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Journal of Chromatography B, 851 (2007) 250–256
JOURNAL OF CHROMATOGRAPHY B
www.elsevier.com/locate/chromb

Arginine, citrulline and nitrate concentrations in the cerebrospinal fluid from patients with acute hydrocephalus☆

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Received 2 June 2006; accepted 16 October 2006
Available online 15 November 2006

Abstract

Citrulline and nitric oxide (NO) are synthesized by NO synthase (NOS) in a 1:1-stoichiometry. In this study, we determined by HPLC arginine and citrulline concentrations by fluorescence detection and nitrate levels by UV absorbance detection in the cerebrospinal fluid (CSF) from patients with acute hydrocephalus that underwent ventricular drainage. We found increased citrulline concentration (50.6 ± 17.2 versus 20.9 ± 2.0 μM) and decreased arginine/citrulline molar ratio (0.42 ± 0.11 versus 1.12 ± 0.16) in hydrocephalus patients, while arginine and nitrate concentrations and citrulline/nitrate molar ratio remained with little change. Citrulline has been determined as a marker of NOS activity in some studies, but it remains to be determined the extent at which this statement holds true, since other biochemical pathways also regulate the concentration of this amino acid. Our results suggest that citrulline is primarily synthesized from NOS in acute hydrocephalus. The evaluation of sample deproteinization by addition of methanol for the analysis of amino acids in CSF is also reported.

Keywords: Sample pretreatment; Inflammation; Cerebrospinal fluid drainage; Argininosuccinate synthetase

1. Introduction

The external cerebrospinal fluid (CSF) drainage is frequently used in clinical neurosurgery for the management of acute hydrocephalus resulting from different etiologies. Due to the patient's disease or the surgical procedure itself an inflammatory response is expected to occur within the central nervous system (CNS).

Nitric oxide (NO) is abundantly synthesized by type II NO synthase (NOS, EC 1.14.13.39) during infection/inflammation in response to cytokines such as interferon (IFN)-γ [1,2] or interleukin (IL)-1β [3,4] in a wide variety of systems, including the CNS. NO has a very short half-life and is oxidized to nitrite and nitrate [5] that are reliable markers for in vivo NOS activity [6,7]. On the other hand, citrulline and NO are both synthesized by NOS in a 1:1-stoichiometry and this amino acid has been analyzed in some studies as a biochemical marker of NOS activity [8–12], although nitrite/nitrate concentration is considered the most reliable marker and is commonly determined to study NO biosynthesis [6,7].

Argininosuccinate synthetase (ASS, EC 6.3.4.5) and dimethylarginine dimethylaminohydrolase (dimethylargininase, DDAH, EC 3.5.3.18) activities also influence citrulline concentration in the CNS [13,14] and thus the reliability of citrulline determination as a marker of NOS activity is still a matter of debate [15,16]. On the other hand, arginine concentration is regulated by other enzymes [13,14].

Both arginine-synthesizing (ASS) and arginine-metabolizing (arginase, NOS) enzymes are co-induced by cytokine or lipopolysaccharide treatment [17–20]. Arginine biosynthesis is important to maintain the levels to support inducible NOS activity [21], thus arginine degradation by arginase should be expected to be a limiting factor for NOS activity. In addition,

☆ This paper is part of a special issue entitled "Analysis of the L-arginine/NO pathway", guest edited by D. Tsikas.

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doi:10.1016/j.jchromb.2006.10.047

NOS activity may increase citrulline levels, but ASS activity utilizes this amino acid for the synthesis of argininosuccinate, and both metabolic pathways are likely to occur during an inflammatory response; thus, it remains to be determined how arginine and citrulline levels are modulated, as well as their possible influence on NOS activity, in the acute phase of an inflammatory response in the human CNS.

We have reported increased CSF citrulline concentration in patients with CNS infectious/inflammatory disorders [8]. In the present study, we measured CSF arginine, citrulline and nitrate concentrations to study the influence of CNS inflammation on the levels of these biochemical markers.

The high protein content of the CSF samples from patients with inflammatory processes is also assessed as a possible interference in amino acids analysis.

