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Original article

Apoptosis and mitochondrial membrane potential changes of T lymphocytes from children with Down's syndrome

Background: *Down's syndrome (DS) patients present a high risk of developing alterations of the immune system. The increased susceptibility to bacterial and viral infection, hematologic malignancies and autoimmune disease, suggest that immunodeficiency is an integral part of DS. Little is known about the mitochondrial damage and tendency to apoptosis in peripheral T lymphocyte cells in DS individuals.*
Objective: *to evaluate the tendency to apoptosis and mitochondrial membrane potential (ΔΨm) changes in peripheral T lymphocytes of DS children both in the presence and in the absence of acute infection.*
Patients and methods: *The present study included thirty children had DS (all of them trisomy 21 of nondisjunction type), and thirty normal children, fifteen of each group had no evidence of acute infection and fifteen had acute infection. Potential apoptosis was measured by flow cytometry using annexin V. The ΔΨm was assessed by the retention of Rhodamine 123.*
Results: *There was no significant difference in the percentage of $CD3^+$ cells or potential apoptotic T lymphocytes between DS children and controls either in presence or in absence of acute infection. However, there was a significant decrease in ΔΨm in the peripheral T lymohocytes of DS children when compared to the controls.*
Conclusion: *The function of T cells not their number is the main mechanism responsible for the impairment of the immune system in DS children. T lymphocytes in peripheral blood from DS patients do not display an increased tendency to undergo apoptosis although a significant loss of ΔΨm was found.*

Keywords: Down's syndrome**,** apoptosis, mitochondrial membrane potential.
Abbreviations: CyQ: Indotricarbocyanine; DS: Down syndrome; FCM: flow cytometry; FITC: Fluorescein isothiocyanate; FSC: Forwarded scatter; GMFI: Geometric mean fluorescence intensity; MPP: Mitochondrial membrane potential; PI: Propidium iodide; Rh123: Rhodamine 123; SSC: Side Scatter.

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INTRODUCTION

Down's syndrome (DS, trisomy 21) is the most common chromosomal abnormality in humans that occurs in 1 out of every 800-1000 births. Many characteristics are commonly seen in DS, including intellectual impairment, heart defects, hypotonia, hyperuricemia, and development of Alzheimer disease[1]. DS patients also present a high risk of developing alterations of the immune system with lack of cell mediated immunty, which are similar to aged people, including increase susceptibility to bacterial and viral infection, hematologic malignancies and autoimmune disease, suggesting that immunodeficiency is an integral part of DS. This contributes significantly to the observed increase in the morbidity and mortality[1-3].

Apoptosis is a genetically controlled process in which the cell actively participates in its own destruction in response to various types of stress. Apoptosis plays a key role in the homeostatic regulation of the hematopoietic system[4]. Apoptosis plays an essential role in T cell development, the shaping of the immune repertoire, and in the ordinate initiation and final resolution of the immune responses to exogenous dangerous signals[5]. The dysregulation of apoptosis in the immune system results in immunodeficiency and autoimmunity[6]. Mitochondria play a pivotal role in the regulation of apoptosis. An early and key event during apoptosis is that the mitochondrial outer

membrane becomes permeable, leading to release of apoptogenic factors into the cytosol[7]. Thus, evaluation of mitochondrial membrane potential (ΔΨm) is of critical importance for the assessment of apoptosis[8].

Changes in mitochondrial functionality and increased apoptosis have been described in neurons of patients affected by DS[9]. These alterations are supposed to be responsible for the precocious onset of Alzheimer disease in patients affected by DS. As regards the peripheral blood cells, Roat et al[10] found alterations in the distribution of several lymphocyte subpopulations and investigated the mitochondrial damage and apoptosis in peripheral blood mononuclear cells from DS children after in vitro treatment with apoptogenic molecules. However, to the best of our knowledge, no one investigated the tendency to spontaneous apoptosis in peripheral T cells from DS patients. Therefore, our aim was to evaluate the tendency to apoptosis and mitochondrial membrane potential changes in T lymphocytes from DS children both in the presence and in the absence of acute infection.

