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Study of the serum levels of polyunsaturated fatty acids and the expression of related liver metabolic enzymes in a rat valproate-induced autism model

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ABSTRACT

To investigate whether the decreased level of serum polyunsaturated fatty acids (PUFAs) in patients with autism is associated with the expression of related liver metabolic enzymes, we selected rats that were exposed to valproic acid (VPA) on embryonic day 12.5 (E12.5) as a model of autism. We observed the serum levels of PUFAs and the expression of related liver metabolic enzymes, including Δ5-desaturase, Δ6-desaturase and elongase (Elovl2), in VPA-exposed and control rats on postnatal day 35 (PND35) and conducted sex dimorphic analysis. We found that the levels of serum PUFAs and related liver metabolic enzymes in the VPA rats were significantly reduced, in association with autism-like behavioral changes, the abnormal expression of apoptosis-related proteins and hippocampal neuronal injury, compared to the control rats and showed sex difference in VPA group. This finding indicated that rats exposed to VPA at the embryonic stage may exhibit reduced synthesis of serum PUFAs due to the down-regulation of liver metabolic enzymes, thereby inducing nervous system injury and behavioral changes, which is affected by sex in the meantime.

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1. Introduction

Autism is a neurodevelopmental disorder. Its hallmark symptoms are abnormalities in social interaction and communication, repetitive behaviors, and narrowed interests (Association, 2013), and these symptoms are accompanied by various neurobehavioral disorders, such as learning disability, hyperactivity and anxiety (O'Brien and Pearson, 2004; De Giacomo et al., 2014; Towbin et al., 2005). Recently, data from the US indicated that the incidence of autism spectrum disorder (ASD) was 14.7‰, with a male to female ratio of 4:1 (CDC, 2014). The causes of autism are complex, involving genetic, epigenetic and environmental factors (Currenti, 2010).

VPA is a common anti-epileptic drug that displays teratogenic effects. From day 20 to day 24 of pregnancy, before closure of the nerve canal, if pregnant women use teratogenic drugs such as VPA, the probability that their offspring develop a neurodevelopmental disorder increases (Rice and Barone, 2000). The intraperitoneal injection of VPA into female rats during early pregnancy results in autism-like changes in the offspring, and the brain structures and the biomarker levels in these offspring are similar to those in autism patients (Kim et al., 2013a; Schneider et al., 2008). Thus, this study adopted this animal model for subsequent research.

There has been increasing evidence that the change of metabolic pathway of PUFAs could affect the normal function of nervous system which is related to pathogenesis of some disease such as autism (Brown et al., 2014; Gumpricht and Rockway, 2014). PUFAs are crucial for proper functioning of cell membranes, particularly in the brain (Caramia, 2008; Extier et al., 2009). As precursors of n-3 and n-6 PUFAs, α-linolenic acid (ALA) and linoleic acid (LA) must be absorbed from food because they cannot be synthesized in the human body. These precursors are synthesized into long-chain PUFAs such as docosahexaenoic acid (DHA) and arachidonic

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acid (AA) by metabolic enzymes after entering the human body. Δ5-desaturase, Δ6-desaturase and Elovl2 are important metabolic enzymes in the synthesis of long-chain PUFAs, which primarily express in the liver (Rapoport et al., 2007), and their functional status directly affects the synthesis of long-chain PUFAs (Nakamura and Nara, 2004). Studies have revealed that autistic patients may exhibit abnormal PUFAs metabolism, which manifests as varying decreases in the levels of PUFAs in samples such as serum and plasma (Sun et al., 2013; El-Ansary et al., 2011a,b; Mostafa et al., 2015a,b; Mostafa and Al-Ayadhi, 2015). Additionally, reduced DHA uptake via food and a decreased serum level of DHA have been observed in autistic children (Al-Farsi et al., 2013).

To explore the possible explanation for the decline in the levels of PUFAs and nervous behavioral changes in autism, we utilized a relevant well-developed animal model of autism, the exposure of rats to VPA on day E12.5, to evaluate the serum levels of PUFAs, the expression of liver metabolic enzymes, the survival rate of hippocampal CA1 region neurons and the protein expression of caspase-3, Bax and Bcl-2 in the hippocampus and used the sex dimorphic analysis.

2. Materials and methods

2.1. Ethical approval

All experimental procedures were conducted in strict accordance with the guidelines established by the Ministry of Health of China and were approved by the Animal Care Committee of Harbin Medical University.