2. Experimental

2.1. Chemicals

HPLC-grade tetrahydrofuran (THF) and absolute methanol (MetOH) were obtained from Mallinckrodt Baker (Kentucky, USA). Bovine serum albumin, *o*-phthalaldehyde (OPA), 2-mercaptoethanol (ME), octylamine and amino acids were obtained from Sigma (St. Louis, MO, USA). Nitrite and nitrate were obtained from J.T. Baker (Mexico). All other reagents were analytical grade.

2.2. Patients

Eight patients (mean age ± S.D., 47.4 ± 14.5 years) that underwent ventricular external drainage for the management of acute hydrocephalus due to hypertensive ($n = 5$) or subarachnoid ($n = 3$) hemorrhage with intraventricular extension were included. Among inclusion criteria, the early placement of an external ventricular drainage and the absence of clinical signs of CNS infection were required. CSF was obtained on the first day after the placement of the external drainage. All the patients included in this group received preventive non-steroidal antimicrobial/anti-inflammatory drugs.

Seven neurologic patients (mean age ± S.D., 39.3 ± 6.8 years) with a normal CSF cytochemical analysis were included as controls. Their diagnosis were controlled peripheral neuropathy (2 cases), ophthalmologic disorders (3 cases) and inactive neurocysticercosis (with negative CSF immunoreactivity) (2 cases). In this group, CSF was obtained by clinically-indicated lumbar puncture.

Routine cytochemical analysis was performed for all the samples collected. Samples were stored at −80 °C until analyzed.

This protocol was approved by our Institutional Ethics Committee. Informed signed consent was obtained in all cases from patients or their relatives.

2.3. Chromatographic analysis

2.3.1. Sample deproteinization

To assay for amino acids recovery following sample deproteinization, a standard solution containing arginine and citrulline (as well as glutamate, aspartate, glutamine and taurine; 25 μM each one) was prepared in deionized water containing different albumin concentrations (either 100 mg/dl or 10 g/dl) similar to those found in human plasma [22–24] and CSF [25,26], as well as plasma from other species [27] in order to simulate high protein content in the CSF samples assayed. Aliquots from this solution were diluted 1:1 with an equal volume of MetOH and centrifuged at 15,000 rpm (ca. 18,500 × *g*) at 4 °C for 15 min. Those aliquots (either processed or not) were analyzed by HPLC (see below) and the percentage of the mean concentrations with or without deproteinization (considering 25 μM as 100%) is reported. Analyte concentrations were determined using a calibration curve freshly prepared in deionized water.

To assay for deproteinization, five aliquots of a solution containing a high albumin concentration (12.5 g/dl) were processed as described above and the protein concentration

