as reported by Summers et al.[25] and Giraldi et al.[17] about dogs with chronic toxoplasmosis with neurological signs, although the most common histopathological changes in CNS are characterized by perivascular cuffing associated to vasculitis, necrosis, malacia, and focal gliosis[11,17]. The characteristics described were different from those of Möhle et al.[18], who observed high counts of inflammatory cells, cysts mostly in the gray matter, neuronal degeneration and hemorrhage in different segments of spinal cord of *T. gondii*-infected mice.

Regarding the average values of shape factor of cytoplasm and nucleus of neurons, we observed more rounded-shape neurons in animals of group 2, as they presented the lowest average values, which can be attributed to the possible cellular edema that gave these cells a rounder shape. Therefore, we could conjecture that these neurons lost their characteristic star shape, because, with support by Summers et al.[25], chronic toxoplasmosis caues areas with cellular edema, as well as loss of division between gray and white matter.

Conclusions

Although the morphological results have not showed differences between the groups and agree with those findings already described in classic literature, the morphometry demonstrated that the neurons of seropositive nonsymptomatic animals presented higher average values than those belonging to control group for all parameters studied, except for shape factor, which were lower. These observations indicate that there were changes in size and structure of these cells, occurring

enlargement and loss of star shape, probably due to a cellular edema process. These results suggest that the neurons of these dogs, although nonsymptomatic, can have lost their conductor function.

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