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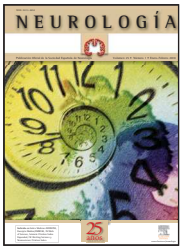

REVIEW ARTICLE

Cerebrospinal fluid cytotoxicity in lateral amyotrophic sclerosis

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KEYWORDS
Lateral amyotrophic sclerosis;
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Abstract
Introduction: The cytotoxicity of cerebrospinal fluid (CSF) in patients with lateral amyotrophic sclerosis in cell cultures that include neurons may be considered as a diffusion mechanism of the disease, due to the proximity of the CSF to the spinal column.
Development: Various literature studies suggest that the motor neurons are more susceptible to cytotoxicity compared to other neuron cells, including glial, in cell cultures. The review of the composition of CSF in lateral amyotrophic sclerosis gives few clues on how this mechanism causes pre-apoptotic and apoptotic changes on the addition on CSF to the cultures, although it could be associated with the glutamate receptors, to a greater extent in those that respond to AMPA/kainate, and have a role in ion channels.
Conclusions: The cytotoxicity of CSF is a peculiarity of lateral amyotrophic sclerosis, which could explain some aspects of how the disease progresses. More studies are required in order to understand more about this mechanism, including better identification of patients from whom samples are obtained, as well as their characteristics, differentiating them into familial or sporadic.

PALABRAS CLAVE
Esclerosis lateral amiotrófica;
Líquido cefalorraquídeo;

La citotoxicidad del líquido cefalorraquídeo en la esclerosis lateral amiotrófica

Resumen
Introducción: La citotoxicidad del líquido cefalorraquídeo (LCR) de pacientes con esclerosis lateral amiotrófica en cultivos celulares que incluyen neuronas puede plantearse como un mecanismo de difusión de la enfermedad, debido a la cercanía del LCR a la médula espinal.

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Citotoxicidad;
Cultivos celulares

Desarrollo: Los diferentes estudios de la literatura indican una mayor susceptibilidad del efecto citotóxico en las motoneuronas, frente a otro tipo de células neuronales y la inclusión de glía en los cultivos. La revisión de la composición del LCR en la esclerosis lateral amiotrófica no permite indicar mediante qué mecanismo se producen cambios preapoptóticos y apoptóticos con la adición del LCR a los cultivos, aunque podría estar relacionado con los receptores del glutamato, en mayor medida, aquellos que responden a AMPA/ kainato, e intervenir en canales iónicos.
Conclusiones: La citotoxicidad del LCR es una singularidad de la esclerosis lateral amiotrófica que podría explicar aspectos evolutivos de la enfermedad. Para el mejor conocimiento de este mecanismo, es necesario que nuevos estudios incluyan una mayor identificación de los pacientes de quienes se obtienen las muestras, así como sus características, y diferenciar si son formas familiares o esporádicas.

Introduction

The possibility that biological materials from patients with amyotrophic lateral sclerosis (ALS), both in its familial and sporadic forms, present some element capable of causing a toxic effect was already described by different authors, who showed the highest cytotoxicity with serum[1-4] or with biochemical or ultrastructural changes in cellular cultures.[5,6] However, subsequent studies could not confirm these effects.[7-11] Cerebrospinal fluid (CSF) toxicity has also been proposed in other neurodegenerative diseases such as Parkinson's disease, where it has been suggested that the CSF of these patients could inhibit the growth of dopaminergic neurons in culture,[12-16] although these studies have not been reproduced. However, the potential adverse effect of the CSF of patients with ALS (ALS-CSF) in various cellular cultures[17-32] has been repeatedly described, although some studies have not found this.[33] Iwasaki et al,[34] in a cell culture from the anterior horn of the spinal cord of embryonic rats, found no differences in the survival produced by ALS-CSF in controls. Gredal et al,[35] in a culture of mouse cortical neurons, did not find differences either in the response to calcium or potassium chloride to ALS-CSF, indicating that no changes take place in calcium homeostasis, which is one of the mechanisms of lesions from excitotoxic mechanism.