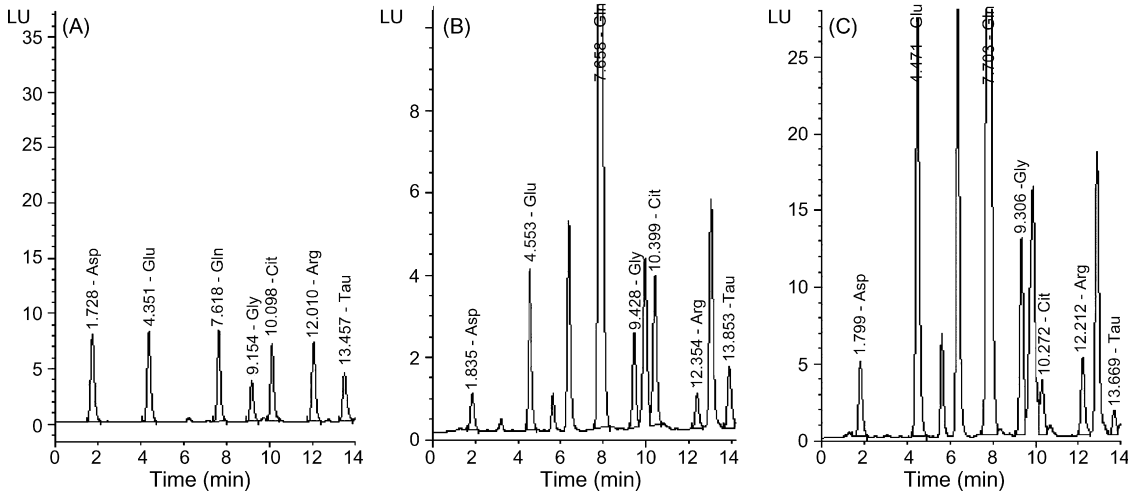

Fig. 1. Representative chromatograms from the analysis of arginine and citrulline in (A) a standard solution (5 μM each) and human CSF samples from (B) control (diluted 1:9, v/v) or (C) hydrocephalus (diluted 1:7, v/v) patients. Retention times for citrulline and arginine are 10.22 ± 0.03 and 11.96 ± 0.02 min, respectively.

in the supernatant following centrifugation was determined by the method of Lowry [28]. To evaluate if the recovery of proteins after the addition of MetOH was dependent on the composition of biological samples and not only on the presence of proteins, five aliquots of human plasma (obtained from a healthy volunteer) were processed as described above and the protein concentration before and after deproteinization was determined by the method of Lowry [28].

2.3.2. Determination of CSF amino acid concentrations

The quantitation of arginine and citrulline by reversed-phase HPLC was performed as we have previously described and validated [8] after minor modification as described below. CSF was thawed and processed as described in Section 2.3.1. Supernatants were collected and diluted as necessary with deionized water. Diluted aliquots of 100 μl were mixed with an equal volume of the OPA/ME reagent, allowed to react for 1 min while stirred in a vortex and injected to a binary pump (LC 200, Perkin-Elmer) with a 20 μl loop. The OPA/ME reagent was prepared as described [8]. The mobile phase consisted of a 50 mM sodium acetate buffer (pH 5.9) with 1.5 vol.% THF and a MetOH gradient, and was filtered through a 0.45-μm nitrocellulose membrane and degassed by sonication prior to use. For chromatographic elution, the program consisted of a linear gradient step from 10 to 35 vol.% MetOH in 15 min, a washing step with 99 vol.% MetOH for 5 min and a reequilibration step with 10 vol.% MetOH for 5 min. Derivatives were separated with an Adsorbosphere OPA HS column (5 μm particle size, 100 × 4.6 mm i.d., Alltech). A flow rate of 1.5 ml/min was used. Signals were recorded with a fluorescence detector (Model 157, Beckman) and an HP 3396 Series II integrator (Hewlett-Packard). Representative chromatograms for these analyses are shown in Fig. 1. Retention times for citrulline and arginine were 10.22 ± 0.03 and 11.96 ± 0.02 min, respectively.

2.3.3. Determination of CSF nitrate concentrations

CSF nitrate concentrations were determined as previously described [29]. Briefly, samples were thawed and processed as described above. Supernatants were injected into the chromatographic system (LC 250, Perkin-Elmer) equipped with a 50-μl sample loop. The mobile phase was a 5 mM octylamine solution (adjusted to pH 6.4 with diluted sulfuric acid) and was pumped at a flow rate of 1.2 ml/min. A Lichrosorb C18 column (5 μm particle size, 250 × 4.6 mm i.d., Alltech) was used. Signals were recorded with an UV/Visible detector (LC-95, Perkin-Elmer) at 228 nm and a ChromJet integrator (Thermo Separation Products). In this HPLC system, nitrite and nitrate eluted at 5.73 ± 0.1 and 6.96 ± 0.1 min, respectively. Representative chromatograms from the analysis of nitrate in standard aqueous solution and CSF samples from one control and one patient are shown in Fig. 2.

