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Effect of time and autologous serum addition on the analysis of cerebrospinal fluid in horses

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Abstract

Background: Cerebrospinal fluid (CSF) is highly labile and delayed processing might alter results of analysis.

Hypothesis/Objectives: To determine the effects of time and addition of autologous serum on cytological evaluation of CSF.

Animals: Ten client-owned adult horses requiring euthanasia.

Methods: Prospective study. Serum and CSF were collected from each horse before and within 10 minutes after euthanasia. CSF samples were divided into 15 aliquots (2 mL each); 1 aliquot was submitted for routine CSF analysis within 60 minutes of collection. Four drops of autologous serum were added to 7 of the aliquots, and stored at 4°C (serum group); the remaining 7 samples were stored unaltered at 4°C (control group). Total nucleated cell count (TNCC) and cell morphology score were done at T4, T8, T12, T24, T48, T72, and T96 hours after collection. Protein concentration was measured in the control group at T0 and T96 hours.

Results: The cell morphology scores were significantly different in the control group at T48 (median 2, range 0-4), T72 (2, 0-4), and T96 (3, 0-4) in comparison to T0 (1). No change was observed in the serum group. TNCC remained stable over time in both groups. No statistically significant difference in CSF protein concentration was found between T0 and T96.

Conclusions and Clinical Importance: The addition of autologous serum to an aliquot of CSF sample before shipping improves the preservation of cell morphology up to 96 hours after collection.

KEYWORDS
autologous serum, cerebro, CSF, horse, spinal fluid, spinal fluid

1 | INTRODUCTION

Cerebrospinal fluid (CSF) analysis is an essential diagnostic tool in horses with suspected neurologic disease.[1] Evaluation of total

Abbreviations: AO, atlanto-occipital; C1-C2, C1-C2 intervertebral space; CSF, cerebrospinal fluid; DCC, differential cell count; FCS, fetal calf serum; LS, lumbosacral; RBCC, red blood cell count; T, time; TNCC, total nucleated cell count; TP, total protein.

nucleated cell count (TNCC), differential cell count (DCC), cell morphology, and CSF protein concentration can provide important information to aid in the differentiation of degenerative, traumatic, inflammatory, neoplastic, and infectious conditions.[2] Different sites of collection are described in horses.[3-5] Time lapse between sample collection and analysis can be variable, ranging from a few hours to 24 hours, or even a few days if the sample is transported to an off-site specialized diagnostic laboratory. Cerebrospinal fluid is a physiologically hypotonic fluid, and its normal low protein and lipid concentration is thought to contribute to its cellular instability and labile nature.[6] In neurologically abnormal dogs, there are significant changes in differential cell percentages and concentration, as well as cell morphology deterioration over time in analysis of unaltered CSF, while protein concentration remained stable.[7] Samples with protein concentration >50 mg/dL are less susceptible to deterioration than those with lower protein concentration.[7] Different methods for extending the diagnostic life span of CSF samples are described such as addition of hydroxyethyl starch (hetastarch), fetal calf serum (FCS), formalin, ethanol, or autologous serum.[7,8] While hetastarch or FCS might not be readily available in a field situation, autologous serum is easy and practical to obtain.[9] Therefore, the aim of this study was to prospectively determine the effect of time and the presence or absence of autologous serum as a stabilizing agent of CSF on routine analysis. We hypothesized that in the unaltered control samples, cell morphology evaluation would deteriorate over time, whereas TNCC and total protein concentration would remain stable. We also hypothesized that addition of autologous serum would improve the preservation of cell morphology, one of the fundamental aspects for a CSF sample to be considered diagnostic.

2 | MATERIALS AND METHODS

2.1 | Animals

Ten adult horses (7-20 years of age, mean 10.8 ± 4.2) from the William R. Pritchard Veterinary Medical Teaching Hospital or part of the research herd from the Center for Equine Health at the University of California Davis that required euthanasia based on published guidelines and not for the purpose of this study were included.[10] There were 4 females and 6 males. Breeds included 5 Quarter horses, 4 Warmbloods, and 1 Icelandic horse. Seven horses were euthanized because of neurologic disease as follows: progressive moderate to severe spinal ataxia caused by cervical vertebral compressive myelopathy (n = 4), equine protozoal myelitis because of *S. neurona* infection (n = 1), intractable idiopathic epilepsy (n = 1), and suppurative meningoencephalitis (n = 1). The 3 horses without neurologic disease had chronic laminitis (n = 1), cardiomyopathy with ruptured chordae tendineae of the mitral valve (n = 1), and occlusive granular cell tumor in mainstem bronchi (n = 1). No Institutional Animal Care approval was required for our study and owner consent was obtained for the collection of samples.

