Relationship Between Argyrophilic Nucleolar Organizer Region Proteins of Pediatric Epilepsy and T Lymphocyte Subpopulation

Hongping Zhong¹, Xiaoning Cheng² and Peiqiang Yan³*

¹Department of Pediatrics, Yan’an University Affiliated Hospital, Yan’an, 716000, Shaanxi, China
²Department of Pediatrics, the Second Affiliated Hospital of Shaanxi University of Traditional Chinese Medicine, Xianyang, 712000, Shaanxi, China
³Department of Pediatrics, Hongze District People’s Hospital, Huai’an, 223000, Jiangsu, China

ABSTRACT

The objective of this study was to investigate the changes in argyrophilic nucleolar organizer region proteins (Ag-NORs) of pediatric epilepsy and T lymphocyte subpopulation and their relationship before and after treatment, 156 children with epilepsy diagnosed at Yan’an University Affiliated Hospital were selected as the research objects. In addition, 100 healthy children were selected and divided into control group. Flow cytometry was utilized to detect T lymphocyte subpopulation. Common silver staining technique was adopted to detect Ag-NORs of T lymphocyte. There were 85 patients in big seizure group (54.48%) and 41 patients in minor vitality loss seizure group, including 12 with infantile spasm (7.69%), 15 with limited motor seizure (9.61%), and 14 with psychomotor seizure (8.97%). Compared with normal control group, there were 46 children with vegetative nervous seizure (29.48%). The expressions of CD3+, CD4+, CD8+ lymphocyte in epileptic children group, primary epilepsy group, secondary epilepsy group, big seizure group, minor vitality loss seizure group, and vegetative nervous seizure group decreased, while that of CD20+ lymphocyte improved (P<0.05). Compared with that of normal group, Ag-NORs content of T lymphocyte among children with epilepsy was lower than that among normal children. It was concluded that T lymphocyte subpopulation could be replaced by the measurement of Ag-NORs content of T lymphocyte to assess the immune function and status of children with epilepsy.

INTRODUCTION

Epilepsy is a chronic cerebral disease caused by multiple pathological factors. It is featured mainly with repeatable, paroxysmal, and transitory central nervous system dysfunction caused by excessive discharge of brain neurons (Sartori et al., 2019; Wongs-Kisiel et al., 2018). Epilepsy occur among population of all ages, regions, and ethnicities. In particular, most patients suffering from epilepsy are children and adolescents (Stewart et al., 2019; Juhász and John, 2020). Children epilepsy is a recurrent nervous system syndrome with complicated causes. So far, the pathogenesis of epilepsy is not completely clear (Quintiliani et al., 2021; Mangunmadja et al., 2021; Arski et al., 2021). In recent years, some scholars find out that most epilepsy children taking antiepileptic drugs for a long time suffer from different levels of the abnormalities in immune function (Operto et al., 2020; Aronu et al., 2021; Johnson et al., 2021). Hence, the improvement of immune function of children with epilepsy may be conducive to improving therapeutic effect and prognosis.

Detection methods and indexes of immune function and status of children with epilepsy are still hot medical topics. Based on consulting a large number of literature research and analysis, researchers found out that the immunoassay system adopted advanced image analysis technique and implemented the quantitative analysis of the transcriptional activity of ribosomal deoxyribonucleic acid (rDNA) of nucleolus formation area in T lymphocyte...
et al. (2021). The system integrates modern oncology, immunology, molecular biology, and computer image analysis technology and quantitatively analyzes argyrophilic nucleolar organizer region proteins (Ag-NORs) of peripheral blood T lymphocyte among children with epilepsy from the perspective of gene transcription level to reflect proliferation ability and activity of cells (Góes Rabelo et al., 2021; Damar and Eroz, 2022; Gupta et al., 2018). In the system, the ratio of silver staining area to nuclear area (I.S%) was set as the detection index of Ag-NORs to reflect the transcriptional activity of rDNA in T lymphocyte nucleus, which indirectly the immune function and status of children with epilepsy (Gajewska et al., 2022).