Nitrite could not be determined in CSF samples from controls and patients. Therefore, the method was validated for nitrate only using a human CSF pool. Individual CSF samples were obtained from several patients being included in different studies in our laboratory. The limit of detection (LOD) for nitrate was determined to be 0.96 μM at a signal-to-noise ratio of 3:1

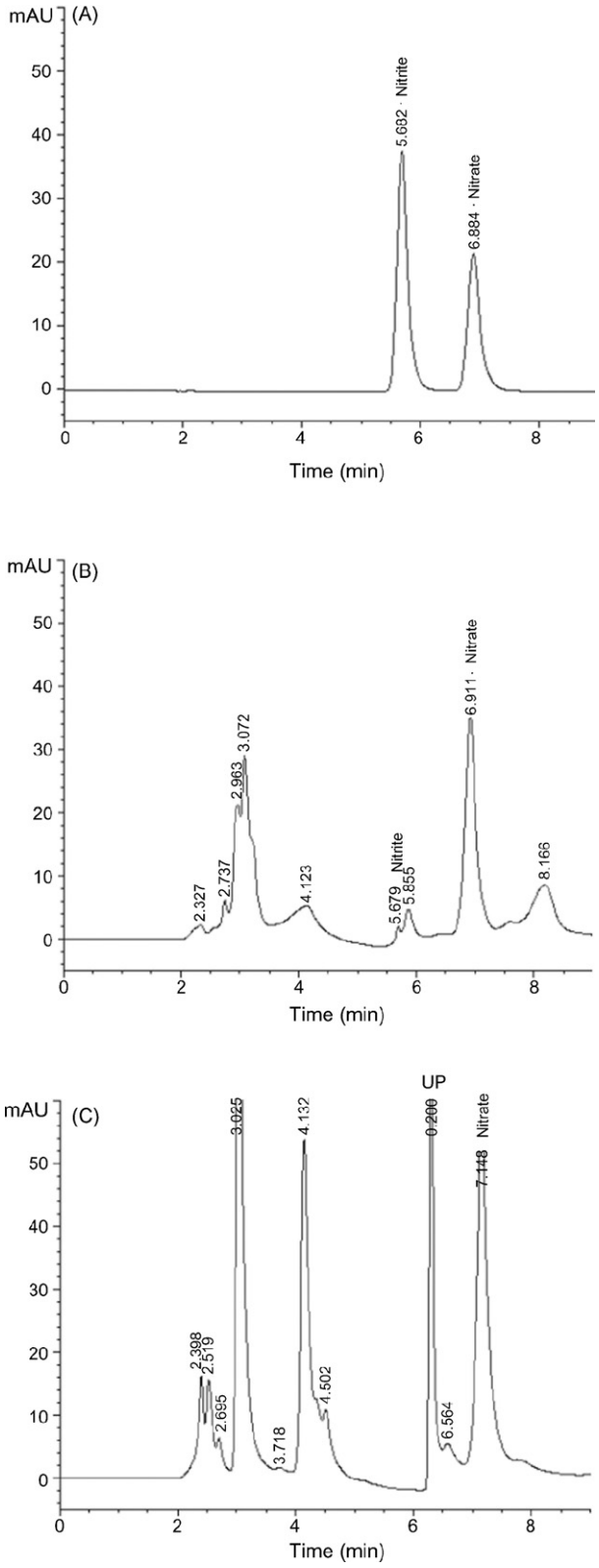

Fig. 2. Representative chromatograms from the analysis of nitrite and nitrate in (A) a standard aqueous solution (5 μM each) and of nitrate in human CSF from (B) control (diluted 1:1, v/v) or (C) hydrocephalus (diluted 1:1, v/v) patients. Nitrite signal was not found in most CSF samples and so it is not shown in panels (B) and (C). Retention times for nitrite and nitrate are 5.73 ± 0.1 and 6.96 ± 0.1 min, respectively. Labelling of "nitrite" and "nitrate" indicates the retention time of nitrite and nitrate, respectively. UP: unidentified peak.

