

Effect of thymectomy on cellular immune function

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1. ABSTRACT

The present study was conducted to evaluate the effect of thymectomy during open heart surgery on immunological function of T lymphocytes in the treatment of children with congenital heart disease (CHD). No significant difference was found in the sjTREC level between pre-thymectomy and post-thymectomy in the non-thymectomy group and the small partial resection group ($P>0.05$). However, the sjTREC level decreased from the pre-surgical level at 1 month ($P<0.01$) and 12 months ($P<0.01$) in the sub-total resection group. No differences were found in proportions of CD3, CD4 and CD8 lymphocytes, proliferative ability of lymphocytes and expression of IL-2, IL-4 and IFN- γ after surgery between controls and three groups of patients ($P>0.05$). In the sub-total resection group, respiratory infection frequency (4.7 ± 1.7 times) did not differ significantly from control group one year after surgery ($P>0.05$); however, mean days of anti-infection were significantly increased ($P<0.01$). In conclusion, sub-total thymectomy leads to a decrease in the sjTREC level in CHD children, whereas the function of peripheral mature T lymphocytes is normal.

2. INTRODUCTION

Children undergoing open heart surgery frequently undergo incidentally partial or complete thymectomy to increase exposure of the surgical field. The effect of thymectomy in humans during a period of postnatal immunological development is unclear. Some studies have demonstrated that the number of T cells is moderately decreased after thymus resection through open heart surgery (1-5). However previous studies have not observed significant clinical manifestations of immune function decline (1, 6).

The thymus is a specialized organ in the immune system. Within the thymus T lymphocytes undergo a series of maturation events, including the T cell receptor (TCR) gene rearrangement where the majority of T lymphocytes express TCR. When TCR has formed, the initial T cells (naïve T cells) can specifically identify the corresponding antigen. Thus, the thymic functions of proliferation and output can be evaluated by the number of naïve T cells. Previous studies have shown that the quantitative determination of T cells receptor rearrangement excision

Table 1. Comparison of the basic characteristics of patients

Variable	Non-thymectomy group (n = 12)	Thymic small partial resection group (n = 20)	Thymic subtotal resection group (n = 15)	Control group (n = 25)
Age, y	2.8 ± 1.2	2.7 ± 1.1	2.5 ± 1.3	2.4 ± 1.6
Male sex	7 (58)	11 (55.0)	9 (60.0)	14 (56.0)
Disease type				
Simple cardiac malformations	9 (75.0)	6 (30.0)	2 (13.3)	
Complex cardiac malformations	3 (25.0)	14 (70.0)	13 (86.7)	

Data are given as mean ± SD or as number (percentage).

circles (TRECs) is a method to assess thymic output function in the short term (7). TRECs is a kind of free gene. It is produced in the process of T cells maturation and stably exists in thymocytes and mature T lymphocytes. TRECs are not copied, backed up and multiplied as cells divide, but are gradually diluted during the cells' constant division (7-10). Therefore, the TRECs level represents the number of naïve T cells. Therefore we can measure thymic output function in the short term by quantitative detection of TRECs using PCR (11, 12). At the present time, δ Rec- ψ J α TRECs – named signal-joint TRECs (sjTRECs) – are the best indicators of thymic output function in the short term because they do not form many TRECs subsets during gene rearrangement, as do other TRECs, resulting in a simpler detection process (13).

We therefore analyzed the influence of thymic removal size on thymus regeneration and output ability in CHD children receiving open heart surgery with thymectomy, by evaluating the level of sjTRECs, proportions of CD3, CD4 and CD8 lymphocytes, proliferating ability of T lymphocytes subsets, and the expression of IL-2, IL-4 and IFN- γ in peripheral vein blood.

3. MATERIALS AND METHODS

3.1.1. Classification

A total of 12 CHD patients in the non-thymectomy group, 20 in the small partial resection group, 15 in the sub-total resection group and 25 age-matched healthy children were enrolled. In the non-thymectomy group, patients with ventricular septal defect (VSD) or atrial septal defect (ASD) did not have any portion of their thymus removed during heart surgery. Patients in the small partial resection group had less than 1/2 of their thymus removed. The patients whose thymus had no clear boundary between right and left and had more than 1/2 but less than 2/3 of their thymus removed were enrolled in the sub-total resection group. The baseline characteristics of the groups are presented in Table 1.

