Acrylamide exposure impairs blood-cerebrospinal fluid barrier function

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Abstract

Previous studies show that chronic acrylamide exposure leads to central and peripheral neuropathy. However, the underlying mechanisms remained unclear. In this study, we examined the permeability of the blood-cerebrospinal fluid barrier, and its ability to secrete transthyretin and transport leptin of rats exposed to acrylamide for 7, 14, 21 or 28 days. Transthyretin levels in cerebrospinal fluid began to decline on day 7 after acrylamide exposure. The sodium fluorescein level in cerebrospinal fluid was increased on day 14 after exposure. Evans blue concentration in cerebrospinal fluid was increased and the cerebrospinal fluid/serum leptin ratio was decreased on days 21 and 28 after exposure. In comparison, the cerebrospinal fluid/serum albumin ratio was increased on day 28 after exposure. Our findings show that acrylamide exposure damages the blood-cerebrospinal fluid barrier and impairs secretory and transport functions. These changes may underlie acrylamide-induced neurotoxicity.

Key Words: nerve regeneration; brain injury; acrylamide; blood-cerebrospinal fluid barrier; tight junction; permeability; thyroid hormone; leptin; cerebrospinal fluid/serum albumin ratio; cerebrospinal fluid; NSFC grant; neural regeneration

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Introduction

Acrylamide is an important chemical and widely used in industrial production of polyacrylamides, which are employed mainly as flocculating agents in water treatment, and as flow control agents in oil well operations[1]. In addition, acrylamide is found in a variety of other sources, such as certain starchy foods cooked at high temperature[2]. Therefore, acrylamide exposure is a serious occupational and public health hazard[3]. Previous studies have shown that acrylamide monomer is a neurotoxin to humans and animals in vivo and in vitro, and can cause central-peripheral neuropathy, which is characterized by ataxia, skeletal muscle weakness, and numbness of the hands and feet[4-6].

Accumulating evidence suggests that acrylamide perturbs neurofilaments and impairs neurotransmission by disrupting presynaptic nitric oxide signaling[7-9]. However, the mechanisms of acrylamide-induced neurotoxicity are still unclear. The blood-brain barrier prevents xenobiotics from entering the central nervous system. Growing evidence indicates that neurotoxins, such as tributyltin, manganese and nanoparticles, may disrupt the function of the blood-brain and blood-cerebrospinal fluid (CSF) barrier[10-12]. However, very few studies have focused on the effects of acrylamide exposure on these barriers.

The brain barriers include the blood-brain barrier and the blood-CSF barrier, which tightly regulate the entry of xenobiotics into the brain. A large amount of research suggests that neurotoxins have a great impact on the blood-brain barrier and a lower impact on the blood-CSF barrier[13-14]. However, this apparent difference might be due to technical issues and an insufficient understanding of the blood-CSF barrier. The blood-CSF barrier separates blood components from the CSF. Compared with the blood-brain barrier, the blood-CSF barrier has a relatively large surface area, and a large and fast blood supply and tight junctions[13-14]. As technical advancements have enabled the direct insertion of miniature video probes into the ventricles, the role of the blood-CSF barrier in neurotoxin poisoning has attracted increasing attention. Blood-CSF barrier dysfunction is associated with neurodegenerative diseases, such as Alzheimer’s disease, where it may result in reduced Aβ clearance[17]. However, the role of the blood-CSF barrier in acrylamide neurotoxicity is unclear.

The blood-CSF barrier regulates the movement, secretion and transport of substances. The selective permeability of the blood-CSF barrier protects the brain from fluctuations in plasma composition and circulating agents through the regulation of tight junctions[18]. Some proteins expressed in the blood-CSF barrier regulate permeability. These proteins include integral membrane proteins (occludin, claudins, JAM) and peripheral proteins (ZO-1, ZO-2, ZO-3). Therefore, blood-CSF
barrier integrity is crucial for maintaining homeostasis in the central nervous system. Evans blue is a useful chemical indicator of barrier leakage, and the level of this dye in the CSF can help assess the degree of blood-CSF barrier permeability.