($n = 10$). Linearity of response was tested by analyzing aqueous solutions between 5 and 400 μM. In this concentration range, the coefficient of correlation was estimated to be 0.999. Lower (LLOQ) and upper (ULOQ) limits of quantitation were defined as the lowest and highest concentrations in the linearity range in pooled CSF that could be determined with an imprecision (R.S.D., $n = 10$) of not higher than 15%. The LLOQ and ULOQ values were determined to be 5 and 400 μM, respectively. Intra-assay precision (R.S.D., %) was determined by consecutive HPLC analyses ($n = 10$) of the human CSF pool. The concentration of nitrate in this pool was determined to be (mean ± S.D.) 58.1 ± 4.1 μM with an imprecision of 7.1%. To determine the accuracy of the method, 10 aliquots of the CSF pool were spiked with a standard aqueous solution of nitrate to reach an added final concentration of 5 μM and were analyzed by HPLC. In parallel, 10 aliquots of the CSF pool were analyzed without external addition in order to determine the basal level of nitrate in the CSF pool. The accuracy (recovery, %) of the method was calculated by means of the equation [(mean measured value − mean basal value)/added value] × 100 and was determined to be 103.5% at the 5-μM nitrate level.

2.4. Statistical analysis

Results from the evaluation of sample deproteinization were compared using paired Student's *t*-tests. Patient groups were compared using Student's *t* or Mann–Whitney *U* tests for independent samples, after checking for normal distribution of data. Correlation analyses were performed using Spearman's correlation. All statistical tests were performed using SPSS 13.0 software. Results are expressed as mean ± S.E.M. if not otherwise specified and were considered significant when $p < 0.05$.

3. Results

3.1. Sample deproteinization

For 100 mg/dl albumin concentration, the addition of methanol did not affect amino acid recovery. Concentrations were near 100% of the nominal value independently of the sample deproteinization procedure (Table 1). On the other hand, when a high protein concentration (10 g/dl) was present in the standard solution, the concentration of most amino acids was 30–45% of the nominal value (25 μM) (Table 1). In contrast, arginine recovery was 86%. After deproteinization, significantly improved recovery values (between 54 and 67% with respect to the nominal value) were obtained for aspartate, glutamate, glutamine, citrulline and taurine, while arginine recovery was not affected.

Protein concentration in an albumin standard solution (12.5 g/dl) was 10.8 g/l after deproteinization (14% efficency of protein precipitation). When human plasma was processed instead of the albumin standard solution, protein concentration (8.32 ± 0.18 g/dl) dropped to 1.46 g/dl after addition of methanol. Correcting for dilution (1:1, v/v), this result represents an efficacy of only 65% for the deproteinization procedure when human plasma is used as the biological matrix.

3.2. CSF protein, amino acid and nitrate concentrations

CSF protein concentration in patients with ventricular drainage (362.1 ± 125.5 mg/dl) was significantly higher than that of neurologic patients (33.6 ± 7.2 mg/dl; $p = 0.021$) (Fig. 3A).

CSF arginine concentration was not significantly different between the groups (neurologic controls: 21.8 ± 1.4 μM; ventricular drainage: 17.4 ± 8.2 μM; $p = 0.616$; Fig. 3C). Citrulline (Fig. 3D) and nitrate (Fig. 3B) concentrations in patients with CSF drainage (citrulline: 50.6 ± 17.2 μM; nitrate: 81.4 ± 30.2 μM) were 242% and 177%, respectively, compared to neurologic patients without inflammatory CNS disorders (citrulline: 20.9 ± 2.0 μM; nitrate: 45.9 ± 13.0 μM); however, only citrulline concentration reached statistical significance compared to neurologic controls (citrulline: $p = 0.027$; nitrate: $p = 0.867$).

The arginine/citrulline (0.42 ± 0.11, Fig. 4A) and citrulline/nitrate (1.39 ± 0.63, Fig. 4B) molar ratios in patients with ventricular drainage were significantly decreased and unchanged, respectively, compared to controls (arginine/citrulline molar ratio: 1.12 ± 0.16, $p = 0.004$; citrulline/nitrate molar ratio: 1.02 ± 0.38, $p = 0.955$) (Fig. 4).