3.1.2. Exclusion criteria

Subjects were excluded if they had a history of recent infections, received blood products or immune inhibitors or had thymic hypoplasia (DiGeorge syndrome).

3.1.3. Informed consent

All the selected children's legal representatives signed the informed consent, which was approved by the medical ethics committee of Shanghai Children's Medical Center.

3.2. Observation schedule

The relative amount of sjTREC was determined before surgery and one month, three months, six months

and twelve months after surgery. The ratio of CD3, CD4, CD8 lymphocytes, proliferative ability of lymphocytes, and cytokines in peripheral vein blood were measured one month after surgery. All subjects were observed every month for 12 months. The frequency of respiratory infections in twelve months after surgery and the number of days of anti-infective therapy were recorded.

3.3. Analytical methods

3.3.1. Measurement of T lymphocyte subsets

All samples were measured on a COULTER Epics X mono-laser flow cytometer (BECKMAN-COULTER, USA), configured with a 488 nm and a 635 nm argon ion laser, allowing four-color data acquisition. Peripheral vein blood was anti-coagulated and incubated with the antibodies CD3-FITC (fluorescein isothiocyanate), CD4-FITC and CD8-PE (phycoerythrin) anti membrane-bound antigens, at 4°C for 15 to 30 min in dark. All samples were incubated with 1 ml hemolysin (Immunotech, France) for 10 min, then fixed with 500 μ l of 2% paraformaldehyde and cells were resuspended. The proportions of CD3, CD4 and CD8 cells were measured. The hemo-subtype IgG of rat was defined as control.

3.3.2. Measurement of proliferative ability of lymphocytes

Proliferation of PBMCs was induced by PHA, and detected by 3 H-Tdr incorporation using 1ml of 1×10^6 /ml PBMC.

3.3.3. Determination of IL-2, IL-4, IFN- γ and IL-2

Serum from fasting venous samples was separated and preserved at the temperature of -20°C until analysis. IL-2, IL-4 and IFN- γ levels were detected by ELISA (enzyme linked immunosorbent assay) with kits (Jingmei BioTech, Shenzhen, China and Bio-Rad, USA) according to protocol.

3.3.4. Measurement of relative amount of sjTREC

3.3.4.1. Design and synthesis of the primer and probe

Because the direction of the signal joint excision circles formed by the recombination between δ Rec and ψ J α , which are deleted, coincides with the original in the opposite direction, in the TCR δ genome sequence, a reverse primer downstream of δ Rec and a forward primer upstream of ψ J α were designed to amplify the PCR product only when DNA circles are formed. In addition, a fluorescent probe was designed for the amplified products (14). A gene was selected in the TCR α chain constant region (C α) as the internal reference gene. Primers and probes were synthesized by Dalian TaKaRa Biotechnology Co., Ltd. (Table 2).

Table 2. The sequences of primers and probes of sjTRECs and internal reference Cα

Gene		Sequence
sjTRECs	Forward primer	5'-CCATGCTGACACCTCTGGTT-3'
	Reverse primer	5'-TCGTGAGAACGGTGAATGAAG-3'
	Probe	5'FAM-CACGGTGATGCATAGGCACCTGC-TAMRA3'
Internal reference Cα	Forward primer	5'-CCTGATCCTTGTCCCCAG-3'
	Reverse primer	5'-GGATTTAGAGTCTCAGCTGGTACA-3'
	Probe	5'FAM-ATCCAGAACCTGACCCCTGCCG-TAMRA3'

3.3.4.2. Reconstruction of TA clone

The aforementioned primer was used to amplify the specific conserved fragment of the sjTREC and Cα gene in a 50 μl-PCR-reaction-system, using the following reaction conditions: 95°C 1 min then 45 cycles of 95°C 15 s, 56°C 30 s, 72°C 30 s. The size of PCR products was 131 bps and 71 bps for sjTREC and Cα, respectively. PCR products recovered and purified by gel and PgemiT-Easy vector (Promega, USA) were combined, transferred into *E. coli* and cultured. White colonies were selected for continued culture in 3ml broth. Plasmids were extracted using a plasmid extraction kit (QIAGEN, Germany) and digested by the restriction enzyme (TaKaRa, Japan). Electrophoresis of the digested products and the initial PCR products at the same time was performed to confirm that the initial target gene was ligated into the T vector. The successfully ligated bacterial culture was selected and re-cultured overnight.