2.2 | Euthanasia protocol

Six milliliters of blood were collected in plain serum tubes from the right jugular vein before sedation, allowed to clot, and centrifuged to obtain serum. As per routine protocol for euthanasia at our institution, an intravenous catheter was placed in the jugular vein, and horses were sedated with xylazine hydrochloride at 1.1 mg/kg IV (XylaMed 100 mg/mL, VetOne, Delray Beach, FL). Euthanasia was performed using a combination of pentobarbital sodium (390 mg/mL) and phenytoin sodium (50 mg/mL) at a dosage of 100 mg/kg IV (Euthasol, Virbac AH, Inc., Fort Worth, TX) and confirmed by the absence of heart beat and corneal reflex.

2.3 | CSF collection and analysis

The atlanto-occipital region was clipped and scrubbed with dilute betadine and alcohol, and 50 mL of CSF per horse were collected within 10 minutes from euthanasia using a 3.5-in. 18 gauge spinal needle as previously described.[3] Two milliliters of CSF were used for determination of initial TNCC, DCC, red blood cell count (RBCC), protein concentration, and cytocentrifuge slide, all done within 60 minutes of collection (T0).

Fourteen 2 mL aliquots of CSF per horse were distributed into plastic cryovial tubes (Self-standing Cryovial, Genesee Scientific, San Diego, California) and split into 2 groups based on the presence or absence of autologous serum. Seven of 14 aliquots were left unaltered without the addition of autologous serum and designated as the control group. Four drops of autologous serum (~200 μL) were added to each of the 7 remaining aliquots (2 drops of serum per 1 mL of CSF) and designated as the serum group. All 14 aliquots were kept refrigerated at 4°C until further analysis at 4, 8, 12, 24, 48, 72, and 96 hours after collection. TNCC and slide preparation for morphologic evaluation at each time point were done after gentle mixing of the aliquot tube by 2 of the authors (blinded for review). The TNCC at 4, 8, 12, 24, 48, 72, and 96 hours after collection was determined using a hemacytometer, counting both sides of the chamber. CSF was loaded into the hemacytometer from a standard nonanticoagulated microhematocrit tube that was first coated with new methylene blue (New Methylene Blue "N", Ricca Chemical Company, Arlington, Texas) to stain nucleated cells in the sample as described.[6]

2.4 | CSF morphologic evaluation and scoring

Slides for morphologic evaluation were prepared by cytocentrifugation (Shandon Cytospin 3, Thermo Fisher Scientific, West Sacramento, California) for 5 minutes at 100 rpm using glass slides (Fisherbrand, Fisher Scientific, Pittsburg, Philadelphia) and disposable plastic chambers (Single CytoSep Cytology Funnels, Electron Microscopy Sciences, Hatfield, Philadelphia). At each time point 500 μL of CSF were used for slide preparation, followed by Wright-Giemsa staining using an

automated cell stainer (Model 7151 Wescor Aerospray Hematology Pro; ELITech Bio-Medical Systems, Logan, Utah).

Protein concentration was evaluated in samples without the addition of autologous serum at T0 (controls) and between 72 and 140 hours after collection using a pyrogallol red microprotein assay (Total Protein [Micro] Assay, DCL, Oxford, Connecticut).

Morphologic scoring was performed by a single board-certified clinical pathologist (blinded for review) blinded to all sample information, as previously described.[7,11] Briefly, all 140 slides were randomized, and then numbered consecutively with a piece of opaque tape that covered the identifying information (time and treatment group) on the frosted end. Each slide was then assigned a morphology score from 1 to 4: (a) no appreciable deterioration in cellular morphology; (b) mildly degenerated cellular morphology, but cell types easily recognizable; (c) moderately to markedly degenerated cellular morphology, but cell types still recognizable; (d) severely degenerated cell morphology, with 95% unrecognizable cells. If there was variation in the degree of degeneration within or between different cell types on a given slide, the morphologic score was assigned according to the most severe change. Once morphologic scoring was completed, the tape covering the identifying information on the frosted end was removed, and the scores were compiled.

2.5 | Statistical analysis

Data analysis was performed using JMP Pro 15.2.0 (SAS Institute, Cary, North Carolina). Normal distribution of the data was assessed using Shapiro-Wilks Normality test. To assess the effect of the treatment (addition of the autologous serum) on cell morphology score and TNCC over time (repeated measurement), a nonparametric Friedman's test was used for each group separately (control and treatment groups). When significant differences were identified, a Steel post hoc test was applied to compare each timepoint to the baseline (T0). The protein concentrations were compared between T0 and T96, using a Wilcoxon signed-rank test. Additionally, samples were divided based on their baseline (T0) protein concentration to high and low groups[7] (<50 mg/dL and >50 mg/dL) and data were re-analyzed for the high and low protein groups separately, using Friedman's test, followed by Steel test. Significance was set at $P < .05$. All data were reported as median with minimum and maximum range.