Neither arginine ($r = -0.099$, $p = 0.736$), citrulline ($r = 0.145$, $p = 0.607$) nor nitrate ($r = -0.225$, $p = 0.420$) concentrations were correlated to total protein content in the samples. Nitrate

Table 1
Recovery (%, mean ± S.E.M., $6 \le n \le 14$) of different amino acids (25 μM each) after sample deproteinization from aqueous albumin solutions of two different concentrations

Amino acid	Recovery (%)			
	100 mg/dl Albumin		10 g/dl Albumin	
	Deproteinized	Non-deproteinized	Deproteinized	Non-deproteinized
Aspartate	97.4 ± 1.7	95.5 ± 2.0	54.7 ± 5.9*	30.2 ± 4.5
Glutamate	115.3 ± 8.2	104.8 ± 2.3	64.3 ± 6.2*	33.4 ± 3.5
Glutamine	108.6 ± 3.6	107.7 ± 1.8	66.3 ± 5.5*	34.3 ± 4.7
Citrulline	104.5 ± 3.3	108.6 ± 1.8	67.8 ± 4.9*	45.2 ± 1.9
Arginine	105.2 ± 8.9	98.2 ± 4.4	87.8 ± 2.5	86.2 ± 3.5
Taurine	100.9 ± 5.3	105.3 ± 3.8	60.1 ± 5.9*	42.6 ± 2.5

* $p < 0.05$ versus non-deproteinized samples for each concentration level.

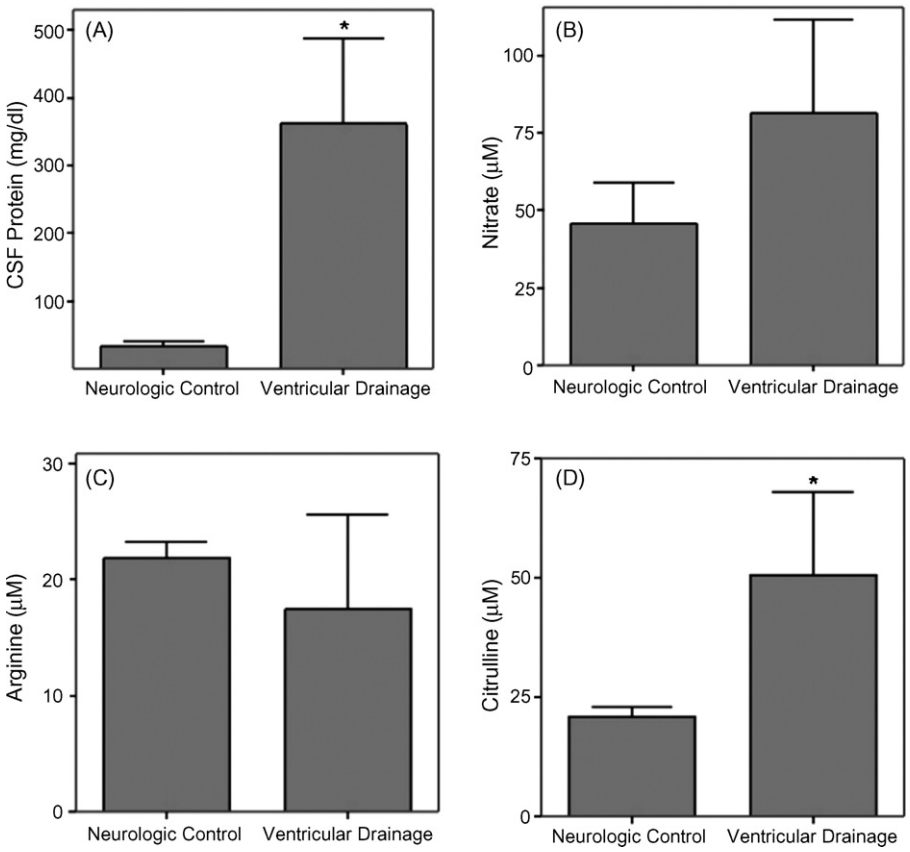

Fig. 3. CSF protein (A), nitrate (B), arginine (C) and citrulline (D) concentrations in patients with ventricular drainage and neurologic controls. $*p < 0.05$.

