SEED Body Fluids

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Cerebrospinal fluid (CSF): a material with special requirements

As a material for analysis, cerebrospinal fluid (CSF) requires particular attention at all times, beginning with the preparations for and performance of sample collection to the transport and subsequent laboratory diagnostics. Once the cerebrospinal fluid sample arrives at the laboratory, it must be processed as quickly as possible by the laboratory personnel, who must proceed with great care spinal taps are not pleasant and patients won't be pleased about having to repeat them, and the results of the analysis are crucial for the subsequent treatment by the physician.

Indications for cerebrospinal fluid analysis

The analysis of a cerebrospinal fluid sample is generally an important component in the diagnosis or exclusion of:

- inflammations involving the central nervous system
- autoimmune diseases, such as multiple sclerosis
- subarachnoid haemorrhages
- neoplasias with possible infiltration into the central nervous system
- idiopathic epilepsies
- traumas

The afflicted patients exhibit severe physical impairments in places and the suspected diagnoses specified require rapid action. In the process, the laboratory diagnostic analysis of cerebrospinal fluid constitutes an important basis for the physician's decision. Which makes it all the more essential to ensure the rapid and at the same time high-quality processing of the material.

Transport preparation and execution

Cerebrospinal fluid is a material which can only be collected in limited quantities. On the average, laboratory diagnostics requires 5–10 mL, in which roughly 4–5 mL should be estimated for the cell count and differentiation for the established emergency programme, and a minimum of 0.5 mL should be estimated for the clinical-chemical determination of the overall protein, albumin, lactate and glucose. There are various analysis programmes for cerebrospinal fluid diagnostics, most of which can be distinguished as emergency, basic or special analysis programmes. The diagnosis and initial analysis results, such as cell count or clinical-chemical test results, determine the selection of the additional parameters, which extend beyond the scope of the basic programme.

The cerebrospinal fluid should be collected in more than two sterile and sealable plastic tubes (polypropylene tubes are generally recommended, since the cells here exhibit the lowest adhesive powers on the wall of the sample vessel) with or without the addition of preservatives; said tubes should be pre-numbered, labelled with the patient information and marked with the specified time the sample was taken. An instruction book on the proper course of action during sampling and transporting samples is helpful and is provided by laboratory facilities in many places.

The following information addresses the particularities of cerebrospinal fluid diagnostics in the scope of the conventional emergency and basic programme at clinical-chemical laboratories, which comprises the visual assessment of the CSF, the cell count and differentiation and a selection of clinical-chemical analytes.

Visual assessment

Prior to the cell count and differentiation, the cerebrospinal fluid is assessed macroscopically and the haemoglobin content and protein concentration are determined qualitatively using a test strip, although the results here are only semi-quantitative. If test strip analysis is consulted, it is generally done for rough orientation purposes without precise analysis results being available, and it is almost always conducted as a POC test.

Cell count determination

Assuming that the cerebrospinal fluid arrives at the laboratory immediately upon being taken and under suitable transport conditions, i.e. under observation of the temperature range to be adhered to, the cells in the cerebrospinal fluid must be counted without delay. A permissible time frame of 2 hours between the collection of the cerebrospinal fluid and the cell count is sometimes specified, but the cells (especially the neutrophil granulocytes) can already lyse within the first hour, which can decrease or falsify the value of the cell count. The shorter lifespan of the cells in the cerebrospinal fluid, especially that of the granulocytes, is attributable to the significantly lower protein content in the cerebrospinal fluid, among other things. Exposure to light or contact with oxygen can shorten the in vitro half life of granulocytes even more [1]. Alone the time elapsed since the cerebrospinal fluid was collected will cause the cerebrospinal fluid to release its carbon dioxide into the air as soon as it is exposed to it. If protein and cell content are low, the cerebrospinal fluid will be lacking further buffer capacities, which keep the pH value stable. Thus the pH will change from values of 7.32 – 7.36 to more alkaline values up to pH 7.8 within seconds, which causes further cell damage and provokes cell lysis [2].

