

Effects of cardiopulmonary bypass on the development of lymphopenia and sepsis after cardiac surgery in children with congenital cardiopathy

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Abstract. The objective of the present study was to investigate whether lymphopenia occurring after heart surgery with cardiopulmonary bypass (CPB) is related to apoptosis and or sepsis in children. The design was a prospective cohort study in a third level care hospital in Mexico City. In total, 68 children (31 girls and 37 boys) with congenital cardiopathy who needed corrective cardiac surgery with or without CPB were included. The samples were obtained from central blood before, immediately after and 24 h after surgery. Complete blood counts and lymphocyte apoptosis were analyzed. Systemic inflammatory response syndrome (SIRS), sepsis and the type of microorganism were recorded. A total of 53 patients received CPB and 15 did not. Lymphocyte count decreased after surgery in both groups ($P < 0.001$). However, neutrophil count increased markedly in both groups. Apoptosis of B (CD19⁺) lymphocytes was higher in the non-CPB group (14, 2 and 21% before, immediately after and 24 h after surgery, respectively) than the CPB group (0, 2 and 3%, respectively), but apoptosis

of cytotoxic T lymphocytes (CD8⁺) was higher in the CPB group (5, 4 and 3% before, immediately after and 24 h after surgery, respectively) than in the non-CPB group (2, 3 and 2%, respectively). However, the extent of apoptosis of T and B lymphocytes after surgery did not differ between groups. The CPB group had more complications than the non-CPB group [38 (71.7%) vs. 9 (60.0%)]. In conclusion, the decrease in lymphocyte count may be related to apoptosis of cytotoxic T lymphocytes in children receiving cardiac surgery with CPB and to apoptosis of B lymphocytes in those not receiving CPB. The decreased lymphocyte counts in both groups suggested that CPB is not the main cause of this decrease. Children who received CPB during surgery had more complications, such as sepsis and cardiogenic shock than did those who did not receive CPB.

Introduction

In Mexico City, for each 1,000 live births, 16.5 to 20 are infants born with congenital cardiac disease. Using birthdate information, we have calculated that 10,000 to 12,000 infants are born with some type of cardiac malformation each year. Mortality caused by congenital cardiopathy represents the sixth leading cause of death in the first year of life and has a 3.2% rate; it represents the ninth leading cause of death in children aged 1 to 4 years (1.6% rate), and the 14th leading cause of death in children aged over 5 years (1.1% rate) (1).

Cardiac correction represents the first place in pediatric surgery. Despite major advances in the past two decades, sepsis is potential problem after surgery and can lead to death. In the Pediatric Hospital at the Instituto Mexicano del Seguro

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Social (National Medical Center), around 100 children per year are seen with congenital cardiac malformations. Most of these infants require cardiac surgery, and more than 60% require cardiopulmonary bypass (CPB). Sixty percent of those who receive CPB may develop an infection (2).

Infants undergoing heart surgery are at increased risk for developing immunosuppression and severe infection (2). These children may also develop various complications caused by the physiological response to ischemia-reperfusion damage (3-4). Many researchers have considered that this damage may be generated by CPB, and some have reported lymphopenia after CPB possibly caused by apoptosis of lymphocytes (5-7). However, only a few studies have reported lymphopenia in children (2-8). Patients who develop lymphopenia often display a characteristic systemic inflammatory response syndrome (SIRS), which may result from the infection itself or may be a secondary response of damage-associated molecular patterns to the original event, such as surgery or hypoxia (9,10).

Bacteremia can cause systemic inflammatory response and sepsis (11). When bacteria reach the blood, a set of mechanisms is activated to eliminate the microorganisms through the release of proinflammatory mediators and chemical agents. These agents stimulate the immune system to act at the vascular endothelial level, which can cause damage to blood vessels and increase permeability. This response activates a plasma cascade of powerful vasoconstrictors, such as cytokines, interleukin 1 (IL-1 β), IL-6, cyclooxygenase, and lipoxygenase, which are also involved in the adaptive immune response and send signals to stimulate apoptosis and produce myocardial depression.

These changes can cause shock, which activates the coagulation cascade and the complement cascade, and can subsequently cause thrombosis in small vessels and hemorrhage at different levels. This plasma cascade simultaneously stimulates several chemical agents, which respond to tissue damage, activation and recruitment of cells such as macrophages, neutrophils, and lymphocytes, and release of inflammatory mediators (12,13).