2.2. Experimental animals

Adult Wistar rats (YiSi, China) were housed overnight in one cage (male:female = 1:1). The next day, the detection of a vaginal plug in the female rats marked the success of copulation and was recorded as E1. The gestational female rats were randomly divided into two groups. The first group, referred to as the model group, received a single intraperitoneal injection of 600 mg/kg VPA (Sigma, USA) dissolved in physiological saline at a concentration of 250 mg/ml on E12.5. The other group, referred to as the saline group, was injected with the same volume of physiological saline. The offspring born from the rats in the model and saline groups were included in the VPA and control groups, respectively (Schneider and Przewlocki, 2005). On PND21, the offspring were weaned and divided into different cages according to sex. The rats were freely provided with the same food and water. The laboratory temperature was 21 ± 1 °C, with a humidity of 55% and a 12-h photoperiod. The experiment was conducted from 9am to 3pm.

2.3. Behavioral tests

2.3.1. Morris water maze test

The Morris water maze test (on PND21) (WMT-100, TME, China) was used to evaluate the learning and memory ability of the rats (Morris, 1984). An acrylic acid platform (with a diameter of 8 cm) was placed at the center of the third quadrant of the water maze and was hidden 1 cm below the water surface. From day 1 to day 3, the rats were trained to locate the hidden platform within 60 s. From day 4 to day 7, the escape latency of the rats to climb on the platform was recorded. On day 8, the hidden platform was removed, and the number of times that the rats crossed the former platform position within 60 s was recorded.

2.3.2. Open field test

This test evaluated the locomotor activity of the rats on PND28. The rats were habituated to the test box for 5 min before the test (Kim et al., 2013a). An auto-tracking camera system (YH-OF, YiHong, China) was used to record the total distance traveled and the duration of movement in the test box within 30 min.

2.3.3. Sociability and social preference tests

The sociability test evaluated the social interaction of the rats on PND31 (Moy et al., 2004). We used three interconnected compartments in this test. The compartments on both sides contained an empty cage. First, the experimental rat was placed in the central compartment for adaptation for 5 min. During this period, the rat was free to enter each compartment. Then, the experimental rat was removed, and a cage containing a conspecific stranger rat to the tested rat was placed in the left compartment, labeled as stranger rat no. 1, whereas an empty cage was placed in the right compartment. Then, the tested rat was placed in the empty central compartment again to begin the 10-min sociability test. Afterwards, the social preference test was immediately performed. A new stranger rat no. 2 was added to the empty cage from the right compartment, which was then swapped with the left cage. The experimental rat was placed in the empty central compartment again according to the same method as described above. The same camera system as that used for the open field test was used to record the duration spent and the distance traveled in the three compartments by the experimental rats.

2.4. Fatty acid analysis

The rats were anesthetized on PND35 after 12-h fasting. Blood was then collected from their abdominal aorta and was immediately centrifuged at 3000 × *g* for 10 min at room temperature. Then, the serum was separated from the blood, and 0.2 ml of serum, 0.2 ml of internal standard solution (200 μg/ml, C17:0) and 2 ml of 10% sulfuric acid–methanol solution were added to a 10-ml test tube (Liu et al., 2010). Then, the tube was suspended for 1 min. The plug was sealed tightly and the tube was incubated in a 62 °C water bath for 2 h. Then the sample was cooled. A certain amount of anhydrous sodium sulfate and 2 ml of hexane were added to the tube, followed by incubation for 1 min. The tube was centrifuged at 4000 × *g* for 5 min, and the supernatant was pipetted into a 2-ml test tube and dried using nitrogen gas. Finally, 0.1 ml of *n*-hexane was used to dissolve the sample, and 1 μl of each sample was employed for analysis using a TRACE gas chromatograph with a Polaris Q mass spectrometer (Thermo Finnigan, Austin, TX, USA). All fatty acid standards were purchased from Sigma Company (USA, ≥99% purity) and were classified into two groups. The n-3 PUFAs included ALA (C18:3n-3), eicosapentaenoic acid (EPA, C20:5n-3), docosapentaenoic acid (DPA, C22:5n-3) and DHA (C22:6n-3), whereas the n-6 PUFAs included LA (C18:2n-6), γ-linolenic acid (GLA, γ-C18:3n-6), AA (C20:4n-6), and docosatetraenoic acid (DTA, C22:4n-6).

2.5. Western blot analysis

On PND35, the rats were narcotized and then killed. Hippocampus and livers were rapidly removed on ice. An aliquot of each collected sample was mixed with RIPA lysis buffer (50 mM Tris–HCl, 150 mM NaCl, 10% glycerol, 1% Nonidet P-40, 5 mM EDTA and 1 mM phenylmethylsulfonyl fluoride) at a ratio of 1:5 (w/v). This mixture was centrifuged at 13,200 × *g* at 4 °C for 20 min, and the supernatant was used for protein concentration estimation using a BCA protein assay kit (Beyotime, China). Then, 30-μg protein samples were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes (Millipore, MA). The membrane was blocked with 5% skim milk for 1 h and incubated in the following antibodies at 4 °C overnight for immunodetection: rabbit anti-FADS1 (1:200, Santa Cruz, USA), goat anti-FADS2 (1:200, Santa Cruz, USA), goat anti-

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