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Original Article

Pathological changes of cochlear in deaf mice at different time after mouse cytomegalovirus infection

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Abstract: Objective: This study aims to observe the pathological changes of inner ear in deaf mice at different time after mouse cytomegalovirus infection. Methods: A total of 60 BALB/C mice were divided into 2 groups randomly: model group (A) and control group (B). In model group, 10 μl of MCMV was injected into the brain of each mouse while 10 μl of physiological saline was injected in control group. 10 cochlear samples were taken from 5 mice selected from each group randomly after infection for 1, 3, 5, 7, 14 and 21 days respectively. They were detected with PCR and HE staining methods. Auditory brain stem response was determined. The apoptosis of spiral ganglion (SGN) cells was detected by apoptosis assay kit. The levels of Bcl-2 and Bax were detected by RT-PCR and western blotting methods. Results: In group A, PCR results were negative after infection for 1 day, they were positive after infection for 3 days to 21 days. In group B, PCR results were negative in the experimental period. Compared with group B, ABR I wave latency and threshold increased while ABR I wave decreased in group A. There were no obvious hyperemia and inflammatory cells infiltration in group B, In group A, hemorrhage of scala tympani and scala vestibule appeared and reached highest peak after infection for 3 days accompanied by inflammatory cell infiltration; the vestibular membrane thickened after infection for 5 days; cell gap of SGN cells widened, arranged more sparsely with cell edema after infection for 7 days accompanied by infiltration of plasma cells; fibroblast proliferation and fibrosis appeared after infection for 14 days. Conclusions: MCMV infection occurred in cochlear after MCMV infection for 3 days and could sustain, the continues pathological changes of inner will bring difficulties to the treatment of CMV deafness, further studies on the specific mechanism of SGN changes caused by CMV infection will provide an important target for the treatment of CMV deafness.

Keywords: Mouse cytomegalovirus (MCMV), cochlea, MCMV-DNA, inflammatory reaction

Introduction

Cytomegalovirus (CMV) is a member of herpes virus, its infection rate is about 0.2%-2.3% in the newborn [1, 2]. Congenital CMV infection will be affected by maternal age, race, economic status and other social factors [3]. Almost all the people faced the risk of CMV infection and the infection rate in populous and economically undeveloped regions is higher. The clinical symptoms such as skin ecchymosis, jaundice, hepatosplenomegaly, chorioretinitis or cerebellar malformation could appear in children with congenital infection or acquired infection of human cytomegalovirus (HCMV) [4, 5]. Dahle [6] first reported progressive hearing loss in children after congenital HCMV infection in 1974, and suggested that subclinical congenital HCMV infection may be one of the causes of sensorineural hearing loss (SNHL) in infant. At present, congenital HCMV infection is thought to be the main cause of infantile non hereditary SNHL [7-11]. Deafness or hearing loss caused by CMV infection can be unilateral or bilateral, they had undalatory, progressive and delayed characteristics, and began in a few months even years after birth [7, 12]. In this study we observed the pathological changes of inner ear in deaf mice at different time with MCMV-induced hearing loss mouse model [13].

Materials and methods

Experimental animals and cells

NIH/3T3 cells and MCMV Smith virus were gifted from Shandong Provincial Academy of

Medical Sciences. A total of 60 SPF grade adult healthy BALB/c mice weighing 20 to 30 g were obtained from the animal experimental center of Shandong University. MCMV Smith virus infected NIH/3T3 cells and the TCID50 was 104 IU/0.1 ml using cytopathic virus titration [15]. The mice were randomly divided into model group (A, n=30) and control (uninfected) group (B, n=30). In model group, 10 μl of MCMV was injected into the brain of each mouse while 10 μl of physiological saline was injected in control group.

