Experimental and clinical evaluation of the cerebrospinal fluid with impact in some canine central neuropathies

(SUMMARY OF THE PHD THESIS)

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INTRODUCTION

The cerebrospinal fluid (CSF) is a plasma ultrafiltrate, which fills the ventricles and the subarachnoid space of the central nervous system (CNS). This is in intimate contact with the glymphatic system, a less-known structure of the blood-brain barrier (LEE, 2021). CSF analysis is considered a less specific diagnostic test in neurological conditions but was proved valuable as a complementary test to reach a diagnosis (BAILEY, 1985; LAMPE, 2020). The clinical utility of the CSF analysis has been evaluated in recent studies, such as in intervertebral disc disease, degenerative myelopathy, steroid-responsive meningitis-arteritis, idiopathic epilepsy, primary brain tumors, immune-mediated or infectious meningoencephalitis. This was proved to be useful in the diagnosis of inflammatory brain disease, but less useful in epilepsy or in primary brain tumors (SRUGO, 2011; OJI, 2007; MAIOLINI, 2012; GONCALVES, 2010; O'NEILL, 2005). In the human literature, CSF evaluation is recommended only in a few neurological conditions, such as intracranial hemorrhages, leukemia, meningitis associated with encephalitis/myelitis, or multiple sclerosis. Based on a joint understanding of physiology and pathology, and the continuous desire to improve daily veterinary practice, we aimed to evaluate experimentally the distribution of carbon nanotubes in the CNS, and the clinical utility of CSF analysis in certain neurological diseases.

AIM AND OBJECTIVES OF THE THESIS

The aim of the research carried out in the chapters of this doctoral thesis, can be summarized in the implementation of a rabbit model in the investigation of the glymphatic system in mammals, and in the experimental and clinical evaluation of the CSF testing in some neurological conditions and canine vestibular diseases, respectively.

The general objectives of this doctoral thesis can be summarized as:

- Evaluation of the potential use of carbon nanotubes as a marker in the investigation of the glymphatic system in mammals;
- Development of procedures for macroscopic detection and characterization by optical microscopy of the distribution spectrum of carbon nanotubes in the subarachnoid space;
- The investigation by immunohistochemical procedures of the toxicity of carbon nanotubes and characterization of their biocompatibility;
- Analysis of the relevance of the CSF examination, as a complementary test in the functional investigation of the CNS and the diagnosis of some canine neurological conditions;
- Associating specific diagnostic tests of CSF analysis to characterize the

clinical evolution of some neurological conditions;

- Analysis of CSF changes in dogs with vestibular disease and their use in the characterization of some clinical forms of vestibular syndromes;
- Evaluation of the level of sensitivity and specificity of CSF in differentiating some clinical forms of peripheral and central vestibular diseases.

THESIS STRUCTURE

This doctoral thesis, entitled „**Experimental and clinical evaluations of cerebrospinal fluid with impact in some canine central neuropathies**" is written and structured accordingly to the recommendations of the USAMV Doctoral School and the Faculty of Veterinary Medicine from Cluj-Napoca, being approved by the Bioethics Committee in the field, via Decision 226/03.08.2020. The thesis included a total of 120 pages, of which 39 (32.5%) belong to the first part, composed of 3 chapters and 81 (67.5%) to the second part, spread over 4 chapters.

PART I - Current stage of knowledge

The first part contains a particularly concise literature review, which synthesizes a comparative analysis of recent literature in the field addressed in the doctoral thesis. Therefore, we focused on the morphophysiology of the glymphatic system, the relevance and methodology of CSF analysis in the diagnosis of CNS diseases with a major impact on canine veterinary neurology, respectively. This current state of knowledge outlines a real monograph structured into three chapters.

Chapter 1, entitled "***Current aspects and perspectives on the study of the glymphatic system***" is presented in two subchapters, containing detailed morphophysiology of this system, as a newly defined structure of the blood-brain barrier. Additionally, it contains details of the methods and results of research put into studying this system in mammals.

Chapter 2, "***Actualities regarding cerebrospinal fluid morphophysiology in canines***", composed of two subchapters, where we discuss the morphology and composition of CSF. Additionally, we comparatively analyze the physiological values of the basic parameters, such as total nucleated cell count and protein level and we detail the current knowledge regarding the mechanism of CSF formation and functions, respectively.