The blood-CSF barrier has a secretory function as well, producing and secreting various proteins into the CSF and parenchyma that are involved in brain development, neuronal survival and neurodegeneration. Transthyretin levels in the CSF and brain parenchyma are derived exclusively from the choroid plexus. By contrast, the liver is the primary source of blood transthyretin. The passive diffusion of serum transthyretin into the CSF is blocked by the blood-CSF barrier\(^{[19-20]}\). Therefore, the transthyretin level in the CSF partly reflects the secretory function of the blood-CSF barrier, and serves as an indicator of blood-CSF barrier secretory impairment\(^{[21]}\).

Furthermore, the blood-CSF barrier has an active transport system, through which nutrients, such as calcium and leptin, are transported into the brain. Under physiology conditions, leptin is secreted by adipose tissue and transported into the central nervous system through the OB-Ra receptor expressed at the brain barriers\(^{[22-23]}\). Thomas et al.\(^{[24]}\) demonstrated that the OB-Ra receptor is mainly expressed in the choroid plexus, and to a lesser extent in brain microvessels (the main tissue of the blood-brain barrier). Thus, leptin levels in the CSF are an indicator of transport function. However, it remains unclear whether acrylamide disrupts the transport of leptin through the blood-CSF barrier.

The present study was designed to examine whether acrylamide exposure interferes with blood-CSF barrier function by increasing permeability, decreasing secretion and reducing the transport of leptin. Our findings should contribute to our understanding of acrylamide-induced neurotoxicity and lay the foundation for future studies on the mechanisms of neurotoxicity.

Results
Quantitative analysis of experimental animals
A total of 128 Sprague-Dawley rats were randomly divided into control group (intraperitoneal injection of saline) and acrylamide exposure group (intraperitoneal injection of acrylamide, 20 mg/kg). Of these rats, 64 were used for assessment of Evans blue and sodium fluorescein content, while the remaining 64 were used to evaluate the CSF/serum albumin ratio, and transthyretin and leptin levels. The rats were used for analyses 7, 14, 21 and 28 days after acrylamide or saline control exposure, with 8 rats at each time point.

Table 1 Effect of acrylamide exposure on gait scores in rats

<table>
<thead>
<tr>
<th>Group</th>
<th>Score (n)</th>
<th>Average score</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1 2 3 4</td>
<td></td>
</tr>
<tr>
<td>Acrylamide exposure 7 days</td>
<td>8 0 0 0</td>
<td>1.00±0.00</td>
</tr>
<tr>
<td>Acrylamide exposure 14 days</td>
<td>5 3 0 0</td>
<td>1.38±0.52</td>
</tr>
<tr>
<td>Acrylamide exposure 21 days</td>
<td>0 7 1 1</td>
<td>2.13±0.35</td>
</tr>
<tr>
<td>Acrylamide exposure 28 days</td>
<td>0 5 2 1</td>
<td>2.50±0.75</td>
</tr>
</tbody>
</table>

Gait scores (range 1–4) were based on evaluation of spontaneous open-field behaviors, which included levels of ataxia, hopping, rearing and hind foot placement. A gait score of 1 indicates no degree of impairment, 4 reflecting complete lameness. Data are expressed as mean ± SD of eight rats at each time point. \(^{*}P < 0.05\), vs. control group (one-way analysis of variance and least significant difference test).

In the end, data from all 128 rats were entered into the final analyses, without dropout.

Acrylamide exposure increased gait scores
After 7, 14, 21 and 28 days of acrylamide exposure, gait scores were measured and recorded (Table 1). Significantly higher gait scores were seen in acrylamide-treated rats at 21, 28 days compared with the control group \((P < 0.05)\). No significant change was seen at other acrylamide exposure time points. Gait scores in rats with acrylamide exposure rose significantly in a time-dependent manner \((P = 0.003)\).

Acrylamide exposure increased blood-CSF barrier permeability
Evans blue concentration in the CSF was significantly increased at 21 and 28 days after acrylamide exposure, to 32.88 μg/mL and 54.62 μg/mL, respectively, compared with the control group \((21.62 μg/mL; P < 0.05; Table 2)\). Additionally, the level of sodium fluorescein in the CSF began to increase at 14 days after acrylamide exposure in comparison with the control group \((P < 0.05)\). Furthermore, the CSF/serum albumin ratio was also significantly increased at 28 days after acrylamide exposure compared with the control group \((P < 0.05; Table 2)\). Collectively, these results indicate that the permeability of the blood-CSF barrier began to increase following 14 days of exposure.