Immune cells display membrane receptors called Toll-like receptors (TLRs). TLRs represent a group of transmembrane proteins that function as pattern-recognition receptors by detecting and responding to microbial ligands, which are defined as pathogen-associated molecular patterns (PAMPs) (14,15). PAMPs are involved in the processes of bacteremia and endotoxemia, promote the release of systemic proinflammatory cytokines, and worsen damage caused by the infection. Bacterial cell wall products, such as lipopolysaccharide (LPS), lipoteichoic acid, and peptidoglycans trigger expression of many inflammatory cytokines in monocytes. LPS, also known as endotoxin, is a major constituent of the outer membrane of Gram-negative bacteria; LPS elicits an immune reaction that is responsible for many of the harmful effects in patients with septic shock (15). This signal pathway activates a variety of transcription factors, such as nuclear factor κ B (NF- κ B), which induces the production of inflammatory mediators that maintain the inflammatory cycle (16,17).

It is clear that LPS cannot be responsible for all clinical features of sepsis and those other factors must also contribute to sepsis (18). The major wall components of pathogenic agents and nonpathogenic agents are considered to contribute

to the development of sepsis, septic shock, and multiple organ dysfunction syndrome (MODS) (19). Similar to LPS, they can interact with TLR4 and CD14 to initiate signal transduction pathways that lead to NF- κ B activation (20,21). Activation of SIRS, which is generally chaotic, causes endothelial damage. Damage to the systemic circulation and microcirculation can cause mitochondrial discharge and increased extraction and consumption of oxygen. These changes can cause tissue hypoxia, and hyperlactatemia. Hypoxia and other types of damage can lead cells to autodeconstruct through mechanisms such as apoptosis, which can elicit sequential organ failure and eventually MODS (22-26).

In the present study, we examined the effects of cardiac surgery with and without CPB on lymphopenia in children with congenital cardiac disease. We sought to determine whether lymphopenia is caused by apoptosis of T and/or B lymphocytes, which is of relevance since it allows us to know a possible mechanism of lymphocyte reduction.

Patients and methods

Patients. The study included for 2 years, all children with congenital heart disease who required cardiac surgery with and without CPB, who were treated at the Pediatric Hospital of the Siglo XXI National Medical Center of the Mexican Institute of Social Security in Mexico City. During this period of time, the group of children that required CPB was greater than the one that did not require it. The control group was the children without bypass. Patients ≥ 15 years of age, with any autoimmune disease, diabetes, cancer or who had received previous treatment with anticoagulants, were excluded from the study. Sixty-eight patients with congenital cardiopathy needing surgery were studied: 53 who received the surgery with CPB and 15 without CPB. There were 30 girls and 38 boys (male-to-female ratio 1.2:1) and their average age was 44.6 months. The present study has been approved by the Ethics Committee of the Health Research Commission by Dr Zamira Apis Hernández (approval no. 2005-3603-0022). Written informed consent was obtained from all parent or legal guardian from patients prior to enrollment in the study.

Central venous blood was taken from the central catheter in most patients; for patients without a central catheter, the samples were obtained using a venipuncture of a peripheral vein.

Blood was collected using disposable pyrogen-free syringes (Becton Dickinson, Franklin Lakes, NJ, USA) into two sterile tubes, one with heparin and the other without heparin.

Methods. The total lymphocyte count and complete blood count were measured before and after surgery. Lymphopenia was defined as a lymphocyte count $<1,000/\text{mm}^3$.

Peripheral blood mononuclear cells (PBMC) were isolated for quantification and cell counts using specific membrane markers to quantify the subpopulations of helper and cytotoxic T lymphocytes and B lymphocytes before, immediately after, and 24 h after surgery. Briefly, PBMC were isolated from central blood samples on a Lymphoprep gradient (Axis Shield, Oslo, Norway), and the concentration was adjusted to 5×10^5 cells/ml in RPMI 1640 medium (Life Technologies, Invitrogen, Carlsbad, CA, USA).