Chapter 3, entitled „***The basics and principles of the general methodology used in CSF analysis***", composed of two subchapters, in which we discuss the basics and principles of the general methodology used in CSF analysis, debating CSF investigation techniques and procedures and their clinical relevance. Additionally, we discuss CSF changes in certain canine conditions.

PART II - Personal contribution

Part II includes, according to the requirements regarding the writing of the doctoral thesis, personal contributions, these being grouped into four chapters, focused on the three studies addressed, that emerge from the established objectives.

The personal contribution begins with the formulation of the hypothesis, the general purpose and objectives, the locations, as well as the particularities in which the research was carried out, to obtain the estimated results.

Chapter 4 is dedicated to the materials and methods used in this doctoral thesis. This chapter describes in detail the necessary biological material, followed by the investigation of the „glymphatic system" by implementing the rabbit model. The experimental protocol details the preparation of carbon nanotubes, followed by the methods used for its intrathecal infusion in the rabbit brain model.

Related to this, the histopathological analysis of the rabbit brain was also described. Last but not least, in this chapter we covered the selection criteria for the canine patients as well.

Therefore, we detailed the methodology applied in the sampling and investigation of CSF, followed by tests applied in its cytomorphological and biochemical analysis. In addition, we covered imaging modalities used before CSF sampling. Finally, we describe and explain the statistical analysis used for the studies.

Chapter 5, includes the first study entitled „***Neurological impact of investigating the glymphatic system***", which was based on the use of carbon nanotubes and the rabbit model in the investigation of the glymphatic system, which facilitates the exchange between the interstitial fluid and the CSF. As a new component of the blood-brain barrier, this system is of great interest, because it can contribute to the understanding of many neurodegenerative diseases, in which its alteration is suspected. We mention that such research has been carried out on laboratory rodents and recently on pigs (BECHET, 2021), followed by our study in rabbits.

Although research using carbon nanotubes for drug delivery in neurodegenerative diseases has been carried out for more than a decade, they have not received much attention in the evaluation of the glymphatic system up until now. In our study on five rabbits, approved by the Bioethics Committee, we used carbon nanotubes (MWCNT-PEG) to evaluate its dispersion in the CNS following intrathecal administration. Additionally, we evaluated their toxicity by the use of neuron-specific enolase immunohistochemistry, and S100, as markers.

The procedure consisted in the injection, under general anesthesia, into the Cisterna Magna of 0.5 ml of MCWCNT-PEG. After 4 hours, the rabbits had a complete histopathological examination of the brain (H-E staining and immunohistochemistry markers with NSE and S100).

The results, revealed accumulations of MWCNT-PEG in the subarachnoid space, caudal to the pituitary gland, lateral to the optic chiasm, at the the bulbar pyramids, ventral to the brain stem, cerebellar vermis and cerebellar hemispheres (Fig.1). Histologically, clear agglomeration with MWCNT-PEG was observed at the level of the subarachnoid space and periarterial. MWCNT-PEG was characterized in the form of amorphous, dark conglomerates in various areas of the subarachnoid space (Fig. 2). Immunohistochemically, there was a lack of any type of tissue reaction in the subarachnoid space (Figs. 3 and 4). Upon completion of this study, we concluded that the rabbit model and the nanotubes lend themselves to implementation as a new procedure in the investigation of the glymphatic system, these markers being macroscopically and histologically identifiable following infusion into the subarachnoid space and lacking local toxicity, respectively.

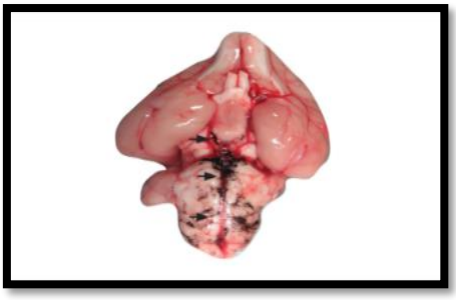

Fig. 1. Highlighting the accumulation of carbon nanotubes in the ventral aspect of the brain and brain stem. Arrows highlight the caudal pituitary region, arterial circle (Circle of Willis; first arrow), the level of the pons (second arrow), and the bulbar pyramids (third arrow).