Determination of auditory brain stem response (ABR)

Determination of ABR was performed according to reference [13]. The mice were anaesthetized with ether after infection for 3 weeks. ABR was determined in the acoustic electric shielding state. The detection electrode was placed under the skin of test ear mastoid, the reference electrode was placed under the skin of the middle of head, the ground electrode was placed on the toe. They were stimulated with rarefaction wave and short sound. The stimulus frequency was 13 Hz and stimulus intensity was 80 dB, scanning speed was 1 ms/D.

Cochlea samples

10 cochlear samples were taken from 5 mice selected from each group randomly after infection for 1, 3, 5, 7, 14 and 21 days respectively. They were detected with PCR, HE staining and apoptosis of spiral ganglion (SGN) cells.

PCR detection

DNA was isolated from the samples using DNA isolation kit. Primers sequence was follows. Forward: 5'-TCAGCCATCAACTCTGCTACCAAC-3'; Reverse: 5'-ATCTGAAACAGCCGTATATCATCTTG-3' [14]. The reaction conditions were 94°C 5 min; 94°C 30 s, 55°C 30 s and 72°C 30 s, 30 cycles; 72°C 10 min. Following amplification for 35 cycles, the products were detected using 1.5% agarose gel electrophoresis and viewed with GIS-2008 digital gel imaging system.

HE staining

Cochlear samples were fixed with 10% formalin for 3 days and decalcified with EDTA for 1 week. They were embedded with paraffin and sliced. They were observed under microscope after routine HE staining.

Detection of apoptosis

The apoptosis of SGN cells was detected by apoptosis assay kit according to the manual. Apoptosis related genes of Bcl-2 and Bax were detected by RT-PCR and western blotting methods. Total RNA was extracted from the cochlear samples using the RNA extraction kit according to the protocol. Samples (1 μg per sample from a total volume of 20 μL) were quantified by spectrophotometry. The expression levels of Bcl-2 and Bax were determined by RT-PCR kit following the manufacturer's instructions. All values obtained were normalized to mouse β-actin. The sequences of PCR primers were as follows: Bcl-2 forward: 5'-GGATTGTGGCCTTCTTTGAG-3', reverse: 5'-CCAAACTGAGCAGAGTCTTC-3'; Bax forward: 5'-TCCACCAAGAAGCTGAGCGAG-3', reverse: 5'-GTCCAGCCCATGATGGTTCT-3'; β-actin forward: 5'-CTGCCGCATCCTCTTCCTC-3', reverse: 5'-CTGTCGCCTTCACCGTTCC-3'. The protein levels of Bcl-2 and Bax were determined by western blotting method. The samples were lysed in RIPA lysis buffer, and the lysates were harvested by centrifugation (12,000 rpm) at 4°C for 5 min. Protein samples were then separated by electrophoresis in a 10% sodium dodecyl sulfate polyacrylamide gel and were transferred onto a polyvinylidene fluoride membrane. After blocking the nonspecific binding sites for 60 min with 5% nonfat milk, the membranes were incubated overnight at 4°C with required primary antibodies. The membranes were then washed three times with Tris-buffered saline with Tween-20 (TBST) for 10 min and were probed with the horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG antibody at room temperature for 1 h. After three washes with TBST, the membranes were developed using an enhanced chemiluminescence system (Applygen Technologies Inc, China). The protein levels were normalized to the level of GAPDH detected using goat anti-mouse GAPDH monoclonal antibody.

Results

ABR determination

Compared with group B after infection for 3 weeks, ABR I wave latency and threshold

Table 1. Comparison of ABR I wave latency, amplitude and threshold in different group after infection for 3 weeks ($\bar{x}\pm s$)

Group	N	I wave latency (ms)	I wave amplitude (uv)	Threshold (dB)
A	30	1.93±0.11*	1.82±0.35*	66.26±4.19*
B	30	1.27±0.13	4.91±0.23	24.52±3.82

*$P<0.05$ vs B group.