Contrary to these few negative studies, a significant number of experiments confirm the cytotoxic effect of ALS-CSF in various cell cultures (table 1). This toxic effect is greater in cultured motor neurons than in those that are not[28] and in cultures containing a higher percentage of glial cells[28] than in those including mostly neurons.[29] The deleterious effect of ALS-CSF may also be mediated by microglia,[25] but Anneser et al[29] have demonstrated the cytotoxic effect in cultures with a very low percentage of these cells. Table 2 shows the studies show ultrastructural changes caused by ALS-CSF in cells, such as an increase in phosphorylation of neurofilaments,[18,19,26] astrocytosis,[21,26,27] vacuolization,[28,31] preapoptotic or apoptotic signs[25,29,30] or signs of cellular death.[28]

Composition of ALS-CSF

The CSF contains proteins and protein fragments discharged by the affected cells, which could serve as biomarkers for neurodegenerative diseases.[36-41] In the case of ALS, regardless of the search for a biological marker, there is an incentive to try to determine what the mechanism is that makes it toxic for cell cultures.[42] The toxic factor could lead to neuronal degeneration directly[24] or be a mediator for the spread of the lesion. The possibility that CSF contains a causal factor is unlikely, but cannot be discarded, because it is considered that the presentation of this disease may be influenced by environmental factors;[43,44] therefore, this factor, which some authors have called mysterious,[42] has been sought extensively.

Glutamate has always been considered as the best candidate to justify a direct effect; different studies have thus focused on the investigation of amino acid neurotransmitters in the CSF. Conversely, it has been found that the glutamate values decrease in severe patients, aspartate values are normal or elevated and glycine values are variable; there are patients with high and low values, while GABA concentration is elevated in patients with advanced or moderate progression.[45] High values of glutamate, aspartate, glycine or beta-N-methylamino-L-alanine were not detected in a study of 17 patients.[46] In another study, the glutamate, isoleucine, leucine, methionine and tyrosine values were normal, while there was an increase in the concentrations of serine, glutamine and alanine in spinal onset patients.[47] The values for kynurenic acid, an endogenous antagonist of AA receptors, appeared significantly elevated in patients, and were higher in more advanced patients.[48] Therefore, although a neurotoxicity mediated by AMPA[17,28] or NMDA[22] receptors of glutamate, as well as by astrocyte GTL1[31] has been suggested to explain the toxic effect of ALS-CSF, it does not seem simple to justify the toxicity from the CSF composition itself. On the other hand, neither Tikka et al[25] nor Anneser et al[29] succeeded in proving that glutamate influences it. Other neurotransmitters and hormones have also been

Table 1 Toxic effect of cerebrospinal fluid (CSF) in amyotrophic lateral sclerosis (ALS) in cell cultures

Author/year	Culture	Result
Courantier et al[17], 1993	Rat neurons in culture	Cellular survival of 47% with ALS-CSF versus 80% of controls
Terro et al[20], 1996	Rat cortical cells	Increase in the rate of neuronal death by almost triple that of controls
Smith et al[23], 1998	VSC-4.1 cells, i.e. cholinergic cells	When the CSF contained elevated values of 4-hydroxinonenal, a lipid peroxidation product produces a 50% increase in cellular death; if there were low contents, there was no increase
Tikka et al[25], 2002	Embryonic rat spinal cells	Reduction of 34% in neuronal survival
SEN et al[28], 2005	Embryonic rat spinal cells	In motor neurons, cellular survival decreased by 50%; in other neurons, by 20%
Anneser et al[29], 2006	Motor neurons from bird embryo spinal cells	Reduction of 10% in cellular survival
Anneser et al[29], 2006	Mixed culture of motor neurons and glia from bird embryo spinal cells	Reduction of 50% in cellular survival. Not modified with the addition of glutamate

Table 2 Ultrastructural or biochemical changes due to the toxic effect of cerebrospinal fluid (CSF) in amyotrophic lateral sclerosis (ALS) in cell cultures