METHODS

This case control study included 30 children with DS (trisomy 21, nondisjunction type) recruited from Assiut University Children's Hospital from January to December 2008. Fifteen of them had no acute infection; they were 11 males and 4 females with a mean age of 14 ± 6.04 months. Another fifteen were studied during acute infections; 10 with bronchopneumonia, 3 with acute gastroenteritis, 1 with both of them and 1 with acute otitis media. They were 9 males and 6 females with a mean age of 10 ± 8.94 months. Thirty age and sex matched children with normal mentality and without any apparent dysmorphic features were studied as controls. Of these controls fifteen had no evidence of acute infection; they were 10 males and 5 females with a mean age of 14 ± 7.39 months. The other fifteen had acute infections during the study: 9 had bronchopneumonia, 3 had acute gastroenteritis, 2 had both of them and 1 had acute gastroenteritis and acute otitis media. They were 10 males and 5 females and their mean age was 12.29 ± 5.59 months. Acute infection was confirmed by clinical examination and the presence of leukocytosis, elevated band counts (increased immature white blood cells) and/or elevated ESR. Children with congenital malformations e.g. congenital heart disease or positive coombs' test were excluded from this study. After the approval of the ethical committee of Faculty of Medicine-Assiut University, an informed written consent was obtained from the parents or care givers. Clinical history, examination and complete blood picture were done for all children included in this study.

To investigate whether peripheral T cells from the patients undergo spontaneous apoptosis, the proportion of cells that underwent apoptosis was measured by flow cytometry (FCM) using annexin V-FITC and propidium iodide (PI) with gating on T cells by staining with CyQ-CD3 monoclonal antibodies (IQ product, Groningen, The Netherlands). The ΔΨm is assessed by the retention of Rhodamine 123 (Rh123), a specific fluorescent cationic dye that is readily sequestered by active mitochondria, depending on their transmembrane potential[11]. Hundred µl of cell suspension were incubated in two tubes with 10µl of anti-CD3 and with 10µl of Annexin V in the first tube only for 20 min at 4°C protected from light. For the negative control isotypic matched mouse IgG antibodies were used in a third tube. The cells were washed and re-suspended in 100µl PBS. The mitochondria were stained by adding to the cell suspension a stock solution of Rh123 (10 mM in ethanol, kept in the dark, 4ºC) to a final concentration of 1 µM. PI is added to discard non-viable cells in the two tubes and analyzed by FCM.

FCM analysis

The flow cytometer (FACSCaliber; Becton Dickinson, San Jose, CA) was calibrated using CaliBRITTE beads (Becton Dickinson) for three-color flow cytometer setup. Data acquisition and analysis was performed using Cell Quest software (Becton Dickinson). The minimum numbers of cells required for analysis were 10,000. The lymphocyte gate (region 1) was determined manually on the basis of forward and side angle light scatter (FSC & SSC respectively). Cells were expressed on a scatter diagram combining SSC with CD3-CyQ fluorescence and a region (region 2) was drawn around positive population. The marker for determining positive and negative cells was set according to the negative control. To select viable cells within R2, region (R3) was drawn satisfying PI negative.

To determine the ΔΨm the fluorescence of retained Rh123 was assessed on a histogram of FL1-fluorescence and the geometric mean fluorescence intensity (GMFI) was recorded (Fig1). Another dot plot of Annexin V-FITC versus PI gated on R2 was done to assess the percentage of potentially apoptotic T cells (Fig 2). Quadrant cursors were set according to the negative controls. For positive control in this work we used the PI positive cells to assess the ΔΨm in dead cells.

Statistical analysis:
Analysis was done using SPSS (version 16). The numerical data were represented as mean ± SD. Student's *t* test was used for comparison. The difference was considered significant if probability (*p*) values were less than 0.05.

RESULTS

The details of demographic, clinical and laboratory data of all children are listed in table 1. The present study revealed that in the absence of acute infection there is no significant difference in the percentage of $CD3^+$ cells in the peripheral circulation of DS children when compared to those of the controls. In addition, there was no significant difference in the proportion of potentially apoptotic T lymphocytes in the peripheral circulation of DS children when compared to those of the controls. As regard mitochondrial membrane potential (ΔΨm), there was a significant decrease in the retention of Rh123 in DS children when compared to the controls (Table 2).