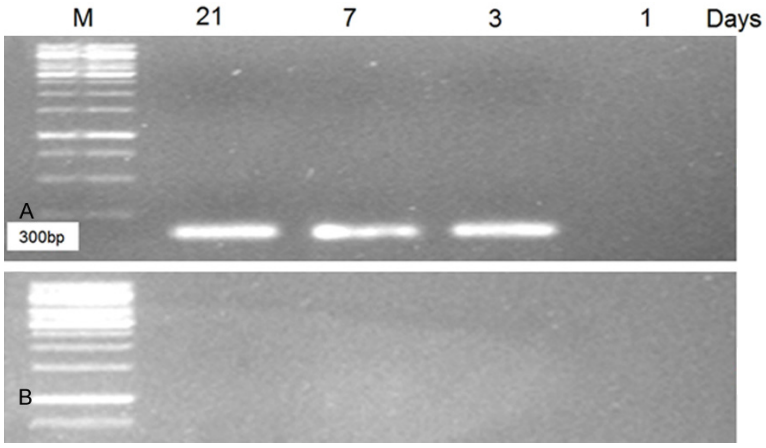

Figure 1. PCR results of MCMV infection in different groups. A: Group A; B: Group B. In group A, PCR results were negative after infection for 1 day, they were positive after infection for 3 days to 21 days. In group B, PCR results were negative in the experimental period.

increased while ABR I wave decreased in group A (P<0.05) (**Table 1**).

PCR results

In group A, PCR results were negative after infection for 1 day, they were positive after infection for 3 days to 21 days. In group B, PCR results were negative in the experimental period. These showed that MCMV infection occurred in cochlear after intracranial injection of MCMV suspension for 3 days and could sustain to 3rd week (**Figure 1**).

Pathological changes of cochlear in different time and groups

There were no obvious hyperemia and inflammatory cells infiltration in group B, no thickening of the vestibular membrane, SGN cells arranged densely without widening cell gap and no fibrosis. In group A, there were no obvious changes after infection for 1 day, massive hemorrhage of scala tympani and scala vestibule appeared and reached highest peak after infection for 3 days accompanied by inflammatory cell infiltration. The vestibular membrane thickened after infection for 5 days; cell gap of SGN cells widened, arranged more sparsely with cell edema after infection for 7 days accompanied by infiltration of plasma cells; fibroblast proliferation and fibrosis appeared after infection for 14 days (**Figure 2**).

Apoptosis results

As shown in **Figure 3**, there were few apoptotic cells and no obvious changes in each time point in control group, while the apoptotic cells increased significantly in each time point after MCMV infection in group A compared to the control group (P<0.05), it reached the peak after MCMV infection for 7 days. RT-PCR and western blotting results showed that the levels of Bcl-2 decreased and the levels of Bax increased in group A after MCMV infection for 3 days when compared to group B (P<0.05, **Figures 4** and **5**).

Discussion

The mechanism of SNHL caused by congenital CMV infection is not very clear. The delayed and progressive characteristics may be due to the persistent inflammation mediated by virus copy or host immune cells. Persistent inflammation such as the cell loss of outer and inner hair, cochlear ROS, increased TNF-α and interleukins could appear in cochlear after inner ear infected by CMV [15-17]. It has been confirmed that, CMV virus can be found in human and animal model of cochlea by PCR or immunohistochemistry [18-21]. In this study we found that MCMV infection occurred in cochlear after intracranial injection of MCMV suspension for 3 days and could sustain to 3rd week. The pathological changes of cochlear also occurred from 3rd day after intracranial injection of MCMV suspension, which including hemorrhage of scala vestibuli and scala tympani with infiltrating inflammatory cells, stria vascular hyperemia, vestibular membrane thickening, spiral ganglion (SGN) cell gap widened and the scala tym-