One hundred fifty-six children diagnosed with epilepsy between May 15, 2020 and April 25, 2022 were selected as the research objects. The immunoassay system was used to measure and analyze CD3+, CD4+, CD8+, CD20+, and CD4+/CD8, and Ag-NORs of T lymphocyte to discuss the change rule of T lymphocyte subpopulation among children with epilepsy. Besides, the effect of immune dysfunction on the pathogenesis among children with epilepsy and the correlation between Ag-NORs content of T lymphocyte and CD3+, CD4+, CD8+, CD20+, and CD4+/CD8+ among children with epilepsy were investigated to provide scientific basis for the adjuvant diagnosis of children with epilepsy and assessment of prognosis.

MATERIALS AND METHODS

One hundred and fifty six children diagnosed with epilepsy at Pediatric Neurology Department and Children’s Hospital Outpatient of Yan’an University Affiliated Hospital between May 15, 2020 and April 25, 2022 were selected as the research objects, including 94 males and 62 females aged between 3 and 10. Their average age was 5.2±1.7. Besides, 100 healthy children with similar age structure were selected as control group. The implementation of this experiment had been approved by Yan’an University Affiliated Hospital between May 15, 2020 and April 25, 2022.

A. All children conformed to the diagnostic standards related to epilepsy (Sansevere et al., 2017).
B. Children aged between 3 and 12.
C. Children didn’t suffer from other neurodegenerative diseases or tumors based on imaging examination.
D. Children were excluded from the research based on the following standards.
   A. Children were less than 3 years old or more than 12 years old.
   B. Children suffered from hereditary diseases or immune system diseases.
   C. Children suffered from heart, liver, and kidney insufficiency.

Flow cytometry method

1mL children’s peripheral blood was extracted and lymphocyte separation fluid was used to separate single nucleoid cell. 0.01mL phosphate buffer solution (PBS containing 0.1% NaHCO₃) was added to rinse single nucleoid cell. Cell density was adjusted to 2×10⁶/mL. 100µL cell suspension was taken from each tube and added with 20µL monoclonal antibody (American Becton, Dickinson and Company). After that, cell suspension and monoclonal antibody were mixed evenly using an oscillator. Rat Ig G (Wuhan Aoke Botai Biotechnology Co., Ltd) was added into negative control group and incubated at room temperature for 30 min. Next, it was washed with 0.01mL PBS and then centrifuged at 1000rpm for 10 min. Supernatant was removed and 20µL fluorescein isothiocyanate (FITC)-goat anti mouse Ig G (Fab') (Shanghai Canspec Scientific Instruments Co., Ltd) was added and then placed away from light at room temperature for 30 min. After being rinsed with 0.01mL PBS, BD FACS Calibur flow cytometer (American Becton, Dickinson and Company) was used to analyze the percentage of positive cells. Lymphocyte hilum was set as usual and 5 to 10×10⁴ cells were detected by each tube.

Detection of T lymphocyte Ag-NORs by common silver staining technique

Firstly, cells were cultured. Under aseptic condition, 0.3 to 0.5mL anticoagulant was extracted and added into culture solution. Cells were cultured at 37°C for 72 h. After that, cells were separated. After being floated in a rotating culture flask, cells were poured into centrifugal tubes. Then, they were centrifuged for 5 min. Supernatant was removed and 5mL hypotonic solution was added before being placed at 37°C for 10 min. Next, 0.5mL stationary liquid was added and mixed evenly. Then, it was centrifuged for 5 min. After that, supernatant was removed, which was repeated twice. 0.3mL stationary liquid was added into sediment and made into cell suspension. The cell suspension was dripped onto pre-cooled slides and then dried naturally for manufacturing. The temperature of water bath rose to 80°C to 90°C. Aluminum plate was used for water separation. Next, silver staining solution was added and coverslip was covered. When the smear turned dark yellow, slides were rinsed with clean water and then dried naturally. Images were analyzed. The stained specimens were observed under high-power microscope.
of T lymphocyte immunoassay system. The ratio of 30 T lymphocyte silver staining nucleolar gray area to gray scale of cell nucleus area (I.S%) was calculated. After that, the ratio was used to reflect the level of Ag-NORs content.

**Statistical methods**

SPSS 21.0 statistical software was adopted for the statistical analysis of result data. The calculate data conforming to normal distribution were expressed with mean standard deviation ($\bar{x} \pm s$) and those that didn’t conform to normal distribution were expressed in percentage (%). The data between groups were compared by t test. Besides, $P<0.05$ indicated that the difference showed remarkable significance.