Acrylamide exposure decreased transthyretin levels in CSF
Transthyretin levels in the CSF were significantly decreased at 14, 21 and 28 days after acrylamide exposure, to 10.67 μg/mL and 8.83 μg/mL, respectively, compared with the control group \((14.44 μg/mL; P < 0.05; Table 2)\). Transthyretin levels in the CSF were significantly decreased.

Table 2 Effect of acrylamide exposure on the permeability of the blood-cerebrospinal fluid barrier

<table>
<thead>
<tr>
<th>Time after acrylamide exposure (day)</th>
<th>Evans blue (μg/mL)</th>
<th>Sodium fluorescein (μg/mL)</th>
<th>The ratio of cerebrospinal fluid/serum albumin (× 10⁻³)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Acrylamide</td>
<td>Control</td>
</tr>
<tr>
<td>7</td>
<td>21.62±10.33</td>
<td>22.37±7.21</td>
<td>2.19±0.20</td>
</tr>
<tr>
<td>14</td>
<td>21.64±8.12</td>
<td>26.64±6.88</td>
<td>2.09±0.20</td>
</tr>
<tr>
<td>21</td>
<td>21.55±8.30</td>
<td>32.88±7.39</td>
<td>2.21±0.21</td>
</tr>
<tr>
<td>28</td>
<td>21.55±8.38</td>
<td>54.62±9.08</td>
<td>3.15±0.07</td>
</tr>
</tbody>
</table>

At 7, 14, 21 and 28 days after acrylamide exposure, acrylamide-exposed rats and control rats were cannulated with dye solution containing Evans blue and sodium fluorescein. The concentration of albumin in the cerebrospinal fluid and serum were assessed by ELISA. Data are expressed as mean ± SD of eight rats at each time point. \(^{*}P < 0.05\), vs. control group (one-way analysis of variance and least significant difference test). The cerebrospinal fluid/serum albumin ratio = cerebrospinal fluid albumin concentration/serum albumin concentration.


Table 3 Effect of acrylamide exposure on transthyretin levels in cerebrospinal fluid

<table>
<thead>
<tr>
<th>Time of acrylamide exposure (day)</th>
<th>Cerebrospinal fluid (μg/mg Pro)</th>
<th>Serum (μg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Acrylamide</td>
</tr>
<tr>
<td>7</td>
<td>27.78±8.76</td>
<td>30.20±3.81</td>
</tr>
<tr>
<td>14</td>
<td>27.71±6.66</td>
<td>29.54±3.15</td>
</tr>
<tr>
<td>21</td>
<td>27.19±6.52</td>
<td>29.01±3.87</td>
</tr>
<tr>
<td>28</td>
<td>27.16±7.52</td>
<td>29.24±4.26</td>
</tr>
</tbody>
</table>

At 7, 14, 21 and 28 days after acrylamide exposure, cerebrospinal fluid and serum were collected to measure transthyretin levels by ELISA. Data are expressed as mean ± SD of eight rats at each time point. *P < 0.05, vs. control group (one-way analysis of variance and least significant difference test).

Acrylamide exposure decreased leptin levels in CSF

Leptin concentration in serum decreased from 5.17 ng/mL to 4.54 ng/mL following 7-day exposure, and a similar change was found at other exposure time points (P < 0.05; Table 4). However, leptin concentration in CSF was reduced 33.33% after 21-day acrylamide exposure compared with the control group (P < 0.05). The CSF leptin to serum leptin ratios were 0.154 and 0.143, respectively, following 21- and 28-day acrylamide exposure, which were significantly lower than those in the control group (P < 0.05).

Discussion

Acrylamide exposure leads to cumulative neurotoxicity in workers and laboratory animals[25,26]. However, the mechanisms of toxicity are not yet clear. Several hypotheses, including interaction with nucleic acids, perturbation of second messenger and neurotransmitter systems, and disruption of oxidative balance, have been proposed[25,26]. LoPachin et al.[28] attempted to explain its neurotoxicity using an organic chemistry approach. However, there is very little research on the effect of acrylamide on blood-CSF barrier function, or on how acrylamide can enter the central nervous system. Our data demonstrates that acrylamide exposure results in the disruption of blood-CSF barrier permeability, and impairs its secretory and transport functions. Our findings offer an alternative mechanism of acrylamide-induced neurotoxicity.