The number of apoptotic lymphocytes cells was assessed using Annexin V-Fluos (BD Biosciences, San Diego, CA, USA) and flow cytometry (FACS Aria IIu, Becton Dickinson, USA). The cells were labeled with specific antibodies: Anti-CD19-allophycocyanin (APC), anti-CD3-phycoerythrin (PE), anti-CD4-PE, and anti-CD8-PE-Cy5 (BD Biosciences). Isotype controls were IgG1-PE, IgG2-APC, and IgG1k-PE-Cy5 (BD Biosciences).

The amount of serum IL-2 was measured by enzyme-linked immunosorbent assay (BD OptEIA™ Set Human IL-2 (BD Biosciences)).

Sepsis was documented based on established criteria (12) and by the presence and identification of microorganisms in cultures. Clinical follow-up was completed until the patients were released from the pediatric intensive care unit or hospital.

Statistical analysis. The qualitative variables were expressed in absolute frequencies and percentages, and the quantitative variables in median and interquartile ranges. Inferential statistics were performed comparing subjects with and without extracorporeal pump using χ^2 test in qualitative variables and Mann Whitney U in quantitative variables. To demonstrate the differences between the pre and post measurements between the groups, Wilcoxon test was used. $P < 0.05$ was considered to indicate statistically significant difference. All statistical analyses were performed using IBM SPSS Statistics software (v. 20; IBM Corp., Armonk, NY, USA).

Results

Demographic and clinical characteristics. Sixty-eight pediatric patients with cardiac congenital alterations needed cardiac surgery; 53 required surgery with CPB and 15 had surgery without CPB. There were 37 (54.4%) boys and 31 (45.6%) girls; 28 (52.8%) boys and 25 (47.2%) girls received CPB, and nine (60%) boys and six (40%) girls did not receive CPB. The male-to-female ratios were 1.12:1 in patients who received CBP and 1.5:1 in those who did not receive CBP. The median age was 34 months in the CBP group and 23 months in the non-CBP group (Table I). The most frequent cardiopathy was shunt (left-right), 64.2% in the CBP group and 33.3% in the non-CBP group. The most frequent surgical correction was definitive repair, 96.2% in the CBP group and 66.7% in the non-CBP group.

Blood count. Neutrophil count in patients with CPB increased significantly ($P = 0.001$), immediately after surgery and counts of lymphocytes decreased significantly (Table II). In the non-CPB group, neutrophil counts also increased significantly, and lymphocyte and monocytes counts decreased significantly after surgery (Table II).

Apoptosis of B and T lymphocytes. We next determined whether the decrease in lymphocyte count observed in patients undergoing cardiac surgery with or without CPB was caused by apoptosis of populations of CD3⁺ (CD4⁺, and CD8⁺) T lymphocytes, and CD19⁺ B lymphocytes. The percentage of apoptotic B (CD19⁺) lymphocytes was higher in patients who underwent surgery without CPB than in those who received CPB ($P = 0.0001$). The percentage of apoptotic cells before

surgery was from 14 to 0% without and with CPB respectively. This percentage was slightly but not significantly higher 24 h after surgery in both groups (Fig. 1A).

The percentage of apoptotic CD3⁺ T lymphocytes before surgery was similar in both groups (34%). This percentage increased slightly after surgery but decreased 24 h later; none of these changes were significant ($P = 0.2$; Fig. 1B). Apoptosis affected 25% of CD4⁺ T lymphocytes in without CPB group before surgery, and this percentage was significantly lower in the CPB group (2%). The percentage of apoptotic T (CD4⁺) lymphocytes was higher in patients who underwent surgery without CPB than in those who received CPB ($P < 0.001$; Fig. 1C). The percentage of apoptotic CD8⁺ T lymphocytes was higher in the CPB group (5%) than in the non-CBP group (2%; $P < 0.001$). Apoptosis of CD8⁺ T lymphocytes increased non significantly immediately and 24 h after surgery in both groups (Fig. 1D). In Fig. S1 we shown the apoptosis analysis dot plots of one patient (patient 10), indicating percentages of apoptosis of B (CD19⁺) and T (CD3⁺, CD4⁺) lymphocytes pre and post-surgery with CPB.