3 | RESULTS

3.1 | Efficacy of autologous serum as stabilizing agent

3.1.1 | Total nucleated cell counts

Comparison within and between unaltered (control) and autologous serum added (serum) samples showed no significant differences in TNCC at all time points. The TNCC of control samples ranged from <1 to 2800 cells/μL (median = 1.5) at T0 (baseline), and from <1 to 3000 cells/μL (median = 1) at T96. The TNCC of the serum group ranged from 1 to 3000 cells/μL at T96 (median = 1). Upon removing the 1 horse with the highest TNCC because of suppurative meningoencephalitis, the median and range were 1, 1-5, respectively at T0, and 1, 0-3 at T96 in the control group, and 1, 0-4 for the serum group at T96 ($P > .05$). Autologous serum was not added to the CSF sample with a TNCC of 2800 cells/μL because the protein concentration was already markedly increased at 1.1 g/dL, and the rationale of adding serum was to produce an increased protein concentration as a preservative.

3.1.2 | Cell morphology

There was a significant effect of time on the morphology scores ($P < .001$) in the control group (CSF samples stored without autologous serum addition). Comparing each timepoints to T0 demonstrated significant differences ($P = .02$, $P = .008$, and $P = .008$) in morphology score at T48 (median 2, range 0-4), T72 (2, 0-4), and T96 (3, 0-4) in the control group compared to the baseline (T0; 1, 1-1); indicating cell degeneration affecting cytological interpretation. In the group with addition of serum, no statistical difference was observed between any of the tested timepoints compared to T0 morphology scores ($P > .05$; Figure 1).

3.2 | Differential cell count

The majority of the horses in this study had a normal CSF cytology. Consequently, 9 out of 10 CSF samples had just 1 major population of cells (small lymphocytes, that also predominate in nonpathologic CSF samples). This finding limited the value of assessing the DCC over time, which consequently was not done.

3.3 | Protein concentration

The protein concentration at T0 ranged from 29 to 1111 mg/dL (median of 55.5). Horses without neurologic disease (n = 3) had

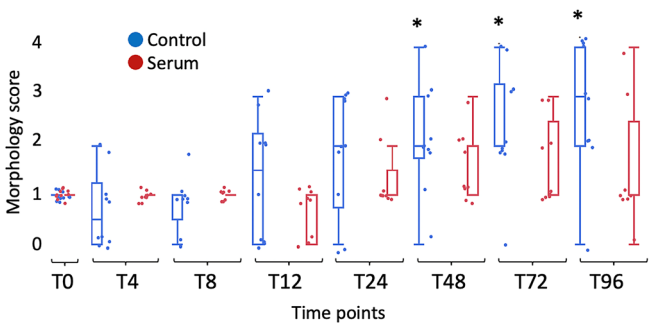

FIGURE 1 The morphology score changes over time in CSF samples with and without autologous serum addition. Comparisons were performed between T0 and subsequent timepoints. There was a significant difference between the morphology scores at T0 and T48, T0 and T72, and T0 and T96 in the control group. Untransformed data are presented for ease of interpretation

normal CSF cytology and protein concentrations within reference limits. All horses with neurologic disease had protein concentrations <90 mg/dL, except for 1 with suppurative meningoencephalitis (1111 mg/dL). There was no statistical difference in protein concentration at T0 and T96 in the control group ($P > .05$). No statistical differences ($P = .74$) were found in cell morphology scores of CSF containing <50 mg/dL (n = 5) and those with >50 mg/dL (n = 5).

4 | DISCUSSION

This study determined some of the effects of storing CSF at 4°C for periods of time before its analysis that might occur in field situations. Without the addition of protein, deterioration of CSF that might result in inaccurate determination of morphologic assessment occurred after 24 hours of storage, while cellular morphologic deterioration was minimized by the addition of autologous serum (2 drops per 1 mL of CSF) and storage at 4°C until further analysis. We investigated these effects for up to 96 hours after collection, also finding that CSF protein concentration does not change over 96 hours.