Author/year	Culture	Result
Nagaraja et al[18], 1994	Spinal cells of a bird species embryo	Increase in neurofilament phosphorylation
Rao et al[19], 1995	Spinal motor neurons from newborn rats	Increase in neurofilament phosphorylation in anterior horn but not in posterior horn
Shahani et al[21], 1998	Spinal cells from newborn rats	Strongly GFAP-positive astrocytosis in grey and white matter
Manabe et al[22], 1999	Rat lumbar spinal cells	Increase in Fos-positive cells in posterior horn
Tikka et al[25], 2002	Spinal cells from rat embryo	DNA fragmentation and preapoptotic and apoptotic signs. Neurofilament dephosphorylation
Shahani et al[26], 2004	Administration in rat CSF, and analysis in spinal tissue	Increase in LDH, in NF phosphorylated antibodies and in reactive astrocytes
Anneser et al[27], 2004	Glial cells from bird embryo	Increase in the astrocytic proliferation and expression of vimentin
SEN et al[28], 2005	Spinal cells from rat embryos	Neuritic retraction, cellular oedema, cytoplasmic vacuolisation and even cellular death. Transient, but pronounced, elevation of calcium in motor and non-motor neurons, although higher in the motor
Anneser et al[29], 2006	Motor neurons from bird embryo spinal cells	DNA fragmentation and preapoptotic signs
Ramamohan et al[30], 2007	Spinal motor neurons from newborn rats	Fragmentation of Golgi apparatus in neurons by the ALS-CSF
Shobha et al[31], 2007	Spinal motor neurons from newborn rats	Vacuolated cells at 48 h. Decrease of GLT-1 expression in grey matter astrocytes, normality in GLAST. Increase in LDH activity
Gunasekaran et al[32], 2009	Spinal motor neurons from newborn rats	Decrease in the expression of Nav1.6 channels. Decrease in the expression of Kv1.6 channels

analysed. In patients with moderate and advanced disease, a CSF ultrafiltrate against acetylcholinesterase (AChE) showed that the activity was diminished.[49] Substance P values in the CSF were increased, especially in patients with duration over two and a half years.[50] The CSF content of 3-methoxy-4-hydroxyphenylglycol (MHPG) in patients was found to be elevated, whereas homovanillic acid and somatostatin were normal.[51] In another study, CSF concentrations of vasoactive intestinal peptide (VIP) were lowered, while those of cholecystokinin (CCK) and neural adhesion cell molecule (NCAM) were normal.[52] Another study showed increased CSF values of adenosine, but unchanged neopterin levels.[53] Prostaglandin E values were high compared with the control group, but unrelated to clinical status, ALS type or disease duration,[54] and these data were reproduced in another study.[55] The CSF T3 values were found slightly increased in one study, whereas those of T4 were normal.[56] Diminished CRF values have been detected.[57] S-100 protein values decreased in the CSF of 20 patients,[58] in a similar situation to that found in serum.[59] The cGMP was found to have decreased in CSF, but with no correlation to the clinical state,[60] although another study found no such alteration.[61]

Several authors have proposed the possibility that the toxic factor is mediated by oxidative metabolism.[20,31] Different studies have analysed its markers in CSF. The concentrations of reduced and oxidised forms of coenzyme Q10 (CoQ10) and 8-hydroxy-2'-deoxyguanosine (8-OHdG) have been determined in ALS-CSF. The percentages of ox-CoQ10 and 8-OHdG were higher than in controls and were related to disease duration. There was a correlation between ox-CoQ10 and 8-OHdG in sporadic ALS.[62,63] The ox-CoQ10 form had already been observed in 20 patients with sporadic ALS in one series,[64] and with no increase in another series of 30 patients with ALS.[65] One study analysed the CSF content of a set of oxidative proteins, the total antioxidant capacity by Fe reduction, the number of 4-hydroxynonenal and the sum of nitrites and nitrates as indicators of oxidation products. In this study, the capacity to reduce Fe was decreased in the CSF and the set of oxidative proteins had increased in CSF and plasma, while the amounts of nitrites and nitrates (contrary to what was found in males in another study[66]) and the figures of 4-hydroxynonenal were similar to those of controls;[67] these data were reproduced in another work[68]. This substance was increased in the ALS-CSF samples that produced the highest degree of toxic effect according to Smith et al.[23] Nitrate values increased in another study on sporadic ALS.[69] No increase in the CSF was demonstrated by HPLC in alphatocopherol (vitamin E) ALS.[70] The values of 3-nitroxityroxine were also diminished.[71] The metabolites of nitric oxide were high and superoxide dismutase (SOD) activity was low in sporadic ALS.[72] Erythropoietin in the CSF was also diminished in ALS, as compared with patients with tension headaches and dementias,[73,74] while it was less in patients with rapid progression.[75] F-2 isoprostane, a marker of oxidative neurodegeneration, was normal in ALS and increased in Alzheimer.[76] Comparing the values of hydroxyl radicals, free ascorbate, SOD1 and SOD2 activities and 8-OHdG showed that, in familiar and sporadic ALS, the concentrations of ascorbate and 8-OHdG in the CSF were higher than in controls, whereas SOD activities were lower. The concentration of copper in sporadic ALS was higher than in the controls.[77]