If no automatic cell counting method is available, the cell count will take place in a counting chamber. The use of the Fuchs-Rosenthal chamber is common practice to do so, since it captures a greater volume than the Neubauer counting chamber, a preference which is recommendable for counting the low cell concentrations in the cerebrospinal fluid. As preparation, the chamber should be clean, lint-free and dry. The cover slide should be positioned so that the Newton's rings are visible. Only then does the space between the cover glass and the chamber ground amount to 0.2 µm. After carefully mixing the cerebrospinal fluid in a plastic tube, the cerebrospinal fluid is filled into the chamber using a pipette. Capillary forces ensure that the cerebrospinal fluid is automatically drawn under the chamber. Care is to be given to make sure that just enough cerebrospinal fluid is drawn up with no air bubbles and that the cerebrospinal fluid does not spill over the edges of the chamber.

The cerebrospinal fluid itself can be filled into the counting chamber without having to be stained first. After a brief sedimentation period (preferably in a moist chamber to prevent drying out), the counting of the cells can begin. In the process, white blood cells and red blood cells are counted without an additional differentiation of the white cell population being conducted or even necessarily being possible. Even the differentiation between red and white blood cells can sometimes be troublesome, especially if the red cells are hardly showing their own colouration or the cells no longer have their textbook appearance. Already swollen or shrunken cells, for example, are undergoing the initial stage of autolysis.

Alternatively, the cerebrospinal fluid can be stained and observed under a microscope after glacial acetic acid is added (ratio 1 + 9). The addition of glacial acetic acid serves in the lysis of the red blood cells and the precise calculation of the white blood cell count. In practice, though, it sometimes happens that the glacial acetic acid does not always cause the red cells to lyse, or only causes lysis in a portion of the red cells, meaning that differentiating between red cells and white cells while counting continues to be difficult. The process of counting white blood cells is complicated in particular if the red blood cells have swollen or become pale from the addition of acetic acid, making them even more difficult to distinguish from the white cells.

The addition of dye solutions such as 0.2 % crystal violet solution or methylene blue should enable improved classification of the white blood cells stained this way, but one will occasionally find that the red blood cells also absorb the dyes, especially if they have already begun the process of lysis. These difficulties may influence the white blood cell count correction, of course, which can decrease in accordance with the red blood cell count (subtraction of 1 WBC/µL per 1,000 RBC/µL).

In order to count the white cells and red cells, the cells are counted in at least 5 group squares of the Fuchs-Rosenthal chamber; if there are fewer than 20 cells in a group square, the cells in the entire chamber should be counted (3.2 µL) in order to improve the quality of the count results [3]. In doing so, however, one must keep in mind that the counted and calculated cell count/µL can still deviate to a certain extent from the real cell concentration in the cerebrospinal fluid being analysed, even if the entire chamber volume of 3.2 µL is evaluated. This means that, even assuming optimum preparation and a trouble-free white blood cell classification process, the counting of 4 cells in a total of 5

group squares (equivalent to 1 µL) in the counting chamber can show a deviation of 50 % from the actual cell concentration in the cerebrospinal fluid (Fig. 1).

A Poisson distribution can be used for the cerebrospinal fluid cells and their distribution in the Fuchs-Rosenthal chamber. The standard deviations and variation coefficients are accordingly defined as:

Standard deviation of the average count result x: $s(x) = \sqrt{x}$
Standard deviation of the total cell count n: $s(n) = \sqrt{n}$
Variation coefficient of the average count result x: $CV(x) = 1/\sqrt{x}$
Variation coefficient of the total cell count n: $CV(n) = 1/\sqrt{n}$

If 5 larger squares of the Fuchs-Rosenthal chamber are counted out and a total of 4 cells are found, then this produces a variation coefficient of $CV(4) = 1/\sqrt{4}$. This means that the count result may deviate from the actual result by as much as 50 %.