3.3.4.3. Establishment of standard curve

To generate the standard curve, we extracted the recombinant plasmids pGEMT-Easy-sjTREC and pGEMT-Easy-Cα and measured their D (λ) values using a protein nucleic acid analyzer. Their initial copy number, after a 10-fold dilution, was calculated and used as the template for real-time FQ-PCR.

3.3.4.4. PCR amplification

Amplification conditions were 95°C for 1 min then 40 cycles of 95°C for 5 s and 60°C for 1 min. The relative quantitative method with two standard curves was used in this study. The sjTRECs and internal reference Cα were detected twice at the same time for each specimen, and the final results were the average of the two detections (ABI Prism 7000, BECKMAN-COULTER, USA).

3.3.4.5. Calculation of the relative number of sjTREC in samples

The absolute quantification of sjTREC was converted into its relative quantification for sample comparability. Because each cell contains two Cα genes, the relative content of sjTREC for per 1000 mononuclear cells was calculated by the formula of (sjTREC absolute content / Cα) × 2 × 1000.

3.4. Statistical analysis

All results were statistically analyzed by SPSS 16.0 software. Measurements were presented as “mean ± standard deviation” ($\bar{x} \pm S$) and means compared among groups using ANOVA (analysis of variance). The t-test was used with homogeneity of variance, while the rank-sum test was used with heterogeneity of variance. The results were statistically significant when P was less than 0.05.

4. RESULTS

4.1. Change of peripheral blood T lymphocyte subsets in CHD patients at 1 month after surgery

The proportions of CD3, CD4 and CD8 cells were not significantly different in peripheral blood of CHD children compared with the control group ($P>0.05$) (Figure 1). The ratios of CD4/CD8 were 1.2 ± 0.1 , 1.3 ± 0.1 and 1.3 ± 0.2 in the non-thymectomy group, small partial resection group and sub-total resection group, respectively, and were not different from the control group (1.2 ± 0.1) ($P>0.05$).

4.2. Lymphocyte proliferation

The proliferative ability of lymphocytes when stimulated with PHA was not significantly different in CHD children when compared with the control group at one month after surgery ($P>0.05$) (Figure 2).

4.3. IL-2, IL-4, IFN-γ and IL-2 expression

There was no significant difference in the expression of IFN-γ and IL-4 in CHD children compared with the controls one month after surgery ($P>0.05$) (Figure 3 and Figure 4).

4.4. sjTREC level

4.4.1. Comparison of the expression level of sjTREC gene before surgery

No significant difference in the expression of the sjTRECs gene was observed between the children with CHD in the three groups and the control ($P>0.05$) (Figure 5A).

4.4.2. Comparison of the expression level of sjTRECs Gene before and after thymectomy

The expression levels of sjTRECs gene at different time-points after surgery are shown in Figure 5B, 5C and 5D. The data in Figure 5B showed that the number of sjTRECs in the non-thymectomy group declined slightly after 1 month, and recovered to pre-surgical levels after 3 months post operation. The levels of sjTRECs at all four points after thymectomy were not significantly different from that before surgery. The number of sjTRECs in the small partial thymic resection group fell to the minimum after 1 month post-thymectomy ($P<0.01$), and began to rise after 3 months but were still below the pre-surgical level (Figure 5C). The levels of sjTRECs were not significantly different between 3 months and before surgery. In the sub-total thymectomy group, the number of sjTRECs fell to the minimum 1 month after surgery ($P<0.01$), and began to rise slightly 3 months post-thymectomy, but was still significantly lower than preoperative levels 1 year after surgery (the number of each point after surgery compared with that before operation were $P<0.01$) (Figure 5D).