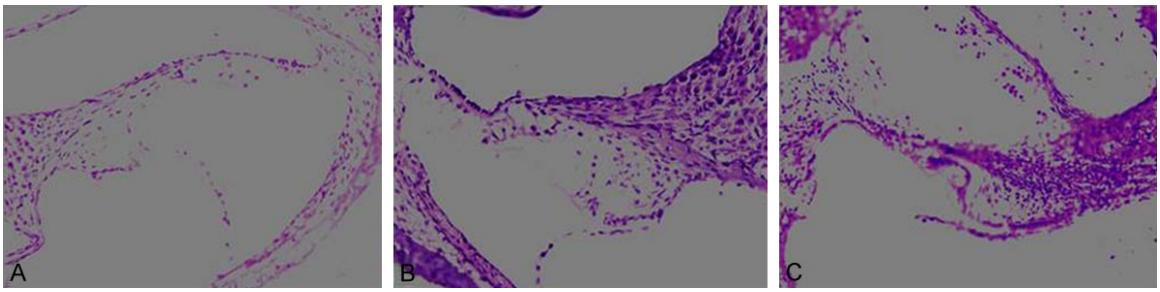

Figure 2. HE staining results (×400). A: HE staining results after infection for 5 day in group A, the vestibular membrane thickened after infection for 5 days; B: HE staining results after infection for 1 day in group A, there was no obvious change after infection for 1 day; C: HE staining results in group B, there were no obvious hyperemia and inflammatory cells infiltration in group B.

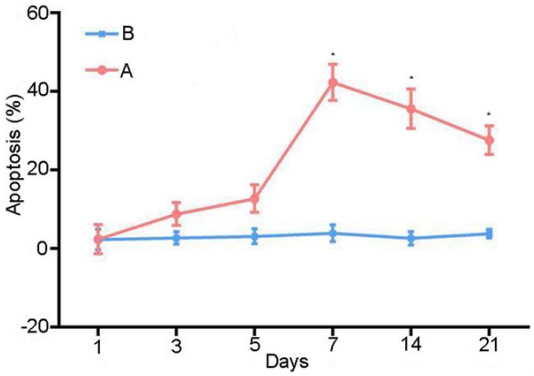

Figure 3. Apoptosis results of SGN cells. A: Group A; B: Group B. There were few apoptotic cells and no obvious changes in each time point in control group, while the apoptotic cells increased significantly in each time point after MCMV infection in group A compared to group B. *Compared with group B, P<0.05.

pani fibrosis. Hemorrhage of scala vestibuli and scala tympani with infiltrating inflammatory cells was more serious after infection for 3-5 days which maybe because of the acute inflammatory reaction in cochlea caused by MCMV infection. However, other studies [22, 23] showed that these occurred after infection for 8 days.

Previous studies showed that SGN and perilymphatic epithelial cells were the main parts of virus infection, while the inner and outer hair cells were not main infection parts [17]. In this study, we found that the intercellular gap of SGN cells changed after infection for 7 days with cellular edema. This was caused by persistent inflammation in the cochlea or by the cytopathic effect (CPE) of MCMV remained unclear. There were few apoptotic cells and no obvious changes in each time point in control group, while the apoptotic cells increased significantly in each time point after MCMV infection in group A. RT-PCR and western blotting results showed that the levels of Bcl-2 decreased and the levels of Bax increased in group A after MCMV infection for 3 days when compared to group B. The relationship between CMV and host cells is very complex, its infection in the body not only can promote the apoptosis of host cells but also can inhibit the apoptosis of the host cells, which maybe correlated with the infection dose [25]. It inhibits the apoptosis of the host cells in the early stage of infection and promotes the apoptosis of host cells in the later stage of infection [26]. The differences existed in different studies because of different modeling methods and different strains of CMV activity.