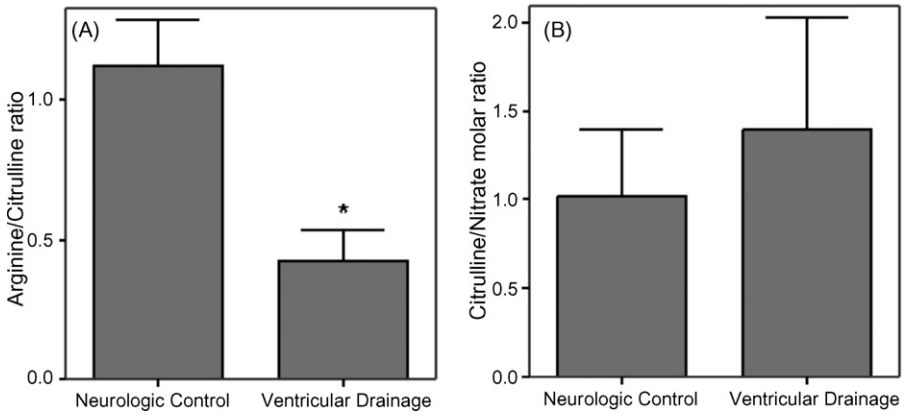

Fig. 4. Arginine/citrulline (A) and citrulline/nitrate (B) molar ratios in patients with ventricular drainage and neurologic controls. $*p < 0.05$.

and citrulline concentrations were positively correlated in hydrocephalus patients, although this result did not reach statistical significance ($r = 0.500$, $p = 0.207$).

4. Discussion

4.1. Sample deproteinization

The addition of organic solvents such as MetOH to biological samples is commonly used as a deproteinization procedure [30–34]. Other methods to achieve this purpose such as the addition of acidic solutions may lead to increased amino acid concentrations [31]. For this reason, the recovery of amino acids after sample pretreatment is very important when amino acid concentrations are to be analyzed in biological samples containing different protein concentrations.

In our study, amino acids recovery after sample deproteinization is not affected by 100 mg/dl albumin concentration, which is even higher than that reported for normal human CSF [25,26]. Furthermore, recovery is around 100% independently of sample deproteinization (Table 1), suggesting that the chromatographic analysis of amino acids in the CSF does not require the addition of MetOH when protein concentration is below 100 mg/dl. On the other hand, in this study CSF protein reached values above

300 mg/dl (Fig. 3). We showed that higher concentrations of proteins (10 g/dl), similar to those present in human plasma [22–24], may influence the quantitation of amino acids. Without deproteinization, amino acid concentrations are reduced (Table 1) compared to the nominal value yielding percent recovery values below 50%, except for arginine. This effect is partially reversed by the addition of MetOH; thus, this pretreatment is suitable for the analysis of amino acids in biological samples containing high protein concentrations.

When the albumin standard solution (12.5 g/dl) was submitted to sample pretreatment only a slight deproteinization was found (14%). In contrast, human plasma yielded a higher deproteinization degree (65%) showing that the efficiency of the addition of MetOH as a deproteinization procedure depends on the composition of the biological matrix and not only on the presence of proteins, indicating that this procedure may not be suitable for all biological samples.

4.2. CSF nitrate and amino acid concentrations

Patients with subarachnoid or hypertensive intraventricular hemorrhage developed an inflammatory response in the CNS especially due to the surgical procedures (ventricular drainage system placement) and the basal pathologies involved. Consistent with this, CSF protein concentration (a biochemical marker of blood-brain barrier integrity) was significantly higher in these patients compared to neurologic controls (without CNS infection/inflammation) (Fig. 3A).

Increased CSF nitrate (Fig. 3B) and citrulline (Fig. 3D) concentrations in our patients are likely to be associated to increased NO biosynthesis during an inflammatory response. This is not surprising since NOS II is induced by cytokines such as IFN-γ [1,2] and IL-1β [3,4], nitrate concentration is a reliable marker for NOS activity [6,7] and citrulline is the co-product of NOS [13,14]. Increased NOS activity in this study is also supported by the decreased arginine/citrulline molar ratio in the patients with ventricular drainage (Fig. 4A).