This assumes a uniform and optimum distribution of white blood cells in the counting chamber, which is not always the case in reality. Each additional volume counted or each additional cell counted thus contributes to improving the result of the analysis.

For a variation coefficient of...	1 %	2.5 %	5.0 %	10 %	20 %
at least this many cells must be counted:	10,000	1,600	400	100	25

Fig. 1 *Statistical accuracy of a count result depending on the number of cells counted*

Cell morphology

As the steps following the cell count, the differentiation of cerebrospinal fluid cells and the microscopic detection of fungus and bacteria, the latter of which is generally conducted in microbiology, constitute an essential part of the cerebrospinal fluid basic programme. The objective of preparing the slide preparation is to have an optimum cell yield in the microscope's field of vision. All cell populations contained in the cerebrospinal fluid must be present in the slide preparation, without their proportion to one another having changed. Problem-free identification must be possible. This means that the morphology of the cells must not have changed significantly and the cells must be arranged in a tight area, but also in a single layer on the slide. The cells should be prevented from overlapping, especially if there are high numbers of red blood cells present. Meeting these requirements is not always easy.

The two conventional methods of slide preparation production for microscopic cell differentiation are the use of a SAYK chamber or a cytocentrifuge.

SAYK method

A filter card for a cytopreparation is placed on a cleansed slide, and a special weighted chamber is placed on top of it. The chamber is filled with cerebrospinal fluid. After approx. 20 – 30 minutes, the cerebrospinal fluid is absorbed by the filter paper and the cerebrospinal fluid cells can be found as sediment on the bottom of the chamber. This means that the supernatant quantity is no longer available for further clinical-chemical analyses. In general, approx. 200 µL of cerebrospinal fluid are filled into the chamber.

However, if a quantity of cerebrospinal fluid is being analysed in which a small number of cells were counted, the laboratory personnel faces the following challenges: The high cell loss (up to 90 %) which is often reported in connection with the SAYK method makes it difficult to find a sufficient number of cells for microscopic evaluation, fluid samples having a low cell concentration. If the cell count is low, the use of a greater volume would suggest itself as a way of obtaining an adequate cell count for the microscopy in the slide preparation. But this can be hard

to accomplish by filling a greater volume of sample into the SAYK chamber, since the filter card would not be able to absorb the liquid at all. This would not produce a suitable slide for the cerebrospinal fluid microscopy. Instead, one may try centrifuging off the cerebrospinal fluid sample as a preparatory step, and then filling the concentrated cerebrospinal fluid into the SAYK chamber. One will have to put up with potential losses in the quality of the morphology which may result from the cytocentrifugation, especially if the cerebrospinal fluid material has low cell concentrations. For improved cell quality, albumin could be added to the cerebrospinal fluid in order to counteract the rapid autolytic process.

Concentrating cerebrospinal fluid which is low in cells is especially significant in order to avoid missing tumour cells in the cerebrospinal fluid, especially in cases of acute myeloid and acute lymphatic leukaemias (AML and/or ALL). In order to obtain a better slide preparation, at least 5 mL of cerebrospinal fluid to which a suitable medium for promoting cell preservation during centrifugation has been added should be used for the pre-centrifugation.

If the cell concentration is high, on the other hand, a small volume can simply be filled into the SAYK chamber.

If the cells in the cerebrospinal fluid are not yet undergoing the process of autolysis, in the finished slide preparation cells will appear which have hardly been morphologically impaired after staining, which is an advantage of this method.

Cytocentrifugation

Special centrifuge attachments with varying diameters (attachments are selected based on the cell concentration in the cerebrospinal fluid and possibly according to the centrifuge manufacturer's recommendations) are here likewise placed on a cleansed slide, upon which a special filter card for the cytopreparation has been placed. Cerebrospinal fluid is filled into the chambers. The volume of the cerebrospinal fluid to be pipetted in depends on the chamber, its diameter and/or volumetric capacity as well as on the cell count, so that a layer with a sufficient count of adjacent cells can ultimately be seen in the slide preparation. There are often recommendations on the centrifuges used regarding the volume of cerebrospinal fluid used and the chamber to be used.