In DS children all the previous parameters were also compared in the presence, versus in the absence of acute infection. Both the percentage of $CD3^+$ cells and the proportion of potentially apoptotic T lymphocytes in the peripheral circulation of DS children were significantly higher in the presence of acute infection. There was no significant difference in ΔΨm in DS children in the presence or absence of acute infection (Table 2).
All these parameters were compared between DS children and the control children in the presence of the acute infection. There was only a significant decrease in the retention of Rh123 in DS children (Table 2).

All studied variables showed no significant correlation with the demographic data of the patients or different types of acute infection.

Table 1. Some demographic, clinical and laboratory data of DS patients and controls.

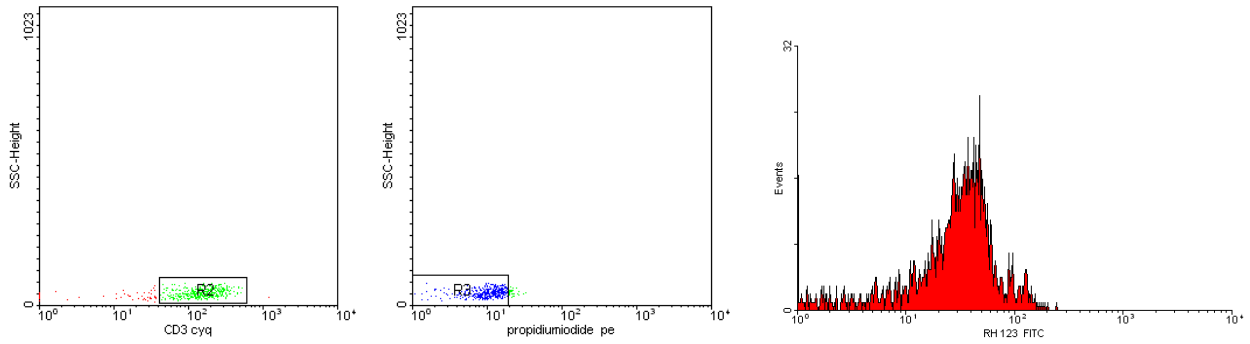
Demographic, clinical and laboratory data	Down syndrome (30)		Controls (30)	
	Without infection (15)	With infection (15)	Without infection (15)	With infection (15)
Age (months)	14.23 ± 6.04	10.13 ± 8.94	14.88 ± 7.39	12.29 ± 5.59
Sex (No. %)				
Male	11 (73.3%)	9 (60.0%)	10 (66.7%)	10 (66.7%)
Female	4 (26.7%)	6 (40.0%)	5 (33.3%)	5 (33.3%)
Weight (kgm) (means+/- S.D)	6.83 ± 4.36	5.20 ± 1.94	9.71 ± 3.41	8.77 ± 2.90
Hb (g/dL) (means+/- S.D)	11.30 ± 1.65	9.79 ± 1.48	11.00 ± 1.39	9.23 ± 2.95
WBC(×10³/ul) (means+/-S.D)	9.57 ± 3.05	10.67 ± 4.12	9.63 ± 3.01	15.30 ± 8.07

Quantitative variables are expressed as mean ± standard deviation.
No = number; Hb = hemoglobin; WBC = white blood cells.

Table 2. $CD3^+$ %, proportion of potentially apoptotic T lymphocytes and GMFI of Rh123 (ΔΨm) among DS children and controls.