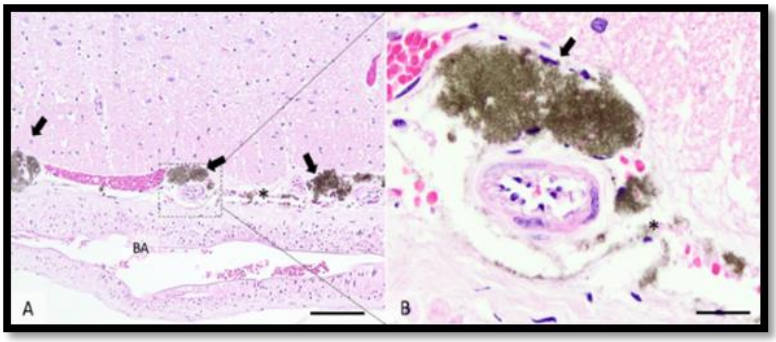

Fig. 2. Brain (brain stem, ventral area). Highlighting the accumulation of carbon particles in the form of aggregates (amorphous, granular, brown-black indicated by arrows), at the level of the periarterial, and subarachnoid spaces (BA-basilar artery). Note the periadventitial accumulation of the carbon nanotubes at the level of collaterals of the basilar artery (Image B, arrow). (Col. H-E, x 20-A; x100-B).

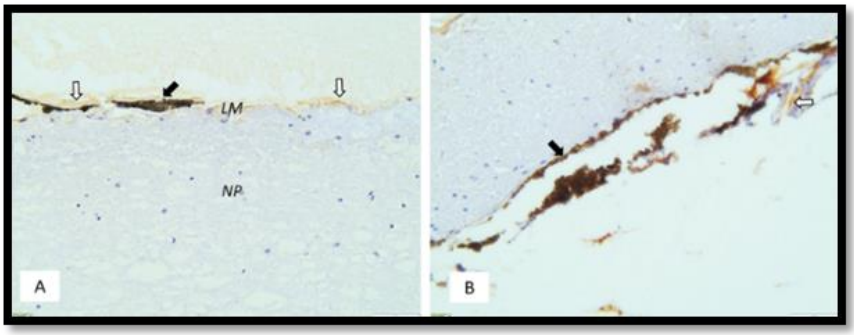

Fig.3. Histological images of nerve and meningeal tissue immunoreaction for S100 protein. At the level of leptomeninges (LM), in the vicinity of the neuropil (NP) (Image A), there is evidence of multifocal carbon nanotube agglomerates (black arrows) (Image B). LM=leptomeninges; NP=Neuropil.

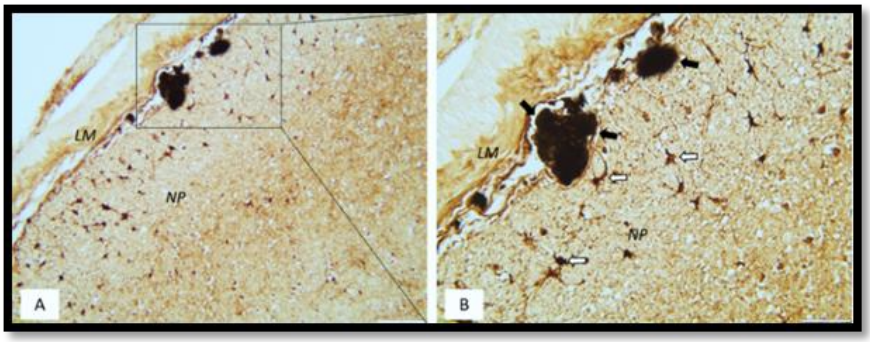

Fig. 4. Histological images of brain tissue immunoreaction for NSE (Neuron-Specific Enolase) (white arrows). At the leptomeningeal (LM) level (Image A), there is evidence of multifocal carbon nanotube clusters (black arrows) focally surrounded by NSE-immunolabelled neurons (white arrows) (Image B). LM=leptomeninges; NP=Neuropil.