The most effective treatment is artificial cochlear implantation for the pre language children with bilateral severe or very severe SNHL. Studies have found that cochlear fibrosis occurred after long-term CMV infection in human [16] and guinea pig [18, 27] and fibrosis could develop into ossification. Although the fibrosis and ossification of cochlear is not a contraindication for cochlear implantation, it is difficult for electrode placement. We found cochlear fibrosis in one mouse after infection for 14 days and in the other mouse after infection for 21 days without obvious ossification. The target cells of artificial cochlea were SGN cells, so the residual SGN cells' number and function in patients determined cochlear transplantation effect. In this study we found morphological changes appeared in SGN cells after infection for 7 days and the cause was not clear. At present, the curative effect of antiviral

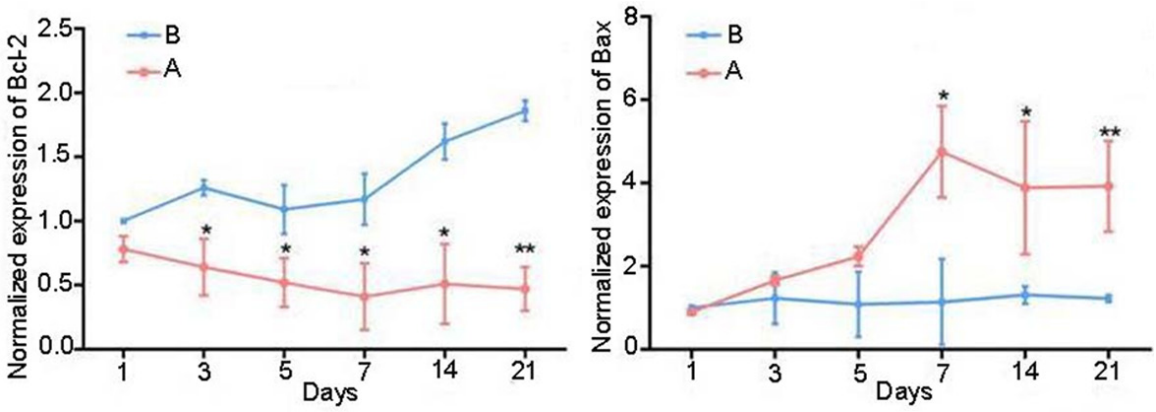

Figure 4. RT-PCR results of Bcl-2 and Bax expression. A: Group A; B: Group B. The levels of Bcl-2 decreased and the levels of Bax increased in group A after MCMV infection for 3 days when compared to group B. *Compared with group B, P<0.05.

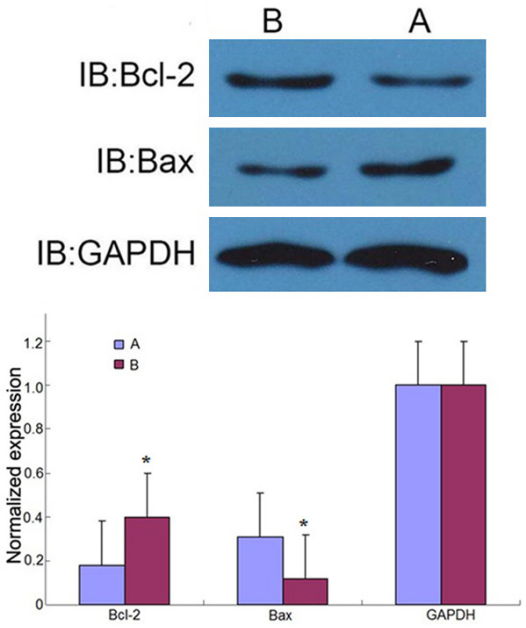

Figure 5. Western blotting results of Bcl-2 and Bax proteins. A: Group A; B: Group B. The levels of Bcl-2 decreased and the levels of Bax increased in group A after MCMV infection for 3 days when compared to group B. *Compared with group B, P<0.05.

therapy alone for CMV deafness was unsatisfactory. Further research on the specific mechanism of SGN changes caused by CMV infection will provide an important target for the treatment of CMV deafness.

In a word, MCMV infection occurred in cochlear after intracranial injection of MCMV suspension for 3 days and could sustain, the continues pathological changes of inner will bring difficulties to the treatment of CMV deafness, further studies on the specific mechanism of SGN changes caused by CMV infection will provide an important target for the treatment of CMV deafness.

Disclosure of conflict of interest

None.

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