In the present study, acrylamide exposure increased barrier permeability, as reflected in higher sodium fluorescein and Evans blue levels in the CSF and an elevated CSF/serum albumin ratio. Blood-CSF barrier function is maintained by the lipid bilayer of endothelial cells and the tight junctions between endothelial cells. Under physiological conditions, chemicals with a molecular weight greater than 180 kDa cannot cross the blood-CSF barrier passively. In the present study, sodium fluorescein (molecular weight: 376 kDa) and Evans blue (molecular weight: 960 kDa) served as markers of blood-CSF barrier leakage[20]. Our study is the first to use CSF samples from animals to assess barrier permeability. Our research group developed a method to directly measure blood-CSF barrier permeability by using a modification of Euser's method[30]. Evans blue and sodium fluorescein solutions were mixed and infused into the femoral artery, and we used a small needle to directly take CSF samples instead of brain tissue. Moreover, the CSF/serum albumin ratio was also measured in the present study. Dorta-Contreas's group[31] found that the CSF/serum albumin ratio is an objective index of the impairment of the blood-CSF barrier. However, this ratio might not be very sensitive due to the high molecular weight of albumin (69 kDa). Our results revealed that sodium fluorescein concentration in CSF significantly increased following 14-day acrylamide exposure, and levels continued to rise until the end of the experiment. The concentration of Evans blue in the CSF was significantly increased to 52.58% at 21 days and 153.46% at 28 days. A higher CSF/serum albumin ratio was observed 28 days after acrylamide exposure compared with control animals. Acrylamide exposure led to an increase in sodium fluorescein concentration in CSF after 14 days of exposure, an increase in Evans blue concentration in CSF starting at 21 days, and an increase in the CSF/serum albumin ratio starting at 28 days. The molecular weights of sodium fluorescein, Evans blue and albumin are 379 kDa, 960 kDa and 69 kDa, respectively, which demonstrates that acrylamide-induced blood-CSF barrier leakage worsened over time.

Current studies on Alzheimer's disease, ischemia, traumatic brain injury and heavy metal neurotoxicity have focused on blood-CSF barrier permeability using in vitro

Table 4 Effect of acrylamide exposure on cerebrospinal fluid (CSF) and serum leptin levels

<table>
<thead>
<tr>
<th>Time of acrylamide exposure (day)</th>
<th>Serum (ng/mL)</th>
<th>CSF (ng/mg pro)</th>
<th>CSF/serum</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Acrylamide</td>
<td>Control</td>
</tr>
<tr>
<td>7</td>
<td>5.17±0.30</td>
<td>4.54±0.42</td>
<td>1.02±0.30</td>
</tr>
<tr>
<td>14</td>
<td>5.17±0.28</td>
<td>4.38±0.26</td>
<td>1.03±0.28</td>
</tr>
<tr>
<td>21</td>
<td>5.18±0.21</td>
<td>4.40±0.39</td>
<td>1.02±0.19</td>
</tr>
<tr>
<td>28</td>
<td>5.17±0.31</td>
<td>4.41±0.31</td>
<td>1.02±0.30</td>
</tr>
</tbody>
</table>

At 7, 14, 21 and 28 days after acrylamide exposure, serum and CSF samples from experimental rats were obtained to measure leptin levels by ELISA. Data are expressed as mean ± SD of eight rats at each time point. *P < 0.05, vs. control group (one-way analysis of variance and least significant difference test).
or in vivo models[32-33]. Our findings demonstrate that acrylamide exposure disrupts blood-CSF barrier permeability, which might disrupt immune surveillance due to perturbed immune cell trafficking across the choroid plexus into the CSF[34]. Therefore, our data provide insight into the mechanism of acrylamide neurotoxicity.

Apart from permeability, the blood-CSF barrier also possesses the ability to secrete CSF and proteins, such as transthyretin, adrenomedullin, vascular endothelial growth factor and apolipoprotein J. The choroid plexus is the main component of the blood-CSF barrier. Transthyretin is the first protein found to be synthesized solely by the choroid plexus. Approximately 20% of the protein newly synthesized in the choroid plexus and about 50% of the newly synthesized protein secreted into the CSF is transthyretin[35-36]. Therefore, transthyretin level in CSF reflects the secretory function of the blood-CSF barrier, and also serves as an indicator for evaluating blood-CSF barrier impairment[31]. We found that transthyretin level in CSF was reduced to 33.6% after short-term (7-day) acrylamide exposure, and this decline was maintained until 28 days after exposure. This indicates that acrylamide acutely disrupts transthyretin secretion. Transthyretin serves as a thyroxine transporter and is involved in beta-amyloid peptide chelation, attenuating neurotoxicity[37-38]. Sousa et al.[39] showed that absence of transthyretin accelerates cognitive decline associated with aging. In addition, transthyretin is required for normal neural function and enhances neural regeneration[40]. The decrease in transthyretin levels induced by acrylamide might be involved in neurotoxicity by affecting nerve regeneration, although further study is required to investigate this possibility.