Considering that neurofilament phosphorylation is one of the observations demonstrated in cell cultures after adding ALS-CSF,[18,19,26] and more so for its potential use as a biomarker, there are different studies that have analysed these proteins in the CSF, as well as those influencing aggregation. Light neurofilament proteins in the CSF were elevated in patients compared with controls and inversely correlated with the evolution, while this determination was lower in familiar ALS-SOD.[78] The values of another neurofilament protein, NfHSMI35, were elevated in ALS and more so than in patients with Alzheimer. The NfHSMI35 values in the CSF were higher in patients with upper motor neuron onset and in those with more rapid evolution and did not correlate with tau protein values. Rosengren et al[79] showed that the filament protein was increased in the CSF. In another case, tau values in the CSF were not found to have increased in 18 patients.[80] Other authors found, however, an increased tau value in the CSF of 20 patients with sporadic ALS, especially in the early stages. The amyloid beta protein 42 is reduced in ALS, according to one study, while tau and phospho-tau proteins were normal in CSF[81]; this was reproduced in another study.[80] The NfHSMI35 determination in the CSF was 5 times higher in 69 patients with ALS than in controls and 10 times higher than in patients with Alzheimer; NfHSMI35 was also higher in patients whose onset was due to upper motor neuron lesion, with no correlation with tau values in the CSF, which were also increased.[82] In connection with TDP-43 in the CSF, no notable increases were found in ALS[83] or in ALS-frontotemporal lobar degeneration, although one study showed elevated levels in patients with early-stage ALS, decreasing with the duration of the disease.[84]

The addition of ALS-CSF represents the appearance of apoptotic or preapoptotic changes[25,28-30] in cell cultures. ALS-CSF contains markers associated with apoptosis, such as matrix 9 metalloproteinase (MMP-9); in a study of 24 patients with ALS and 15 controls, this marker was shown to be slightly lower in patients without reaching statistical significance, with a tendency to decrease with evolution.[85] In another study,[86] MMP-2 and MMP-9 values and the MMPs inhibitor, TIMP2, values were normal, while those of TIMP1 were elevated In the evaluation of cytochrome C in the CSF of 40 patients with ALS, it appeared decreased in 46% of patients compared with controls, and there was no significant variation in the serum or a relation with clinical characteristics.[87]

Shahani et al[24] have suggested the possibility that there may be an immunological mechanism to explain ALS-CSF toxicity. Different studies have measured immunological markers in the CSF of patients with ALS. The determination of RANTES (a beta-chemokine), which is a chemoattractant for lymphocytes and monocytes, showed higher values in ALS than in non-inflammatory neurological processes.[88] Flt3 is a cytokine with neurotrophic and antiapoptotic activity, which promotes neuronal survival and has been found significantly elevated in ALS.[89] An increase of CD4 complement in the CSF has been described.[90] Antibodies against anterior horn cells of the spine are present in the CSF of most patients with ALS,[91] and antibodies against

structures of the glia and axons were detected in a high percentage in the CSF of patients, as well as antibodies against cells.[92] Increased titres of antibodies against ganglioside GM1, AGM1 and sulphatides have been observed in a percentage of patients with ALS.[93] The results on the values of interleukin-6 in CSF are discordant, given that for some authors it is elevated,[94] while other studies have found it normal,[95] although it has also been said that interleukin-6 is elevated due to hypoxemia.[96]