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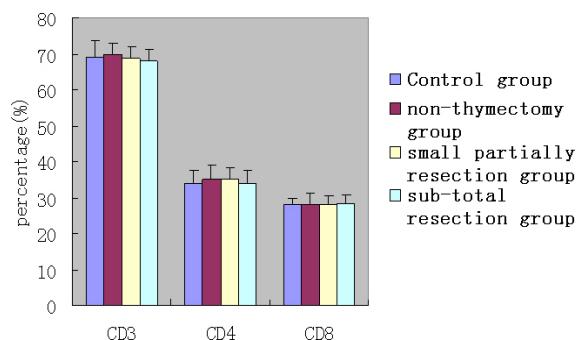


Figure 1. Peripheral lymphocyte subset counts in children with CHD and control group ($\bar{x} \pm S$, %).

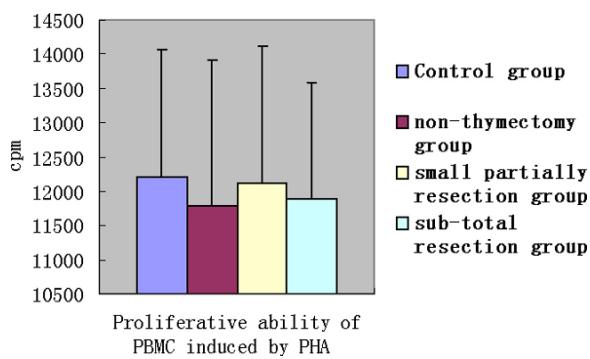


Figure 2. Result of lymphocyte proliferation test in children with CHD and control group ($\bar{x} \pm S$, cpm).

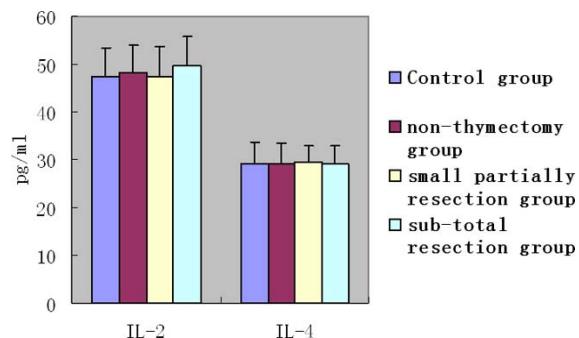


Figure 3. Result of IL-2 and IL-4 in children with CHD and control group ($\bar{x} \pm S$, pg/ml).

4.5. Respiratory infections in every group 1 year after surgery

The average numbers of respiratory infections were 4.3 ± 1.5 in the control group, and 4.7 ± 1.7 , 4.5 ± 1.4 and 4.5 ± 1.4 in the sub-total resection group, small partial resection group and non-thymectomy group, respectively. No significant difference was found among these four groups. The average duration of anti-infective therapy was 9.9 ± 3.1 days in the control group, and 14.1 ± 3.5 , 11.1 ± 3.1 and 10.2 ± 2.9 days in the three experimental groups, respectively. There was a significant difference between the sub-total resection group and the control group ($\chi^2 = 16.59$, $P < 0.01$).

5. DISCUSSION

TRECs accurately indicated the change of thymus output function caused by thymus resection. In the present study, we tested the expression of TRECs though FQ-PCR and found that the number of sjTREC in children with congenital heart disease before surgery did not differ from normal children of the same age. The decreased numbers of sjTRECs in children with CHD one month after surgery could be due to the blood loss in surgery and the dilution of blood from cardiopulmonary bypass. A previous study showed this was associated with the proliferation of T cells (3). Our results confirmed this, and further demonstrated that the decrease in the number of sjTREC was related to the size of left thymectomy, by showing that the number of sjTRECs began to rise after 3 months post surgery and reached the pre-surgical level 1 year after operation in the small partial resection group; while in the sub-total resection group, the number of sjTRECs still remained significantly lower than that before surgery ($P < 0.01$). This suggests that sub-total thymic resection in cardiac surgery for congenital heart disease leads to long-term or permanent decline in SjTREC levels.