Another major role of the blood-CSF barrier is to transport nutrients in blood into the CSF and to transport waste from the CSF into the blood for clearance. Under physiological conditions, some substances synthesized peripherally are transported into the central nervous system. Leptin is one of those substances and can enter the brain through the OB-Ra receptor in the choroid plexus[41]. In the present study, leptin concentration in CSF was significantly reduced at 21 days after acrylamide exposure. Leptin level in serum was reduced 7 days after exposure, which needed further research to demonstrate. At present, there is no direct evidence showing that acrylamide exposure disrupts appetite in experimental animals or occupational workers. Our body weight data showed that a little body weight loss was present at 28 days of acrylamide exposure; however, the difference in weight was not significant (data not shown), similar to the previous study. In order to clarify whether the reduction in leptin level in CSF was mediated by disruption of leptin transport across the blood-CSF barrier or by a reduction in serum leptin level, we calculated the CSF/serum leptin ratio. The CSF/serum leptin ratio was significantly lower at 21 days in exposed rats compared with the control group, suggesting that acrylamide exposure disrupts leptin transport across the blood-CSF barrier.

Leptin possesses the ability to promote the development and maturation of the brain, and prevents apoptosis of neurons by increasing levels of the anti-apoptotic factor Bcl-2, as well as by decreasing levels of the pro-apoptotic factor caspase-3. Therefore, acrylamide-induced apoptosis of cerebellar granule neurons might partly be due to a decrease in leptin levels[42-43].

In summary, our findings firstly suggest that acrylamide exposure does impair blood-CSF barrier functions, including permeability, secretion and transport, which may be an alternative mechanism of acrylamide-induced neurotoxicity.

Materials and Methods

Design

A randomized controlled animal experiment.

Time and setting

This study was performed at the Central Laboratory, School of Public Health, and the Experimental Animal Center, Hebei United University, China from August 2011 to July 2012.

Materials

Animals

A total of 128 male Sprague-Dawley rats, aged 12 weeks, weighing 180–220 g, of specific pathogen free grade, were purchased from Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China; animal license No. SCXK (Jing) 2006-0009). Upon arrival, the rats were housed in the animal housing facility (a temperature-controlled room, under 12-hour light/dark cycle) of the Experimental Animal Center of Hebei United University, China (license No. SYXX (Ji) 2010-0038). They were acclimated for 1 week prior to experimentation. All animals were allowed free access to standard rat chow and tap water. The experiments described in this study were performed in accordance with the Guidelines for the Care and Use of Laboratory Animals of Hebei United University, China.

Drugs

Acrylamide, C3H5ON, white powder, was purchased from Amresco (Solon, OH, USA), batch No. 20110620. In our experiments, acrylamide was dissolved in saline at 20 mg/mL, and was fresh-made before injection.

Methods

Acrylamide administration

Acrylamide was intraperitoneally administrated to rats at a dose of 20 mg/kg per day, as previously described[44]. The injection schedule was 5 continuous days of injection and 2 days of withdrawal for 7, 14, 21 and 28 days of acrylamide exposure. Control rats were given the same volume of saline. The injection was performed using a syringe (5 mL).

Gait score evaluation

At 7, 14, 21 and 28 days after acrylamide exposure, the exposed and control rats were evaluated for walking ability, and gait score was assessed with a range of 1–4, as described previously[45-46]. A gait score of 1 indicates no degree of impairment, with 4 reflecting complete lameness. The evaluators performed gait scoring in a blinded manner and were not involved in animal care after acrylamide exposure.

Sample collection

At different time points after acrylamide exposure, serum and CSF samples were collected from the rats for albumin, transthyretin, and leptin measurements. CSF samples were obtained through a 26-gauge needle inserted between the protuberance and the spine of the atlas, and were centrifuged at 3,000 × g for 5 minutes, immediately after collection, then aliquoted, frozen at −80°C and stored for further analysis.
Blood samples were obtained from the abdominal aorta with a 5 mL needle syringe and placed at 4°C for 4 hours, then centrifuged at 4,000 × g for 10 minutes, aliquoted, and frozen at −80°C for use.