Gunasekaran et al[32] have pointed out the potential protective effect of certain growth factors such as BDNF and CNTF. The figures for BDNF are not altered in ALS, in contrast to GDNF, which are increased.[97] In relation to other growth factors, increased levels of monocyte chemotactic protein 1 (MCP-1) have been found in the CSF of patients with ALS, as well as decreased values of vascular endothelial growth factor (VEGF), although without significant demonstration. According to a study proposing that the MCP-1/ VEGF ratio in the CSF could differentiate ALS from other neurodegenerative diseases,[98] MCP-1 is positively correlated with the Norris scale. This protein is also found to be increased in another study.[99] The concentrations of granulocyte colony-stimulating factor (G-CSF) and MCP-1 in the CSF are increased in comparison with controls.[100] In contrast, another study shows that VEGF values in the CSF are increased significantly in long-term diseases and in those with onset at extremities.[101] No correlation was found between VEGF and the degree of hypoxia, although the figures are high in hypoxemic patients.[102] Another study found a decrease in VEGF values in early-stage ALS.[103] The epidermal growth factor (EGF) is reduced in the ALS CSF, according to one study.[104] The beta 1 transforming growth factor can protect neurons from oxidative damage and inhibit apoptosis; its value in the CSF has been found to be within normal limits, although it has appeared elevated in long-term patients.[105] A significant decrease in growth hormones, insulin and IGF-1 was observed in the CSF,[106] as well as a drop in nitric oxide, IGF-1 and IGF-2, which appeared normal.[107]

Discussion

The CSF is located very near many of the pathological lesions that can develop in ALS, especially spinal. For this reason, it may possible to visualise in it specific biochemical changes more easily than in other neurodegenerative diseases. However, this does not justify the fact that CSF could contain a factor that generates pathological processes in unspecific cell models. This would support the assumption that the disease mechanism development may involve more environmental factors than those of genetic susceptibility. Although they may be clinically similar, familial ALS (especially SOD1-related forms) and sporadic ALS may have different mechanisms[108] and a different response to treatment; this could explain the recent failure of clinical trials with minocycline,[109,110] although there may be other reasons.[111] Familial forms associated with SOD1 base the experimental model of the most commonly used transgenic animal for research on the disease.[112] It is not possible to know whether different ALS subtypes have a different cytotoxic activity, since few articles describe this information.[23,25,32] The study by Tikka et al[25] included CSF from 26 patients, of which 5 were familial-SOD1, by D90A mutation; 5, familial non-SOD1; and 16 sporadic. There were no differences among the 3 groups in terms of DNA fragmentation or neurofilament phosphorylation degree, suggesting that the behaviour of the groups in terms of cytotoxicity may be similar. Although some studies suggest that ALS-CSF cytotoxicity does not correlate with disease duration,[25,29] most do not indicate this information, so it is not possible to rule out that the toxic factor may appear or disappear at a given time in the evolution.

An important aspect to consider is the variable susceptibility of cell cultures in which the cytotoxic effect has been observed, since the majority is constituted by cells obtained from embryonic, adult or cortical spinal marrow (tables 1 and 2). In any case, it seems that the toxic effect would have a greater affinity for motor neurons[28] and be enhanced by glial cells in the culture.[25] This observation is consistent with the data of Clement et al,[113] which showed that for neuronal degeneration to occur in the transgenic mouse model with SOD1 mutation, it is necessary for there to be, along with the accumulation of abnormal SOD1 in neurons, participation of non-neuronal cells, which could be through an alteration of glutamate transporter proteins GTL1 and EAAT2.[114-117] This is one of the arguments in favour of cell therapy in the disease.[118] Tikka et al[25] have reported that the cytotoxic effect could be mediated by microglia, consistent with the hypothesis of Boillee et al,[119] although Anneser et al[29] has found it in cultures with a scarce presence of these cells.