Chapter 6, entitled „***Correlative evaluation of CSF in dogs with central neuropathies***", grouped together a set of microscopic evaluations analyzed in the second study focused on the complex investigation of six canine patients with complex neurological pathologies. The hypothesis of this study reiterates the nonspecific paraclinical character of CSF evaluation and the need to correlate it with complementary imaging investigations. The study was carried out over a period of 6 months in the Faculty of Veterinary Medicine Cluj-Napoca clinics and laboratories, grouping mixed breed dogs (n=3), French Bulldog (n=1), Argentinian Dogo (n=1) and Mioritic Shepherd (n=1), between 1.5 and 7 years.

They presented neurological signs compatible with: altered state of mentation, head tilt, lack of menace response, lack of palpebral reflex, circling and cervical hyperesthesia. Corroboration of reported neurological signs, indicated multifocal and focal neuroanatomic localizations. CT scan of the brain revealed a hyperattenuating mass in the right lateral ventricle in one dog. CSF analysis results revealed increases in the median total nucleated cell count (TNCC) at 105 (20-358) cell/μL and total protein (TP) levels at 50 (30-100) mg/dL. CSF cytology revealed the predominance of mixed pleocytosis (monocytic and lymphocytic) in three of the cases, and the remainder had monocytic or lymphocytic pleocytosis. Based on recent literature, monocytic and lymphocytic pleocytosis can be associated with granulomatous meningoencephalitis or Distemper encephalomyelitis (O'NEILL, 2005). Hence, the Distemper virus was confirmed via immunofluorescent testing in one case. In the case of the dog with intraventricular mass and mixed pleocytosis (Fig. 5), choroid plexus papilloma was histologically confirmed.

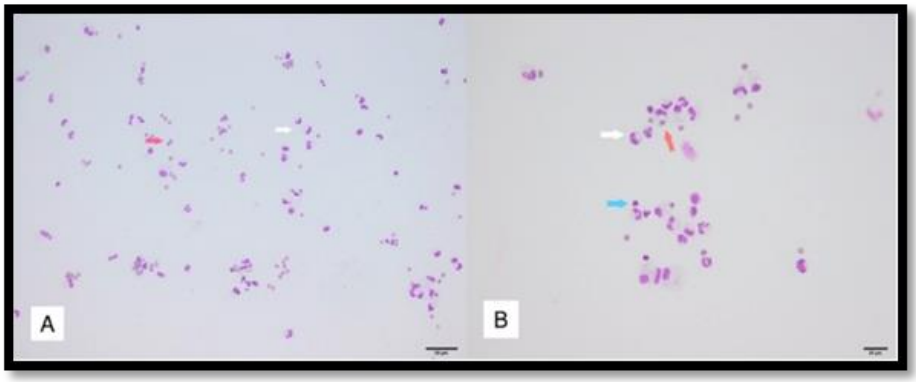

Fig. 5. Cytological changes of CSF indicating: A. Mixed pleocytosis with activated macrophages (white arrow) forming conglomerates next to neutrophils (red arrow); Diff-Quik stain, x50; B. Mixed pleocytosis with phagocytosed erythrocyte material (red arrow), with the predominance of macrophages (white arrow), alongside lymphocytes (blue arrow) (Col. Diff-Quick, x100).

The CSF changes of this patient revealed mixed pleocytosis with activated macrophages forming conglomerates alongside neutrophils and lymphocytes. We also mention that, in the other canine patients, we suspected the evolution of some forms of immune-mediated meningoencephalitis, as revealed by changes in the CSF, reported by other researchers before (O'NEILL, 2005). The results of this study allowed the formulation of some conclusions, which outline the conditions for the application of cytomorphological and biochemical analyzes of CSF in the veterinary clinic.

In this context, we recall the probability that the monocytic and/or lymphocytic pleocytosis shown in the CSF cytology is correlated with the evolution of meningoencephalitis of unknown origin (MUO) or meningomyelitis caused by Distemper viral infection. It is also considered that the corroboration of the data provided by CT and CSF investigations can be useful in clarifying the suspicion of CNS neoplasia.

We appreciate that the information provided by this study is comparable to many of those consulted in the veterinary literature, arguing that CSF analysis is useful in the characterization of inflammatory diseases of the CNS. However, given that CSF has little diagnostic value on its own, its interpretation should be correlated with other ancillary diagnostic tests (MAIOLINI„2012; GONCALVES,2010).