Clinical complications. Clinical complications developed in a higher percentage of patients in the CPB group (71.3%) than in the non-CPB group (60%). A lower percentage of patients in the CPB group developed SIRS [25 (47.2%) vs. 10 (66.7%)]. Importantly, sepsis occurred only in the CPB group [7 patients (13.2%)]. Seven patients (13.2%) died after surgery with CPB, two because of sepsis (28.6%). Thirteen patients in the CPB group (24.5%) but only two patients in the non-CPB group (13.3%) exhibited cardiogenic shock (Table I).

Sepsis and mortality. The microorganisms isolated are listed in Table III. Seven patients in the CPB group (13.2%) died after surgery (Table I); two (28.6%) died of sepsis and five (20.0%) died of SIRS. By contrast, in the non-CPB group, only two patients (10%) died of SIRS (Table I). The mortality rate was similar in both groups (13.2 and 13.3% in the CPB and non-CPB groups, respectively). The mortality rate was higher in boys (85.7%) than in girls (14.3%) in the CPB group.

Interleukin-2. Serum concentration of IL-2 was of 5 to 9 pg/ml, did not change significantly from before to immediately and 24 h after surgery and did not differ significantly between groups (data not shown).

Discussion

Despite advances in technology and the development of surgery to correct congenital heart defects, cardiopathy still occurs at a rate of 5.2 to 12.5 per thousand live births in the pediatric population. Currently in Mexico, six in 1,000 live births per year present with congenital cardiopathy disease (1). It was previously thought that lymphopenia in these patients is caused by the exclusive use of CPB secondary to the effects of ischemia-reperfusion. The devices used in surgery for these patients have evolved with advances in technology. However, this situation continues because of factors occurring during surgery, such as severe hypothermia, aortic cross-clamping, duration of CPB, metabolic response to trauma, hypoxia, or sudden changes in temperature. Other factors may also be

Table I. Patient information.

Characteristic	Total, n=68		Without CPB, n=15		With CPB, n=53		P-value
	Frequency	%	Frequency	%	Frequency	%	
Sex							0.08
Male	37	54.4	9	60.0	28	52.8	
Female	31	45.6	6	40.0	25	47.2	
Age (months) ^a	33	(10.5-61)	23	(12-30)	34	(9-62)	0.05
Weight (kg) ^a	11.1	(7.6-18)	11	(8-19)	11.2	(6.8-18)	0.70
Size (cm) ^a	87	(65.5-110)	84	(66-110)	88	(66-110)	0.80
Extracorporeal derivation time (min)					105	(87-125)	
Type of congenital heart disease							0.002
Right ventricle obstruction outflow	21	30.9	4	26.7	17	32.1	
Shunt (left-right)	39	57.4	5	33.3	34	64.2	
Anomaly connection pulmonary veins	4	5.9	3	20.0	1	1.9	
Left outflow obstruction	4	5.9	3	20.0	1	1.9	
Procedure							0.001
Palliative	7	10.3	5	33.3	2	3.8	
Definitive repair	61	89.7	10	66.7	51	96.2	
Complications							
SIRS (yes)	35	51.5	10	66.7	25	47.2	0.7
Sepsis or septic shock (yes)	7	10.3	0	0.0	7	13.2	0.7
Cardiogenic shock (yes)	15	22.1	2	13.3	13	24.5	0.7
MODS (yes)	6	8.8	1	6.7	5	9.4	0.8
Mortality	9	13.2	2	13.3	7	13.2	0.9

^aMedian (percentile 25-percentile 75), Test U Mann Whitney. CPB, cardiopulmonary bypass; SIRS, systemic inflammatory response syndrome; MODS, multiple organ dysfunction syndrome.

Table II. Complete blood count.

Cell/mm ³	Without CPB				With CPB				P-value (paired) ^a
	Median	Percentile 25	Percentile 75	P-value (paired) ^a	Median	Percentile 25	Percentile 75	P-value ^b	
Neutrophils pre	4.0	3.0	5.8	0.001	3.5	2.3	5.1	0.02	0.001
Neutrophils post	9.9	6.0	13.9		8.3	5.0	10.5		
Lymphocytes pre	3.1	1.9	4.6	0.001	3.5	2.5	4.8	0.40	0.0001
Lymphocytes post	1.5	0.9	1.9		1.6	1.1	2.1		
Monocytes pre	0.7	0.4	1.1	0.03	0.7	0.6	1.0	0.04	0.7
Monocytes post	1.1	0.5	1.2		0.6	0.3	1.0		
Eosinophils pre	0.2	0.1	0.49	0.6	0.2	0.1	0.3	0.70	0.7
Eosinophils post	0.0	0.0	0.01		0.0	0.0	0.05		
Basophils pre	0.0	0.0	0.10	0.7	0.1	0.0	0.1	0.90	0.8
Basophils post	0.0	0.0	0.001		0.0	0.0	0.01		