Based on our results, we recommend dividing CSF into 2 aliquots if time until analysis is uncertain, 1 aliquot being unaltered CSF and the second having autologous serum added. Veterinary field work circumstances, shipping time and delays, and laboratory hours are some of the factors that can influence time elapsing between CSF collection and analysis. Therefore, preservation of CSF samples to maintain the diagnostic value is essential. Unaltered CSF can be used for the determination of TNCC, protein concentration and other protein-based assays such as those used for the detection of antibodies for diagnostic purposes (eg, equine protozoal myeloencephalitis).[12] Although no significant differences in CSF protein concentration were observed between baseline samples and those 96 hours after collection, possible effects on specific antibody testing need to be investigated. The second aliquot with autologous serum added can be used for the preservation of CSF and more accurate interpretation of cytological analysis.

The recommendation to analyze CSF as soon as possible after collection, preferably within 30 minutes,[2,7] is pervasive in the literature, but based mostly on theoretical considerations, not evidence. A delay of 4 to 8 hours from CSF collection to analysis is unlikely to alter the interpretation of CSF in dogs, especially if the protein concentration is >50 mg/dL.[7] At this protein concentration and above, only 6% of cells became unrecognizable because of degeneration when CSF was analyzed within 12 hours.[7] This was in contrast to 33% of unrecognizable cells at the same time point when the CSF protein concentration was below 50 mg/dL in dog samples.[7] The authors concluded the likelihood of cytological misinterpretation because of cell deterioration depended on initial protein concentration and time until analysis.[7] Using cut-off protein concentrations of above or below 50 mg/dL in our study, there were no statistical differences in cell preservation of the sample over 96 hours. However, our testing population was small (10 equine samples vs 30 dog samples) in comparison, and also had less variation in DCC.[7] CSF protein concentrations in normal dogs and cats are lower compared to those of normal horses, and this might help post-collection cellular preservation over time in equine CSF versus dogs and cats.[2,13,14]

The addition of 2 drops of autologous serum (~100 μL) per 1 mL of CSF produced a final serum dilution of 9% to 10%. This dilution was chosen based on the study in dogs in which FCS was added at 20%.[7] Fetal calf serum has about half the protein concentration of normal horse serum, therefore a dilution of approximately 10% was chosen for this study. Furthermore, 2 studies in calves showed that the addition of autologous serum to CSF samples for a final concentration of 11% aided in the cytological preservation of the sample.[6,9] We chose the 2-drop method rather than pipetting an exact volume of serum for ease and simplicity under field conditions.

Although ultrasound-guided C1-C2 thecal puncture is now 1 of the most commonly used approach in horses in field situations and facilities with no diagnostic capabilities, CSF in our study was collected from the AO space because it allowed the collection of a larger volume of fluid to perform the study.

Limitations of this study included the small number of CSF samples used and lack of diversity of disease processes causing CSF cytological alterations, especially those with increased TNCC and varying DCC, to further investigate the effects of collection delay on cell morphology. The low sample size in our study could lead to a potential type II error in our analysis, by not identifying the differences among the different timepoints and T0 in the group with the addition of serum (false-negative). However, in the control group with similar sample size, we found changes in cell morphology in 3 timepoints in comparison to T0. Furthermore, despite no statistical difference noted over time in cellular morphology scores of samples containing autologous serum, some cell deterioration was indeed noticed. Therefore, a larger sample size would be necessary to further assess the clinical relevance of this observation. Also, the effects of increased initial CSF protein concentrations were not fully investigated in this study because only 1 horse had an initial protein concentration above 90 mg/dL. Furthermore, although the overall CSF protein concentration was stable until 96 hours after collection, the effects of time until analysis on specific antibody types routinely measured for diagnostic purposes were not investigated here. Additionally, a study in dogs found that mononuclear cells in CSF deteriorated more rapidly over time than did neutrophils.[7] It is still unknown if this is the case in equine CSF.

In conclusion, equine CSF stored at 4°C can likely be analyzed up to 24 hours after collection without changes in cellular morphology. If the sample cannot be analyzed within 24 hours of collection, autologous serum might be added at 2 drops per 1 mL to a split aliquot of CSF that can be used for slide preparation and morphological assessment. A separate aliquot of CSF without protein addition can have TNCC and protein concentration determination up to 96 hours after collection. CSF samples with and without autologous serum addition should both be kept refrigerated at 4°C. The effects of delayed analysis on specific antibody measurement in equine CSF remains to be

determined and further studies are required to evaluate the influence of morphology score changes on clinical interpretation of the sample.

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CONFLICT OF INTEREST DECLARATION

Authors declare no conflict of interest.

OFF-LABEL ANTIMICROBIAL DECLARATION

Authors declare no off-label use of antimicrobials.

INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE (IACUC) OR OTHER APPROVAL DECLARATION

No IACUC was needed because horses were euthanized due to their medical condition and not for purposes of this study. Owner consent was obtained for collection of samples.

HUMAN ETHICS APPROVAL DECLARATION

Authors declare human ethics approval was not needed for this study.

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