Chapter 7, is dedicated to the third study, entitled "***The relevance of CSF analysis in the characterization of vestibular disease***", also based on published scientific work (DANCIU CECILIA-GABRIELLA et al., 2020). This study is focused on evaluating the value of CSF investigations in differentiating vestibular system dysfunction from central vestibular syndromes and peripheral ones.

The investigations undertaken, it is mainly based on the evaluation of the sensitivity and specificity of CSF analyzes in differentiating some clinical forms of vestibular disease with peripheral localization from those with central localization. In this study, we hypothesized that CSF abnormalities are more frequent in inflammatory vestibular conditions.

This study is retrospective in nature, and was carried out at a single institution (Royal Veterinary College) over an 8-year period. Patients included in this study were divided into eight groups according to their diagnosis: idiopathic vestibular disease, otitis media/interna, neoplasia affecting the middle/inner ear, meningoencephalitis of unknown origin (MUO), brain neoplasia, ischemic infarction, intracranial empyema and metronidazole intoxication.

Thus, a total of 102 dogs met the inclusion criteria. Of these, 53 dogs, with a median age of 69 months (10-192) were diagnosed with central vestibular syndrome, and 49 dogs, with a median age of 101 months (21-163) were diagnosed with peripheral vestibular syndrome. No statistical significance was found between age and location of the vestibular syndrome ($p>0.05$). The frequency of diagnosed conditions had the following distribution: idiopathic vestibular disease (44; 43.13%), MUO (36; 35.29%), ischemic infarct (8; 7.84%), otitis media/interna (4; 3.92%), brain tumor (4/102, 3.92%), intracranial empyema (3/102, 2.94%), metronidazole intoxication (2; 1.96%) and neoplasia affecting the middle/inner ear (1; 0.98%). The TNCC of the CSF ranged from 0 to 2480 cells/μL.

As expected, dogs diagnosed with central vestibular syndrome had significantly higher TNCC (p=0.001) reaching 201 (0-2480) cells/μL, compared to those with peripheral vestibular syndrome, in which the average was only 2 (0-14) cells/μL.

Pleocytosis was also identified in 26 dogs (49%) with central vestibular syndrome and in 5 dogs (10%) with peripheral vestibular syndrome. From a statistical point of view, pleocytosis was significantly increased (p=0.003) in dogs with central vestibular syndrome.

The total protein in CSF varied between 0 and 253 mg/dL, and the value in dogs with central vestibular syndrome (50;12-253 mg/dL) was significantly higher (p=0.004) than in those with peripheral vestibular syndrome (23; 9-66 mg/dL). CSF TP increased in 31 dogs (58%) with central vestibular syndrome and in 19 dogs (38%) of those with peripheral vestibular syndrome.

The difference between the two categories is statistically significant (p=0.046). When estimating the sensitivity and specificity of the CSF TNCC to differentiate central from peripheral vestibular syndrome, we assigned values of 49% and 90%, respectively, based on the level of increase of this parameter. Comparatively, the sensitivity/specificity of the CSF TP to differentiate these two syndromes was also estimated to be 58% and 39%, respectively.

Our results suggest that the CSF analysis is not intended for the express differentiation of the two forms of vestibular syndrome, but nevertheless, it can reveal useful information in clarifying specific etiologies.

Thus, the values of TNCC and TP were more elevated, and the CSF cytology revealed a predominance of lymphocytic pleocytosis in cases diagnosed with MUO, and macrophagic activation in those diagnosed with vestibular idiopathic disease and ischemic infarction.

We believe that CSF analysis may be more frequently changed in dogs with inflammatory conditions of the vestibular system and rarely in dogs with non-inflammatory causes of vestibular disease.

The general conclusions and recommendations, derived on the basis of the grouped analysis of the obtained results, constituted a succinct assembly that completes the content of the thesis.

The originality and innovative contributions of the thesis, summarize the main novelties and elements of originality brought by the studies undertaken, analyzing the innovative character of the experiments, tests and clinical evaluations carried out, as well as the contribution brought to the enrichment and diversification of knowledge in the field.

References include a rich set of titles (221), appropriate to documentation, results obtained, discussions and interpretations performed.

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