Even though cell loss from cytocentrifugation is not as great as it is in the SAYK chamber, this procedure does not always yield a slide preparation which permits the simple morphological analysis of the cells. Even if the recommended g-force for centrifugation is complied with, cells may appear to be larger or deformed as a result of centrifugal force. With lymphocytes, for instance, the nucleus may appear more prominent, which complicates distinguishing malign cells from normal lymphocytes. Since finding cells in the cytopreparation can be hindered in different ways, such as through cell loss during sedimentation, uneven distribution in the cytopreparation, morphological impairments or different volumes used, cell differentiation specifications are generally only given in percent form.

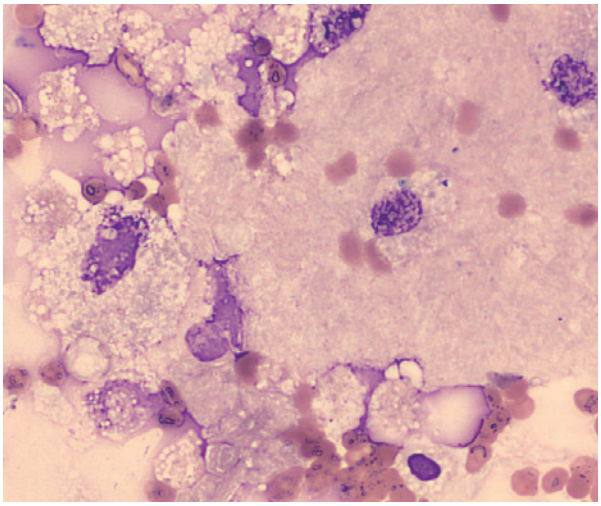

Fig. 2 *Example of a cytopreparation in which the cells have not been preserved well*

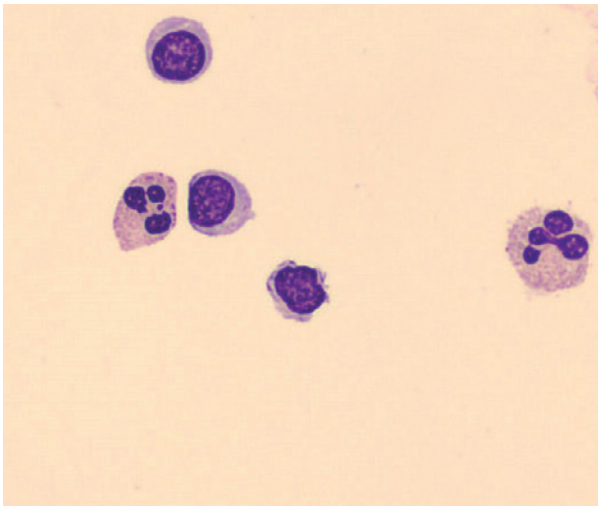

Fig. 3 *Example of a cytopreparation with well-preserved cells*

The cell count and differentiation of the cerebrospinal fluid are supplemented by the results of the clinical-chemical diagnostics.

Clinical-chemical wet chemistry

Lactate and glucose

In healthy individuals, L-lactate present in the cerebrospinal fluid originates from the cerebral parenchyma and serum, in which the lactate value is independent of the serum. There are age-specific reference values, though.