**RESULTS**

General data of the group were displayed in Table I. Among 156 children with epilepsy, there were 94 males (60.25%) and 62 females (39.74%) aged between 3 and 10. Their average age was 5.2±1.7, including 45 children aged between 3 and 5 (28.84%), 58 children aged between 5 and 7 (37.17%), and 53 children aged between 7 and 10 (33.97%).

<table>
<thead>
<tr>
<th>Items</th>
<th>Number of cases</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Gender</strong></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>94 (60.25)</td>
</tr>
<tr>
<td>Female</td>
<td>62 (39.74)</td>
</tr>
<tr>
<td><strong>Age</strong></td>
<td></td>
</tr>
<tr>
<td>3-5 years old</td>
<td>45 (28.84)</td>
</tr>
<tr>
<td>5-7 years old</td>
<td>58 (37.17)</td>
</tr>
<tr>
<td>7-10 years old</td>
<td>53 (33.97)</td>
</tr>
<tr>
<td><strong>Classification of cause of disease</strong></td>
<td></td>
</tr>
<tr>
<td>Primary epilepsy</td>
<td>95 (53.84)</td>
</tr>
<tr>
<td>Secondary epilepsy</td>
<td>61 (28.84)</td>
</tr>
<tr>
<td><strong>Type of seizure</strong></td>
<td></td>
</tr>
<tr>
<td>Generalized tonic-clonic seizure</td>
<td>85 (54.48)</td>
</tr>
<tr>
<td>Infantile spasm</td>
<td>12 (7.69)</td>
</tr>
<tr>
<td>Limited motor seizure</td>
<td>15 (9.61)</td>
</tr>
<tr>
<td>Psychomotor seizure</td>
<td>14 (8.97)</td>
</tr>
<tr>
<td>Vegetative nervous seizure</td>
<td>46 (29.48)</td>
</tr>
<tr>
<td><strong>Course of disease</strong></td>
<td></td>
</tr>
<tr>
<td>1 month to 1 year</td>
<td>45 (28.84)</td>
</tr>
<tr>
<td>1 to 3 years</td>
<td>54 (34.61)</td>
</tr>
<tr>
<td>3 to 5 years</td>
<td>25 (16.02)</td>
</tr>
<tr>
<td>5 to 7 years</td>
<td>28 (17.94)</td>
</tr>
<tr>
<td>7 to 11 years</td>
<td>21 (13.46)</td>
</tr>
</tbody>
</table>

The cause of disease is classified into primary epilepsy (95 cases, 53.84%) and secondary epilepsy (61 cases, 28.84%). To be specific, there were 21 neonates with asphyxia (13.46%), 5 neonates with intracranial hemorrhage (3.2%), 4 neonates with birth trauma (2.56%), 6 neonates with febrile seizure (3.84%), 1 neonate with postencephalitits (0.64%), and 2 neonates with cerebral palisy (1.28%).

According to seizure types, children with epilepsy were divided into big seizure group (generalized tonic-clonic seizure), minor vitality loss seizure group, vegetative nervous seizure group. In big seizure group, there were 85 children (54.48%). In minor vitality loss seizure group, there were 41 cases, 12 suffering from infantile spasm (7.69%), 15 suffering from limited motor seizure (9.61%), and 14 suffering from psychomotor seizure (8.97%). Besides, there were 46 children with vegetative nervous seizure (29.48%).

Before the detection of drug use, there were 38 cases without taking antiepileptic drugs (24.35%). 124 patients ever took one or more antiepileptic drugs (79.48%).

The longest course of disease was 11 years and the shortest was 1 month. The course of disease of 45 cases ranged between 1 month and 1 year (28.84%), that of 54 cases ranged between 1 and 3 years (34.61%), that of 25 cases ranged between 3 and 5 years (16.02%), that of 28 cases ranged between 5 and 7 years (17.94%), and that of 21 cases ranged between 7 and 11 years (13.46%).