Significant decline of TRECs level in CHD children caused by sub-total thymectomy may increase the susceptibility to new bacteria (15). However, data from E. Mancebo (insert reference number or year of publication) indicated that children who received thymectomy in cardiac surgery during the neonatal period did not suffer more infection compared with a healthy control group, and this study further indicated that the frequency of respiratory infection in children did not increase sharply after sub-total thymectomy. The ratios of T-cell subsets and the expression of IL-2, IFN- γ and IL-4 in the present study demonstrated the impact of thymectomy in cardiothoracic surgery on the immunological function of peripheral blood T lymphocytes.

The reaction and balance between CD 4+T cells and CD8+T cells maintain a normal immune response. The numbers of mature T lymphocytes and its subsets in peripheral blood also play an important role in anti-infective immunity (16). This study found that proportions of peripheral blood CD3+T cells, CD4+T cells and CD8+T cells and the ratio of CD4+/CD8+ in children with CHD tend towards normal levels in three groups. Proliferation of lymphocytes is a major determinant of immune response intensity and reflects the status of cellular immunity (17). In this study, T cell proliferation was normal in CHD children after thymectomy. This suggests that extensive thymectomy does not influence the proportion and proliferation of T cell subsets.

IL-2 produced by active T cells mainly mediates and regulates the specific immune response, and is a leading regulated cytokine in the complicated immune network (18). IL-2 can activate many immunocytes, and is especially required in inducing T cell proliferation and activating naïve T cells to differentiate into effector cells (19). The results of this study demonstrated that the

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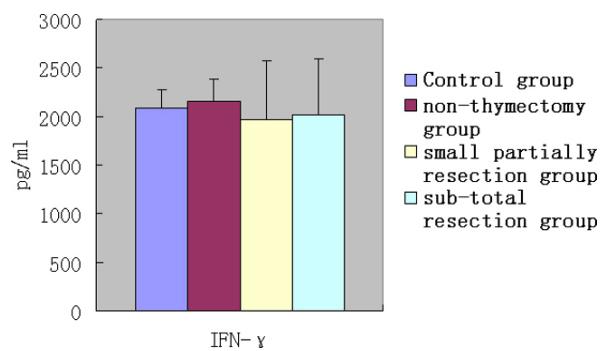


Figure 4. Result of IFN- γ in children with CHD and control group ($\bar{x} \pm S$, pg/ml).