L-lactate in the cerebrospinal fluid is increased in the event of neurological diseases. If the glucose level in the cerebrospinal fluid is simultaneously diminished (depending on the serum level and thus to be determined from both sample materials), suspicion arises of a mycotic or bacterial inflammation involving the central nervous system. Most microorganisms (except for viruses) engage in glycolysis temporarily, in the absence of oxygen, which is normally necessary for their cellular respiration. Glycolysis can take place in the presence or absence of oxygen.
The main steps in the decomposition of glucose up to pyruvate in glycolysis, in the presence or absence of oxygen, are:

- activation of the glucose via glucose-6-phosphate to fructose-1, 6-biphosphate, followed by the division of the C6 body into triosephosphate (2 C3 bodies)
- dehydration to glyceraldehyde 3-phosphate (GADP) and dihydroxyacetone phosphate (DHAP)
- decomposition of the glyceraldehyde 3-phosphate into pyruvate (pyruvic acid)

Under anaerobic conditions, such as in cerebrospinal fluid, the pyruvate generated is reduced to L-lactate via L-lactate dehydrogenase. The salt of the dextrogyratory lactic acid is the final product of glycolysis under anaerobic conditions [4].
Lactic acid fermentation:
$C_6 H_{12} O_6$ ---> 2 $CH_3CH_2OCOO^-$ + energy

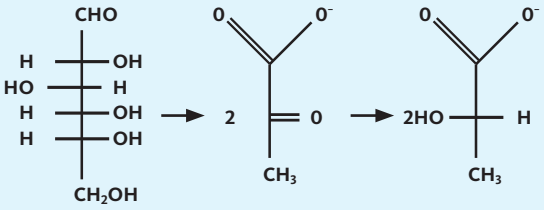

Fig. 4 *Glycolysis under anaerobic conditions*

But the microorganisms are not the only thing that contributes to an increase in L-lactate and reduction in glucose concentration through their metabolic activity in the cerebrospinal fluid as time passes. The red blood cells and granulocytes also fulfil their energy requirements through anaerobic glycolysis. In order to prevent losses in the quality of the analysis, emphasis must be placed on processing the sample material quickly due to this aspect. If this proves impossible, the addition of glycolysis inhibitors to the cerebrospinal fluid must be taken into consideration.

Albumin and total protein

As a rough means of orientation, the total protein determination provides an initial indication of the status of the barrier function. An increase in the total protein in the cerebrospinal fluid is an unspecific indication of a disease of the central nervous system. The supplementary determination of albumin in the cerebrospinal fluid which is exclusively produced in the liver provides helpful information on potential disorders of the blood-cerebrospinal fluid barrier. Diagnostics based on these analytes is hindered in the event of an artificially bleeding puncture. Red blood cells (RBC) and/or haemoglobin present increase the protein result, and with some protein determinations, at over 3,000 RBC/µL the result is no longer even reliable [5].

Conclusion

Considering the aforementioned potential challenges, which are not uncommon, it becomes clear that, as a result of the physiological processes in the cerebrospinal fluid and other factors, the difficulties of cerebrospinal fluid diagnostics can lead to secondary changes to the cell count, the cells themselves and to the clinical-chemical parameters. Additionally, the variability of the results is increased even further, starting with the problems in cell counting or subsequently as a result of cell alterations during cell acquisition, as described using the conventional methods of preparing a cytopreparation as an example, as well as from deviations in staining. When viewed as a whole, the dependence of the quality on the preanalytics, rapid processing and method of processing, among other things, is one of the reasons why automation and standardised analysis are desirable in cerebrospinal fluid diagnostics, even in consideration of all available recommendations.