**Determination of blood-CSF barrier permeability using Evans blue and sodium fluorescein**

In the present study, Evans blue (molecular weight: 961 kDa; Sigma, St. Louis, MO, USA) and sodium fluorescein (molecular weight: 376 kDa; Sigma)[4][49] were used to assess the permeability of the blood-CSF barrier. Rats were anesthetized with ketamine HCl (100 mg/kg) via intraperitoneal injection. The skin of a lower limb was cut and the femoral artery was separated. A minimal incision at the femoral artery was made and a plastic PE50 tube was placed inside the artery. The tube was then connected with a syringe containing dye mixture solution (4 mL/kg 2% Evans blue + 0.6 mL/kg 10% sodium fluorescein) with an injection rate of 0.45 mL/min. After 30 minutes of cannulation, heart perfusion was performed to wash off the dye in the vessels using saline for 30 minutes, 3 mL/min. CSF samples were then collected to measure Evans blue and sodium fluorescein concentrations.

Detection of Evan blue concentration in CSF: CSF sample and Evans blue standard solution were mixed with two volumes of N,N-dimethylformamide, and then incubated in water at 50°C for 48 hours. Samples were centrifuged at 16,000 × g for 10 minutes to remove precipitated protein. The supernatants were put on a microplate and absorbance values were read in an ELISA reader (Bio-rad, Hercules, CA, USA) at 635 nm within 10 minutes. A standard curve was made based on the absorbance value of standard Evans blue solution. The sample concentration was calculated according to a standard curve and expressed as μg/mL.

Detection of sodium fluorescein in CSF: CSF sample and sodium fluorescein standard solution were mixed with three volumes of PBS and centrifuged at 15,000 × g for 10 minutes. The supernatant was isolated, diluted with 20% trichloroacetic acid (1:2), and centrifuged again at 100,000 × g for 15 minutes to precipitate protein. The supernatant was neutralized with 5 mol/L NaOH and transferred to a microplate, and its fluorescence intensity value was read by a spectrophotometer (RF-5301PC, Shimadzu Corporation, Japan) at an excitation wavelength of 480 nm and an emission wavelength of 525 nm within 5 minutes. A standard curve was plotted based on the fluorescence intensity value of standard sodium fluorescein solution. The sample concentration was calculated according to a standard curve and expressed as μg/mL.

Quantification of albumin, transthyretin and leptin using ELISA

Albumin concentrations in CSF and serum were quantified using an albumin ELISA kit (R & D Systems, Minneapolis, MN, USA; lower detection limit of 1.0 ng/mL). The concentrations of transthyretin and leptin in CSF and serum were determined using a transthyretin ELISA kit (R & D Systems; detection limit of 1.5 μg/mL) and a leptin ELISA kit (R & D Systems; detection limit of 0.25 μg/L), respectively. Albumin, transthyretin and leptin rabbit anti-rat polyclonal antibodies were used. Briefly, a 96-well polystyrene microplate coated with a rabbit anti-rat polyclonal antibody against rat albumin, transthyretin or leptin were used at room temperature. 50-μL aliquots of standards at different concentrations and 5-fold diluted serum and 10-fold diluted CSF samples were added into wells. After 1 hour of incubation, samples were rinsed and incubated with 50 μL of chromogen solution A and B for 15 minutes. When optimal blue color was seen, the termination solution was added to terminate the reaction. The absorbance values of the samples were immediately read on a microplate reader (Bio-Rad, Hercules, CA, USA) at 450 nm. The total protein concentration of the sample was measured using a Bradford protein assay kit and read on a microplate reader at 595 nm. A standard curve was plotted based on the absorbance value of standard albumin, transthyretin and leptin solutions. The sample concentration was calculated according to standard curves.

**Statistical analysis**

All data are represented as mean ± SD. SPSS 13.0 software (SPSS, Chicago, IL, USA) was used for data analysis. One-way analysis of variance was used to examine the differences among groups for albumin, transthyretin and leptin concentrations in CSF and serum. Intergroup pairwise comparison was performed using least significant difference test. All P values were two-tailed, and P < 0.05 was considered significant.

**References**


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