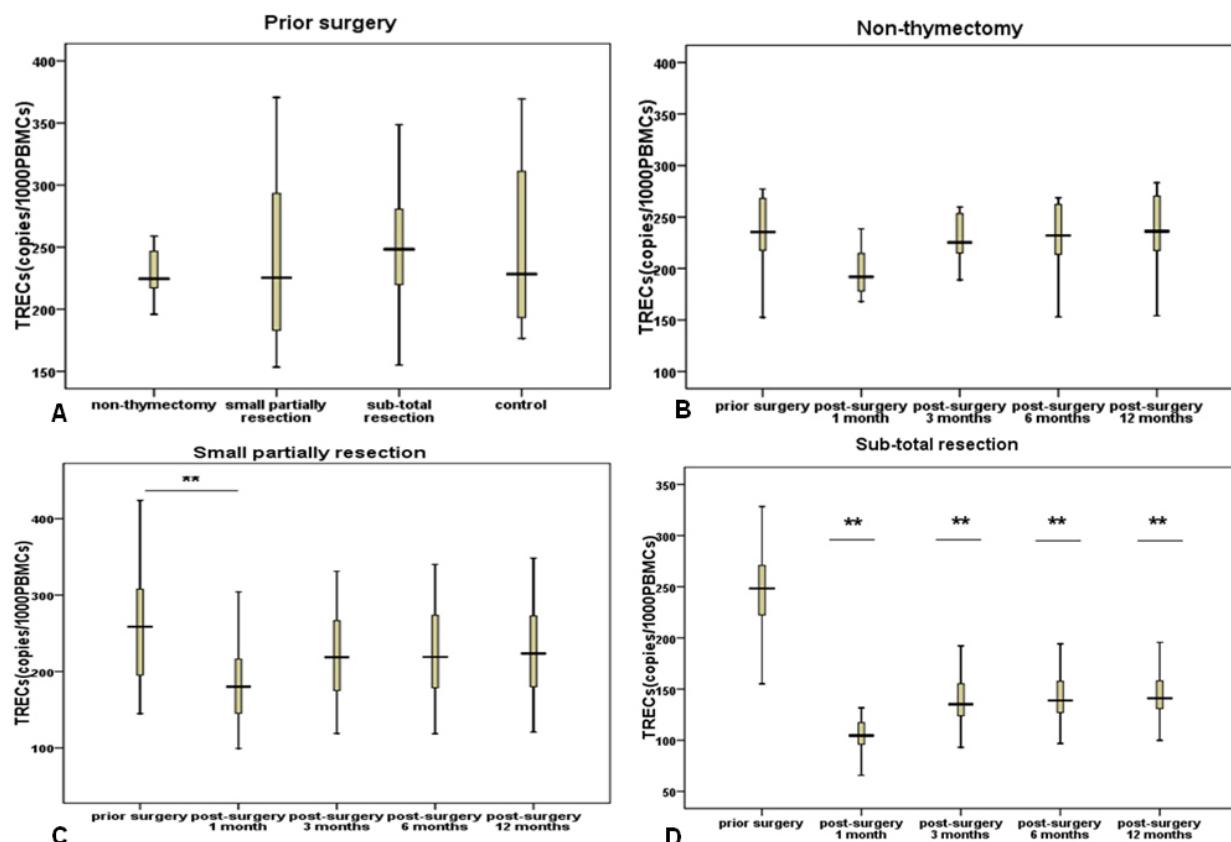


Figure 5. (A), Comparison of the expression level of sjTREC gene before surgery. (B-D). Comparison of the expression level of sjTRECs gene before and after thymectomy .The vertical axis represents the relative number of sjTRECs (copies/103PBMCs), the horizontal axis represents time before and after surgery.

immunobiological activity of T cells mediated by IL-2 were normal in children with extensive thymectomy.

IFN- γ was primarily excreted by Th1 cells while IL-4 was produced by Th2 cells. The ratio of Th1-type cytokines and Th2-type cytokines could affect the balance between Th1 and Th2 cells. This study showed that the ratio of IFN- γ /IL-4 was normal in post-extensive thymectomy children. In response to an immune challenge,

CD 4+T cells were activated, proliferated and finally differentiated into Th1 and Th2 cells, which then mediated cellular immunity and hormonal immunity, and regulated the inflammatory response to intracellular and extracellular bacteria infection. Th1 and Th2 cells produced cytokines which induced specific immunocytes to reach areas of inflammation by chemotaxis and induced them to grow up into effector cells which could eliminate specific antigens (20, 21).

Previous studies found that, although the expression of TREC genes and the number of T lymphocyte declined, the incidence of clinical infection did not increase dramatically (3, 6). Our study found that the proportion of mature T lymphocyte subsets and their proliferation were normal in children with post sub-total thymectomy even though the sjTREC level was low, which confirmed that peripheral lymphoid tissue played a key role in reconstruction of T cells post sub-total thymectomy (22). Douek suggested that pre-T cells might reconstruct the TCR gene in lymph nodes, but not exert great impact on the total level of TREC (23). This could provide a reason that despite the decline in the level of sjTRECs, the function of mature peripheral lymphocytes was not obviously affected. Therefore, the incidence of clinical infection was not higher in the sub-total thymectomy group than the healthy control group despite sjTREC decreasing. However, the time of recovery from respiratory infection in the sub-total thymectomy group was found to be longer than that of normal controls one year after surgery in this study. We believe a more reliable result would be obtained from a larger sample size and longer follow-up time. Considering the declined level of the TREC gene in CHD children after sub-total thymectomy, the thymus should be removed as rarely as possible or even preserved during surgical therapy of CHD children.

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Abbreviations: CHD: congenital heart disease, sjTREC: signal-joined T cells receptor rearrangement excision circles, VSD: Ventricular septal defect, ASD: Atrial septal defect

Key Words: Congenital heart disease, thymus, TRECs, T cells

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