The changes in CD3+, CD4+, CD8+, CD20+, and CD4+/CD8+ among children with epilepsy were shown in Figure 1. The expressions of CD3+, CD4+, and CD8+ cells in epileptic children group (Group II, 156 cases), primary epilepsy group (Group III, 95 cases), secondary epilepsy group (Group IV, 61 cases), big seizure group (Group V, 85 cases), minor vitality loss seizure group (Group VI, 41 cases), and vegetative nervous seizure group (Group VII, 46 cases) reduced, while the expression of CD20+ cells in the above groups increased. The comparison with normal control group (Group I, 100 cases) demonstrated that the difference showed statistical meaning ($P<0.05$).

The expression of CD4+/CD8+ cells increased. The comparison of CD4+/CD8+ cell expression between minor vitality loss nerve group as well as vegetative nerve seizure group and normal control group indicated that the difference revealed statistical meaning ($P<0.05$).

Ag-NORs detection results of T lymphocyte in peripheral blood in all groups were displayed in Figure 2. Compared with that of normal group, Ag-NORs of epileptic children group, primary epilepsy group, secondary epilepsy group, big seizure group, minor vitality loss seizure group, and vegetative nerve seizure group was apparently reduced, and the differences were statistically significant ($P<0.05$).
The changes in lymphocyte in peripheral blood of children with epilepsy in all antiepileptic drug groups, including CD3+, CD4+, CD8+, CD20+, and CD4+/CD8+, were illustrated in Figure 3. Compared with that of normal control group (100 cases), the expressions of CD3+, CD4+, and CD8+ cells in peripheral blood lymphocyte subpopulation of the group without taking antiepileptic drugs (46 cases), the group taking antiepileptic drugs (110 cases), phenobarbital group (42 cases), and phenytoin sodium group (68 cases) were remarkably reduced, while the expressions of CD20+ and CD4+/CD8+ cells in the above groups were significantly enhanced. The differences were statistically significant (P<0.05).

The changes in Ag-NORs of peripheral blood lymphocyte of children with epilepsy in all antiepileptic drug groups were demonstrated in Figure 4. Compared with that of normal control group (100 cases), Ag-NORs of peripheral blood T lymphocyte of the group without taking antiepileptic drugs (46 cases), the group taking antiepileptic drugs (110 cases), phenobarbital group (42 cases), and phenytoin sodium group (68 cases) was remarkably reduced. The differences were statistically significant (P<0.05).
DISCUSSION

Epilepsy is a chronic nervous system disease that usually affects the development of pediatric nervous system. After standard treatment, 80% of pediatric epilepsy can be effectively controlled. The final therapeutic effect on a small number of children with epilepsy is not significant after the treatment by antiepileptic drugs. As a result, the disease can’t be effectively controlled (Perucca et al., 2018; Holmes, 2021; Braams et al., 2019). The cause of the abnormalities in immune status may be as follows. When epilepsy occurs, excessive discharge of neurons in the brain tissue results in ischemia and hypoxia in brain tissue and the abnormalities in neuroendocrine system, which further leads to the regulation of immune system by neuroendocrine system (Wong-Kisiel et al., 2018). Besides, repetitive epilepsy causes long-term stress state of body, which results in the changes in body endocrine system.

Since the immune mechanism hypothesis of epilepsy was put forward, a large number of animal experiment and clinical study results and data demonstrated that some children with epilepsy really suffered from the abnormalities in immune function. Some of the abnormalities were caused by antiepileptic drugs, which was drug-induced immune abnormalities. Some occurred before the use of antiepileptic drugs, which was called non-drug immune abnormalities (Pitsch et al., 2021). The results of the detection of 156 included children with epilepsy revealed that the expressions of CD3+, CD4+, and CD8+ cells in peripheral blood among children with epilepsy decreased, while the expression of CD20+ cells and the ratio of CD4+ to CD8+ increased. The comparison with normal control group suggested that the differences were statistically significant ($P<0.05$). The reduction in CD3+, CD4+, and CD8+ cells in peripheral blood of children with epilepsy indicated the deficiency of cellular immune function among children. The growth of the ratio of CD4 to CD8 might be the index of a disturbed immune system. The findings of a study show that the serum cellular immunity of children with epilepsy decreased and CD3+ and CD4+ levels in peripheral blood reduced before treatment (Machado-Santos et al., 2018). After the treatment by phenytoin sodium, the levels of CD3+ and CD4+ lymphocyte rose, which indicated the abnormalities in immune function among children with epilepsy. The conclusion was consistent with the research result. Another study reported 62 epileptic children who took phenytoin sodium, phenobarbital, and other antiepileptic drugs (Tröschter et al., 2021). The conversion rate of lymphocyte among 35 cases (58.33%) decreased, which confirmed that children with epilepsy also suffered from the abnormalities in cellular immune function.