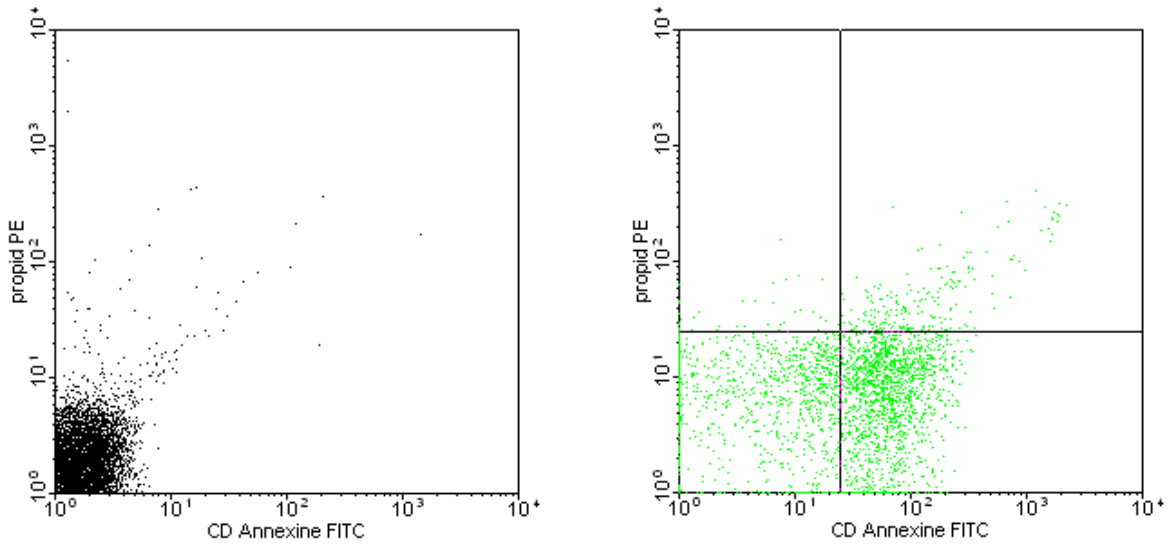
	$CD3^+$ (%)	Potentially apoptotic T lymphocytes (%)	GMFI of Rh123 (ΔΨm)
DS without infection	73.16 ± 4.99	4.39 ± 1.09	6.75 ± 2.97
DS with infection	82.77 ± 9.82	35.59 ± 11.01	5.22 ± 2.52
Controls without infection	70.44± 5.30	4.28 ± 1.24	11.40 ± 4.38
Controls with infection	84.01 ± 10.44	35.47 ± 24.53	11.02 ± 3.80
***P* value A**	0.235	0.854	0.008*
B	0.006*	0.000*	0.148
C	0.794	0.990	0.000*

Quantitative variables are expressed as mean ± standard deviation.
DS = Down's syndrome; GMFI = geometric mean fluorescence intensity; Rh123 = Rhodamine 123; ΔΨm = mitochondria membrane potential; * = statistically significant result.
A= *p*-values when DS without infection group compared with controls without infection group.
B= *p*-values when DS without infection group compared with DS with infection group.
C= *p*-values when DS with infection group compared with controls with infection group.



(a) **(b)** **(c)**

Figure 1. (a) A scatter diagram combining SSC with CD3-CyQ fluorescence and a region (R2) is drawn around positive population for CD3 (T lymphocytes). (b) Region (R3) is drawn satisfying PI negative population to select the viable cells. (c) A histogram showing Rh 123-fluorescence that retained in the mitochondria of those viable T lymphocytes.



(a) **(b)**

Figure 2. Representative of flow cytometric data revealed that T cells from a healthy control were Annexin V and PI negative (a), where as a proportion of T cells from a patient were Annexin V positive and PI negative (apoptotic cells) (b).

DISCUSSION

The immune function in individuals with DS has been shown to be defective, especially regarding T cell compartment[9]. The investigation of lymphocyte population in peripheral blood has afforded insights towards understanding and assessing possible immune deficiencies, malignancies, and autoimmune diseases[2]. The present study revealed that there was no significant difference of the percentage of $CD3^+$ T lymphocyte in the peripheral blood from DS children and the control children in the absence of acute infection. The percentage of $CD3^+$ T lymphocyte increased significantly in DS children in the presence of acute infection, but remained comparable to those of control children in the presence of acute infection. The data of the present study are different from those observed by Cuadrado and Barrena[11], who found that quantitative studies of peripheral T lymphocytes in DS children revealed a reduction, often quite small in the percentage. However, they are in agreement with other authors who concluded that the number of T lymphocytes both in secondary lymphoid organs and in circulation is kept under strict control[12], so that the $CD3^+$ number is maintained[13,14].