The experiment by Anneser et al[29] noted that the toxic factor should have a low molecular weight and be resistant to heat. The proteomics analysis of CSF represents a model for research on neurological diseases,[120,121] is ideal for small molecules and, therefore, could provide additional information. To our knowledge, there are only four proteomic studies on CSF in ALS in the literature. Ramstrom et al[122] found no different protein in the controls using mass spectrometry. Ranganathan et al[123,124] found 3 different proteins, 1 decreased and 1 increased. One of the decreased proteins was cystatin C and the other was transthyretin (TTR); the increased protein was a fragment of the neuroendocrine protein 7B2. Transthyretin has also been found in Alzheimer disease,[125,126] although the decrease is greater in ALS, and related to the final stages. Alpha and beta haemoglobin chains also appear to increase in the CSF of deceased patients obtained at autopsy, probably due to the blood-brain barrier breakdown. In the third study, Pasinetti et al[127] found 3 proteins different from those of the controls. These proteins were identified as cystatin C, which was diminished, one of 4.8 kDa, which could be beta-2-microglobulin (also increased in the Alzheimer disease[128]) and another that was a peptide fragment of neurosecretory protein VGF; Ranganathan et al[124] also made this discovery *in vivo*. Brettschneider et al[129] also analysed the CSF of 14 patients and observed 2 overregulated proteins and 3 underregulated. Two of the latter were Zn-alpha-2-glycoprotein and ceruloplasmin precursor protein. The role of cystatin C in ALS has previously been discussed in another article.[108]

Table 3 Effect of substances on the toxicity of cerebrospinal fluid (CSF) in amyotrophic lateral sclerosis (ALS)

Author/year	Culture	Potential protective effect	Without effect
Courantier et al[17], 1993	Rat neurons in culture	AMPA/kainate receptor antagonist	NMDA receptor antagonist
Terro et al[20], 1996	Rat cortical cells	Vitamin E. Allopurinol	—
Smith et al[23], 1998	VSC-4.1 cells, which are cholinergic	Allopurinol. Vitamin E. Glutathione	—
Manabe et al[22], 1999	Rat lumbar spinal cells	MK801, an NMDA antagonist	CNQX, an AMPA/kainate antagonist
Shahani et al[24], 2001	Spinal motor neurons from newborn rats	Cyclophosphamide	—
Tikka et al[25], 2002	Spinal cells from rat embryos	MK-801 minocycline, NMDA antagonist. CNQX, AMPA/kainate antagonist	—
Anneser et al[27], 2004	Glial cells from bird embryos	AIDA, a mGluR antagonist	DCG-4, mGluR agonist
Shahani et al[26], 2004	Administration in rat CSF, and analysis in spinal tissue	Deprenyl	—
SEN et al[28], 2005	Spinal cells from rat embryos	APV, NMDA antagonist. NBAX, AMPA/kainate antagonist, stronger effect of the latter	—
Anneser et al[29], 2006	Spinal motor neurons from bird embryos	AIDA, an mGluR antagonist. DHPG, an mGluR agonist, but which has a dual effect and can be antagonist	Glutamate. CPPG, an mGluR antagonist. AMPA antagonist
Shobha et al[31], 2007	Spinal motor neurons from newborn rats	Nitro-L-arginine methyl ester (L-NAME), a nitric oxide synthetase inhibitor	—
Gunasekaran et al[32], 2009	Spinal motor neurons from newborn rats	BDNF. CNTF	—

Table 3 presents the results of pharmacological interventions on ALS CSF culture and the response on the cytotoxic effect. The effect of the toxic factor appears to be mediated by increased calcium, which is directly correlated with the degree of cell death caused by the ALS-CSF,[28] but also by Nav1.6 or Kv1.6 channels.[32] Both AMPA/kainate receptor antagonists[17,28] and glutamate NMDA receptor antagonists[22] (but with greater intensity by the first) partially reduced the cytotoxic effect, although this has also been associated with the presence of free radicals[20] or with nitric oxide mediation.[31] In any case, the response to these interventions on the culture may be related more to cell injury mechanisms than to the very composition of ALS-CSF. Gunasekaran et al[32] point out the potential benefit of applying BDNF or CNTF to the culture.

Undoubtedly, the CSF may make it possible to identify biomarkers in ALS[130,131]. However, its cytotoxicity is also a singularity that could explain the evolutionary aspects of the disease. Studies completed and included in this review lack clinical information, so the cytotoxic effect cannot be correlated with clinical patient conditions or especially if the forms were familial or sporadic. It seems clear that, to understand this mechanism, further studies need to include better identification of those patients from whom the samples were obtained.

Conflict of interests

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