^aFriedman test, ^bWicoxon test. CPB, cardiopulmonary bypass.

involved, such as gene regulatory mechanisms including the responses of sepsis genes (26), stress induced by the disease and/or surgery, the patient's inherent susceptibility, and stress to hematopoietic tissue and cells, which accelerates the

biological cycle and can cause cell self-destruction. Several studies of adults have reported multiple complications secondary to the use of CPB. More recent studies of children have been reported, but only up to 61 months of age. Shi *et al*

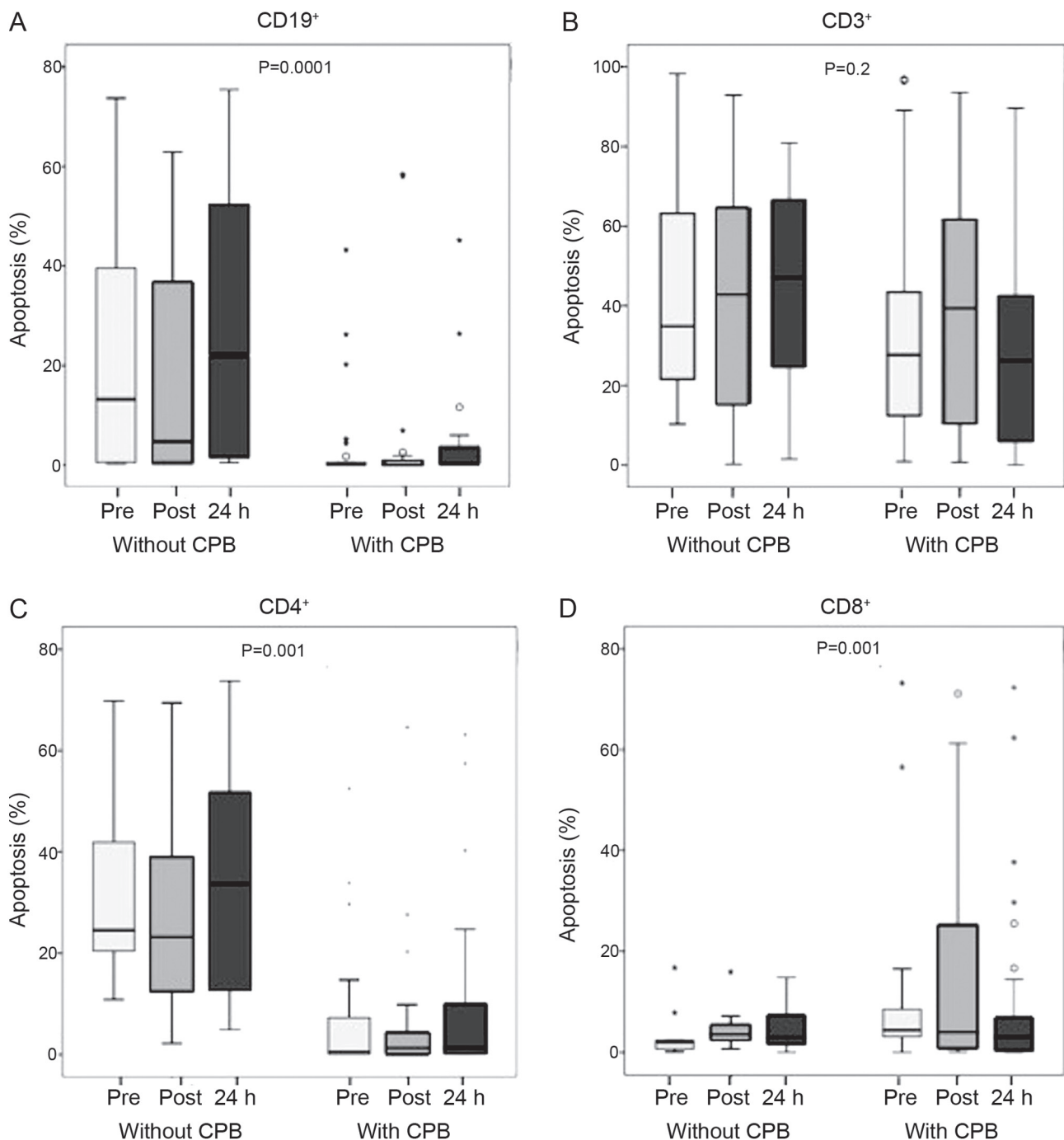


Figure 1. Apoptosis of B (CD19⁺) and T (CD3⁺/CD4⁺ or CD3⁺/CD8⁺) lymphocytes of patients operated on with and without CPB. (A) Histogram of apoptosis of B lymphocytes before and after surgery in the CPB and non-CPB groups. P=0.0001. (B) Graphic representation of apoptosis of T (CD3⁺) lymphocytes before and after surgery in the CPB and non-CPB groups. P=0.02. (C) Histogram of apoptosis of T (CD4⁺) lymphocytes before and after surgery in the CPB and non-CPB groups. P=0.001. (D) Graphic representation of apoptosis of T (CD8⁺) lymphocytes before and after surgery in the CPB and non-CPB groups. P=0.001. CPB, cardiopulmonary bypass.

included only pediatric patients and found decreased lymphocyte counts regardless of the use of CPB (2). Our findings support those of Shi *et al.*

The complete blood counts showed similar cell distributions in both groups of patients. However, despite this similarity in cell counts, they should not be considered as only one group before surgery because of differences in their presurgery physiological condition, which may influence the decision of cardiologists and surgeons about which patients need or do not need CPB.

After surgery, patients in both groups showed a significant increase in neutrophil count, which was probably caused by an immediate immune response to the damage caused by the surgery and was independent of the use of CPB. Most studies agree that the increase in neutrophil count reflects an important change in cells central to the inflammatory response and the recognition of pathogens or their ligands. By contrast, lymphocyte count decreased significantly in both groups, which was unexpected because studies of adults have reported that lymphopenia occurs only in patients undergoing

Table III. Sepsis etiology.

A, Surgery with CPB		
Isolate microorganism	Frequency	%
<i>Staphylococcus sp.</i>	2	3.8
