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A METHOD FOR ESTABLISHING AN ANIMAL MODEL OF LIKE-AUDITARY NEUROPATHY

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Abstract

Objective: To establish an animal model of like-auditory neuropathy in neonatal rat. **Methods** The animals were injected with phenylhydrazine hydrochloride or saline at 7-day of age. ABR and DPOAE were performed to assess the auditory function. The cochlea basilar membrane stretched preparation and cochlear frozen sections were prepared for immunohistochemical staining to examine the morphological change of hair cells and spiral ganglion cells (SGNs). **Results** At 7-day age the ABR waveI, III, V, latencies andI- III,I-V IWIs in the experimental group were significantly prolonged compared with those in the control group. The ABR thresholds were also elevated in the experimental group. We found there is no significant difference in DPOAE in phenylhydrazine hydrochloride exposure group compare to control group. The cochlear hair cells showed no signs of loss in both group, but the total number of neurofilaments positive cells in SGNs were significantly reduced in the phenylhydrazine treated animals. **Conclusion** Our study suggests that phenylhydrazine hydrochloride can change the auditory function and induce peripheral nerve pathology by targeted mainly the SGNs in neonatal rat.

Keywords: Animal model; Auditory brainstem response; Distortion - product otoacoustic emission; auditory neuropathy

Introduction

Neonatal hyperbilirubinemia has been accepted as one of the main risk factors leading to hearing loss in children. Previous epidemiological studies have shown a close connection between hyperbilirubinemia and auditory impairment [1]. studies have shown the incidence of auditory neuropathy/dyssynchrony (AN/AD) from different population was between 0.23% and 1.3%. In children with severe hearing loss, this ratio is increased between 1.8% and 14%. Furthermore, the hyperbilirubinemia is one of the most important risk factors for AN/AD, which is characterized by normal OAEs and/or cochlear microphonics (CM), but abnormal auditry brainstem responses (ABRs).

In 2008, Mejia et al started a neonatal rat hyperbilirubinemia model by intraperitoneal injection of phenylhydrazine hydrochloride to induce hemolysis, but they only studied the role of bilirubin and iron in the encephalopathy secondary to a hemolytic disease [2].We could not completely understand how lesions in the auditory nervous system from the hair cell to the brainstem and cerebrum contribute to the development of AN/AD. Here, we establish a hyperbilirubinemia model induced by hemolytic process to study the auditory system physiopathology of the neonatal hyperbilirubinemia.

Material and methods

Animal and treatments

In this study, thirty five 7-day-old Wistar rats at 11 to15grams of both sexes were used. All animals were purchased from Beijing Weitonglihua laboratory animal Technology co., LTD. All the animal procedure was con-

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ducted in accordance with the Guidelines for Animal Experiments at PLA General Hospital. The animals are randomly divided into the experimental (n=21) and the control group (n=14).The animals in experimental group were treated with subcutaneous injection of phenylhydrazine (Tianjin Guangfu fine chemical industry research institute) at 50mg/kg for two consecutive days. The animals in control group were given subcutaneous injection with the same amount saline. After treatment the animals were placed in the warmed heating pad to keep constant temperature in humidity condition.

Measurement of Bilirubin concentration

To determine the Bilirubin concentration in blood after after injection of phenylhydrazine, we collected the blood from 5 rats in the control group and 10 rats in the experimental group.We given intraperitoneal injection of 10% chloral hydrate(0.45 ml/100g) to euthanize the animals at 24 hours after the last administration. In brief, we collected 1 ml blood from each animal,centrifuged at 10000 xg 4 ℃ for 5 minutes, then the serum at upper level was collected and immediately sends to the people's liberation army general hospital-clinical laboratory with sample covered to avoid direct light explosion. We determined the concentrations of serum total bilirubin and direct bilirubin using ROCHE MODULAR P800/ D2400 automatic biochemical analyzer.

Electrophysiological Recordings

At 7th days after the treatment respectively,bilateral ABR was performed. The rats firstly anesthetized by intraperitoneal injection of 10% chloral hydrate (0.45 ml / 100 g) . And then alternating short sound (click) at 3-4 kHz was generated using TDT RX6 MULTIFUNCTION PROCESSOR instruments, TDT system III (Tucker-Davis - technologies system III),overlaied 1024 times, 10ms duration (band-pass filter: 300-3000 Hz).Subdermal needle electrodes were placed at the vertex (non-inverting), the ipsilateral mastoid (reference), and contralateral mastoid (ground). Stimuli were presented through a ES1 microphone placed at 0.5 cm from the test ear. The presentation level was attenuated in 10-dB steps from 90 down to 10 dB (SPL).Replications were obtained at stimulus levels near threshold. The lowest stimulus level that elicited a repeatable was considered as the threshold.After treatments in 7th days ,American respectively,DPOA- Es at 2f1-f2 were recorded using the TDT RX6 MULTIFUNCTION PROCESSOR and ER-10b + LOW NOISE MIC SERIAL# 829. The EC1earpiece was inserted into the ear canal. Two primary tones, f1 and f2 (L1=70dB SPL,L2=70dB SPL,f2/f1=1.2), delivered to the ear canal via flexible tubes connected to the earpiece. Respectively test the DPOAE amplitudes at 2,4, 6 and 8 kHz,four different frequency Estimate.the DPOAE passed if response amplitude is 3 dB higher than the background noise, and record the amplitude at 2f1-f2.

Tissue processing and immunohistochemistry

The animals were euthanized with by intraperitoneal injection of 10% chloral hydrate and perfused with 4% polyformaldehyde.Cochlear basement membranes were isolated from the cochlea specimen under an anatomical microscopy.The basement membrane samples were blocked with 5% sheep serum in room temperature for 30 min, washed three times by 0.01M phosphate buffer solution (PBS) for 5 min each time.Anti-Neurofilament200 (Sigma, rabbit, 1: 200) and Anti-Myosin7a (PROTEUS BIOSCIENCES INC, rabbit, 1: 200) were used as a primary antibody and incubated in 4 ℃ refrigerator overnight.Next day,the samples were washed 3times by 0.1% PBS for 5 min/time. Gout anti-rabbit Alexa flour488 (1: 200) or Texas Red (1:200) were used as a second antibodies and incubated for 1 hour in room temperature. The samples were washed 3 times by 0.1% PBS for 5min/ time. DAPI (beyotime) was added to the samples to label the nucleus for 10min in room temperature and washed again for 3 time in PBS. The samples were coversliped with glycerin. The inner and outer hair cells, spiral ganglion and nerve fibers were examined under the laser confocal microscope (OLYMPUS FLUO VIEW 1000) to identify the morphological changes after treatment.

Other half of the cochlea specimen were fixed by 4% paraformaldehyde and placed in 4 ℃ refrigerator overnight.After the cochlea were decalcified by 10% EDTA for 72h and dehydrated with 15% and 30% sucrose for 2 to 3 days. The cochlea were embedded with OCT glue and sectioned at 10um thickness with the cryomicrotome The staining and image capture were performed as indicated above.

Statistical Analysis

Values are reported as mean ± SD.Differences were compared by one-way analysis of variance (ANOVA). was considered to indicate statistical significance.

Results

Bilirubin concentration

The concentrations of total serum bilirubin、direct serum bilirubin and indirect serum bilirubin in experimental group are significantly higher than that in the control group.

Electrophysiological Recordings