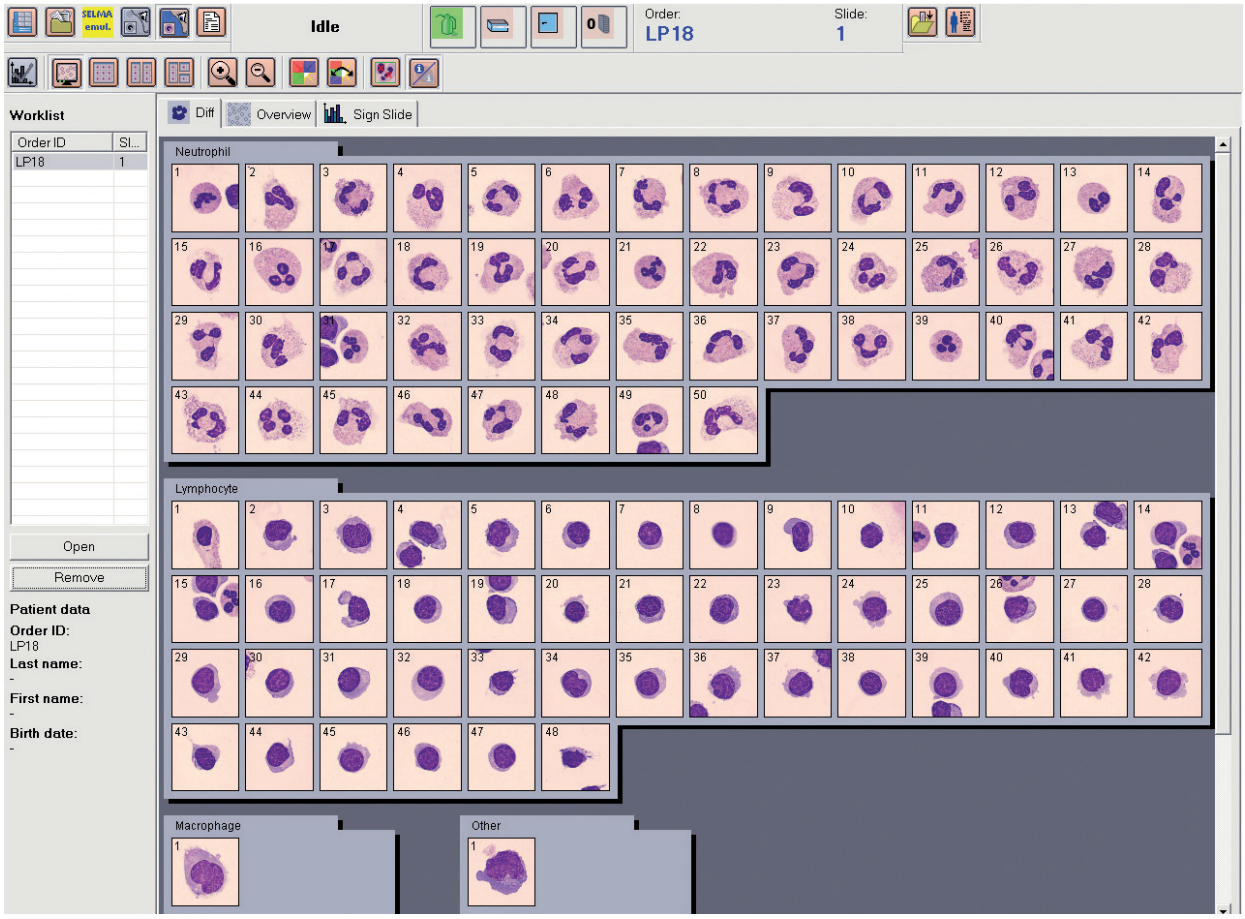

Fig. 5 *Example of the differentiation of cells from cerebrospinal fluid using CellaVision® DM1200*

References

[1] Zettl U, Mix E, Lehmitz R. *(2005): Klinische Liquordiagnostik. De Gryter publisher's 2nd edition (German).*
[2] Kluge H et al. *(2007): Atlas of CSF Cytology. Thieme publisher's 1st edition.*
[3] Wildemann B et al. *(2010): Laboratory Diagnosis in Neurology. Thieme publisher's 1st edition.*
[4] Horton R et al. *(2006): Principles of Biochemistry. Pearson Store 4th updated version.*
[5] Halbach J. *(2006): Klinische Chemie und Hämatologie für den Einstieg. Thieme publisher's 2nd edition (German).*

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