<i>Enterobacter cloacae</i>	1	1.9
<i>Escherichia coli</i>	1	1.9
<i>Enterococcus faecalis</i>	1	1.9
<i>Streptococcus pneumoniae</i>	2	3.8
Cultivo negativo	46	86.7
Total	53	100.0
B, Surgery without CPB		
Isolate microorganism	Frequency	%
Cultivo negativo	15	100.0
Total	15	100.0

CPB, cardiopulmonary bypass.

surgery with CPB. As in our study, a previous study that included pediatric patients reported reduced lymphocyte counts in both patients operated on with and without CPB, although the decrease was greater in the CPB group (2,27). In other study that included infants, it was compared the clinical effectiveness and biocompatibility of poly-2-methoxyethyl acrylate (PMEA)-coated and heparin-coated CPB circuits in elective cardiac surgery with CPB for ventricular septum defects, finding that the leukocyte counts were significantly lower 5 min after CPB in the PMEA group than the heparin group, showing that PMEA-coated circuits cause transient leukopenia during pediatric CPB (28). However, this study was different from ours, because they did not compare cardiac surgery with and without CPB and did not determine the apoptosis of the lymphocytes. We also found that the counts of all hematopoietic cell populations decreased after surgery in the CPB group, which indicated sensitivity to surgical stress in this group. Eosinophil count decreased in patients undergoing surgery without CPB. The differences in hematopoietic cell counts may reflect the inflammatory response and/or synthesis of cytokines following surgery (29). Different cytokines are produced to stimulate the production of specific hematopoietic lineages after damage to tissues (30).

The decrease in lymphocyte count may increase the risk of developing an infection, which may lead to severe sepsis, the precursor of MODS that can lead to death. In tertiary-level hospitals, sepsis remains an important issue because it is the leading cause of death in patients surgery to correct congenital heart defects; it is also the main cause of admission to post-operative therapy in heart-surgery patients. In this study, we observed a higher frequency of cardiogenic shock and sepsis in patients with CPB than in patients without CPB. However, sepsis developed only in patients who received CPB. These findings suggest the need for further understanding of the

cellular, biochemical, and molecular mechanisms underlying the responses of pediatric patients who undergo surgery with CPB.