Immunological studies suggest that T lymphocyte play an important role in immune function. The occurrence of epilepsy may be negatively correlated with the function and status of T lymphocyte (Helmstaedter et al., 2021; Ouedraogo et al., 2021; Xu et al., 2018). When epilepsy occurs, the release of cytokine may be utilized to inhibit the transcription of rDNA of lymphocyte to reduce the number of Ag-NORs. Compared with that of normal group, Ag-NORs of epileptic children group, primary epilepsy group, secondary epilepsy group, big seizure group, minor vitality loss seizure group, and vegetative nerve seizure group was remarkably reduced. The difference were statistically significant ($P<0.05$). The conclusion was similar to the research results obtained by (De Francesco et al., 2021). Because the conversion of T lymphocyte is blocked, cytokine can’t be secreted normally and invasive pathogens can’t be removed in time to control disease.

Ag-NORs is an acidic nonhistone protein and an essential index of regulating the transcriptional activity of rDNA. Silver staining technique of nucleolus formation area can be adopted to observe the number, size, and intensity of Ag-NORs clearly, which reflect the transcription of active proteins by rDNA and synthesis status of proteins as well as the proliferative activity of cells (Levite et al., 2020). The body’s specific defenses include humoral immunity and cellular immunity. T lymphocytes (T cells) are active in cellular immunity. T lymphocytes proliferate upon binding to the antigen, producing a variety of T cells, including a number of lethal Ts and a number of T cells (Zhu et al., 2020; Ni et al., 2020; Hasan and Elmeshhedany, 2021; Chen et al., 2020; Wang et al., 2020; Xie et al., 2020). Deadly T cells directly attack virus-infected cells and cancer cells, producing A special protein called perforin and an enzyme (programmed death) make pores in these cells and cause them to die. This is why this type of immune response is known as cellular immunity. T cell types are lethal T, memory T, helper (h.c) and inhibitor, cytotoxic T lymphocyte and CD8 suppressor positive, but the other two groups are CD4 positive (Lu et al., 2022; Min et al., 2021).

Therefore, the detection of expression activity of Ag-NORs of T lymphocyte in peripheral blood could help understand the immune activity of T lymphocyte and even the immune function of the whole body. According to the research results, Ag-NORs content of T lymphocyte among children with epilepsy was statistically correlated with CD3+, CD4+, CD8+, CD20+, and CD4+/CD8+. Ag-NORs content of T lymphocyte reflects the immune function of children with epilepsy. Hence, expensive and technically demanding T lymphocyte subpopulation can be replaced by Ag-NORs content of T lymphocyte to assess

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the immune function and status of children with epilepsy.

The immunoassay system and advanced image analysis technique were utilized to quantitatively analyze Ag-NORs of T lymphocyte. Traditional manual microscope visual counting method was replaced. Images were collected, displayed, and processed intelligently to make the detection results more accurate.

The disadvantages of this research lie in limited time, insufficient sample size, and the unclear cause of abnormal T lymphocyte and its action mechanism in epilepsy, which need to be further researched in subsequent studies.

CONCLUSION

T lymphocyte subpopulation can be replaced by the detection of Ag-NORs content of T lymphocyte to assess the immune function and status of children with epilepsy. The research results suggest that immune dysfunction might exists in the whole pathological process of epilepsy, which shows significance in the further detection of the pathological change of epilepsy, the investigation into the pathogenesis of epilepsy, the guidance on treating pediatric epilepsy, and the estimation of prognosis.

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IRB approval
Ethics Committee approval was obtained from the Institutional Ethics Committee of Yan’an University Affiliated Hospital to the commencement of the study.

Ethical statement
The study was approved and all patients provided written informed consent.

Statement of conflict of interest
The authors have declared no conflict of interest.

REFERENCES