Since only CSF citrulline concentration was significantly increased compared to controls (Fig. 3D) this amino acid in CSF may be a more sensitive biochemical marker of the acute inflammatory response following subarachnoid or intraventricular hemorrhage, although we can not exclude a type II error regarding CSF nitrate concentration due to the small sample size in this study. The citrulline/nitrate molar ratio remained almost unchanged between the groups although it was slightly higher in the patients with ventricular drainage (Fig. 4B). This result suggests a parallel increase in the concentration of both analytes which is consistent with the synthesis of both NO and citrulline by NOS. However, CSF citrulline and nitrate concentrations were not significantly correlated, perhaps as a result of the small sample of patients included in the study. Also, other pathways leading to increased citrulline concentration should not be ruled out. Since only NOS, DDAH and ASS metabolize citrulline within the CNS [13] the possible role of those enzymes in acute hydrocephalus is discussed below.

The urea cycle does not occur in the brain, then, any contribution of this cycle to produce citrulline from sources other than NOS activity should be mainly due to liver metabolism. Since liver dysfunction was absent in patients, even when the urea cycle might lead to an increased plasma citrulline concentration, which in turn might enter the CNS through an impaired blood–brain barrier, the urea cycle is not the most likely explanation of our results.

DDAH is expressed in the mammalian brain [35] and synthesizes citrulline from methylated arginines [13]; some authors have suggested that this enzyme could be responsible for citrulline accumulation during CNS infection/inflammation [15]. Its activity is inhibited by *S*-nitrosylation [36,37] and thus, this inhibitory mechanism is expected to occur during inflammation-induced high output NO release, although both cytokine-induced increases and decreases in DDAH activity have been reported in smooth muscle [38] and endothelial cells [39], respectively; also, methodological considerations regarding the analytical detection of *S*-nitrosothiols should be taken into account [40,41].

On the other hand, the slight increase in the citrulline/nitrate molar ratio in the patients with ventricular drainage (Fig. 4) suggests that citrulline accumulation may not only be associated to NOS activity and thus other citrulline-metabolizing enzymes might be involved. ASS inhibition could also contribute to increased citrulline levels since this enzyme needs citrulline and aspartate to catalyze the rate-limiting step in arginine biosynthesis [13,14]. ASS is inhibited by *S*-nitrosylation following lipopolysaccharide injection in mice [42]; thus, this enzyme might be inhibited during CNS inflammation and might lead to a slight decrease in arginine concentration (as shown in Fig. 3C) and, along with NOS, to increased citrulline levels (Fig. 3D). However, the possible involvement of DDAH and ASS in acute hydrocephalus remains speculative.

As suggested by the slight decrease in arginine concentration, ASS inhibition might contribute to the increased citrulline levels to some extent, but is not likely to be the main enzyme involved, thus citrulline accumulation is more likely to be due to increased NOS activity during inflammation as shown by the increased concentration of both nitrate and citrulline (Fig. 3), leading to an unchanged citrulline/nitrate molar ratio between the groups (Fig. 4B).

We found increased CSF citrulline concentration in patients with CNS infection and/or inflammation in a preliminar report [8]. Since some studies analyze citrulline concentration as a marker for NO biosynthesis [8–12], it is important to determine the physiological or pathophysiological conditions in which this suggestion holds true. In order to achieve this goal, complementary biochemical markers of the different pathways regulating citrulline concentration (such as asymmetric dimethylarginine, dimethylamine and ornithine) should be considered. The present results support the conclusion that CSF citrulline concentration may be considered a complementary biochemical marker to study NO biosynthesis during acute hydrocephalus.

Acknowledgements

The authors wish to thank Dr. Esther Talavera (Institute of Neurobiology, Qro., Mexico) for the citrulline standard, Luis Tristán (MSc) for his valuable cooperation and Antonio Bueno

for his technical assistance. I. Pérez-Neri is recipient of a grant from the National Council for Science and Technology (CONACyT, Num. 186343).

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