The decrease in lymphocyte count observed in our study seemed to be caused by apoptosis of B lymphocytes (CD19⁺) in the non-CPB group and of T lymphocytes (CD8⁺) in the CPB group, although no significant differences were found in the rate of apoptosis of B and T lymphocytes after surgery in both groups. Several studies have reported that CPB causes cytotoxic effects in several hematopoietic lineages, such as a decreased mitogenic activity of lymphocytes or morphological changes in neutrophils (29,31,32). However, it seems that the effect may be greater in certain cell lineages, although, to our knowledge, this has not been reported in any previous work. We observed increased apoptosis of CD8⁺ T lymphocytes after surgery with CPB and increased apoptosis of B lymphocytes after surgery without CPB. The decreased number of cells may be caused by a cytostatic effect given that hematopoietic tissue is one of the most sensitive tissues to environmental changes, such as temperature changes, contact with foreign surfaces, or cell damage. It is probable that all of these factors contribute to the decreased number of hematopoietic cells. This is the first study to show that the decreased in lymphocyte count in children undergoing corrective heart surgery, with and without CPB, may result from increased apoptosis of CD8⁺ T and B lymphocytes. Then, in addition to analyzing the effects of cardiac surgery with and without CPB in the reduction of lymphocytes in children with congenital heart disease, we sought to establish if the reduction of these lymphocytes was caused by the apoptosis of the T lymphocytes and B lymphocytes, which is of relevance since it allows us to know a possible mechanism of lymphocyte reduction.

In addition, sepsis, and cardiogenic shock were also higher in the CPB group. The rates of sepsis, cardiogenic shock and MODS were higher in patients who underwent surgery with CPB. These children also had the highest neutrophil counts and lowest lymphocyte counts after surgery.

In this study, lymphocyte count decreased after surgery in both groups, but the reason for this decrease seemed to differ between the CPB and non-CPB groups. That is, the decrease in lymphocyte count was probably related to apoptosis of B lymphocytes (CD19⁺) in the no-CPB group but to apoptosis of T lymphocytes (CD8⁺) in the CPB group. Apoptosis can be activated by both intrinsic and extrinsic factors and hematopoietic tissue is one of the most sensitive tissues to extrinsic factors, such as temperature changes, contact with foreign surfaces, and cell damage. These factors may be involved in the decreased number of hematopoietic cells observed after cardiac surgery in infants. However, it must be considered that the number of patients included in the groups was different, which may be a limitation of the study.

In conclusion, this is the first study to determine the effects of cardiac surgery with and without CPB on leukocyte subsets in children where it was observed that the decrease of lymphocytes can be due to the increase in the apoptosis of the T and B lymphocytes. The decreased lymphocyte count after heart surgery may be caused by some of the events occurring during surgery, but not necessarily the effects of CPB. Patients who received CPB exhibited a large increase in neutrophil counts and a decrease in lymphocyte counts. These cells

are important mediators of the inflammatory response and recognition of pathogens and/or their PAMPs. Leukocytosis seen after cardiac surgery with and without CPB may have been caused by the increase in neutrophil count. Lymphocyte count decreased after cardiac surgery in both the CPB and non-CPB groups, this probably reflects apoptosis of B and T lymphocytes in response to surgical stress and not to CPB by itself.

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Availability of data and materials

The datasets used during the present study are available from the corresponding author on reasonable request.

Authors' contributions

RJA, ERM and CMB contributed to the conception and study design. RJA and NSZ contributed to the acquisition of data. CMB, RJA and NSZ performed the cytometry analysis and interpretation of data. RTF performed the statistical analyses of the data. JZ, ASG and CR analyzed and interpreted the patient data regarding the cardiology diseases. RJA, NSZ and CMB drafted the manuscript. RJA, NSZ, ERM and CMB revised the manuscript critically for intellectual content. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The present study has been approved by the Ethics Committee of the Health Research Commission by Dr Zamira Apis Hernández (approval no. 2005-3603-0022). Written informed consent was obtained from all parent or legal guardian from patients prior to enrollment in the study.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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