As the immunodeficiency is an integral part of DS that makes a significant contribution to increased morbidity and mortality, so it remains questionable whether normal of T- lymphocyte counts in children with DS reflects populations with normal phenotype and function[3]. Many authors supported the concept that there is an abnormality in circulating T lymphocyte subset in children with DS and there is abnormal release of immature T cells in peripheral blood of DS subjects[2,3,11]. A decreased proportion of mature thymocyte and inefficient release of mature T cell in the periphery provide a mechanism of impaired cellular responses of individuals with DS[11]. So the function of T cells not their number- is the main mechanism responsible for the impairment of the immune system in DS children and may further add to the known fact that cellular immunity is more severely affected than humoral immunity in these children[15].

Apoptosis is the mechanism by which the body removes both the ineffective and the potentially damaging immature cells[16,17]. The measurement of apoptotic activity may greatly enhance possibilities for staging the disease[18]. Flow cytometry- based Fluorescence labeling Annexin V has become one of the mostly used methods of detecting apoptosis. It provides a simple, rapid, sensitive and immediate detection method and offers the possibility of detecting early phases of apoptosis[18,19]. The current study, revealed that T lymphocytes in peripheral blood from DS patients and healthy controls have a similar tendency to undergo apoptosis. This is in agreement with Roat et al[10] who found that although different types of cells from DS patients have an increased susceptibility to cell death; peripheral mononuclear cells do not display an increased tendency to undergo apoptosis. However, a recent study[15] observed increase tendency to apoptosis in peripheral T lymphocytes in DS children without the exclusion of infection which might be the cause of this increase. On the other hand, autoimmunity can result from a decreased apoptosis of potentially auto reactive T lymphocytes and increased resistance to variety of apoptogenic stimuli often characterizes tumor cells[20].

The tendency to apoptosis of T lymphocytes in DS children in the current study, increased significantly in the presence of acute infection. This finding can be explained by the fact that apoptosis can be triggered by a huge variety of stimuli (e.g. toxins, cytokines) via intrinsic and extrinsic pathways[17,21]. Therefore it is not surprising that apoptosis is also a response that is found during the engagement of the human body with invading microbial agents (viral or bacterial) [22,23,24]. So in the current study we can suggest that this phenomenon occurs normally in DS children, evidenced by that there was no significant difference of apoptosis of peripheral T lymphocytes in both DS and normal children in the presence of acute infection. This is in agreement with Cochii et al[25] who reported that in DS children, the immune cellular status is similar to normal population.

Regarding mitochondria, they are essential to multi cellular life; without them, a cell ceases to respire aerobically and quickly dies, a fact exploited by some apoptotic pathways[16]. The mitochondrial membrane impermeability is necessary for maintaining the proton gradient which is required for oxidative phosphorylation. An early and key event during apoptosis, that the mitochondrial membrane becomes permeable, leading to release of apoptogenic factors into the cytosol. Mitochondrial membrane permeability to small ions and water causing transmembrane potential[17] ($\Delta\Psi m$) represent a sensitive parameter of the effectiveness of the mitochondrial bio-energic function[26]. Several cationic dyes distribute electrophoretically into the mitochondrial matrix in response to the electric potential across the inner mitochondrial membrane. These dyes have been extensively employed to measure the mitochondrial electric potential ($\Delta\Psi m$)[27]. In the current study, Rh-123 were used for measuring $\Delta\Psi m$. The use of Rh-123 for measuring $\Delta\Psi m$ is of great interest; it is a sensitive and reliable probe of membrane potential in isolated mitochondria because the method is simple and direct and can be employed using a standard fluorometer[26]. The present study revealed that cells from DS patients show a significant loss of $\Delta\Psi m$ than the control group either in the presence or in the absence of acute infection. This finding is consistent with previous reports[10,28]. Thus, since DS cells with altered mitochondrial function do not undergo apoptosis, it hypothesized that DS patients tend to maintain damaged cells, or that they have a higher capacity to repair functional damages. It remains to be established if and how this phenomenon can be linked to development of autoimmunity or neoplastic disorders in DS individuals[10].

In conclusion: The function not the number of T cells is responsible for the impairment of the immune system in DS children. T lymphocytes in peripheral blood from DS patients do not display an increased tendency to undergo apoptosis and show a significant loss of $\Delta\Psi m$ than the control group. Further studies on a wider scale may be needed to assess the T lymphocyte function and other

parameters related to mitochondrial functionality in DS children.

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