Plasma endothelin-1 and circulating endothelial progenitor cell levels after cardiopulmonary bypass in children with congenital heart diseases: a preliminary study

Angélica Rangel-López (ragn62@prodigy.net.mx)
Instituto Mexicano del Seguro Social https://orcid.org/0000-0001-7040-9379
María Eugenia Paniagua-Medina
Instituto Mexicano del Seguro Social
Héctor Jaime González-Cabello
Instituto Mexicano del Seguro Social
Ricardo López-Romero
Instituto Mexicano del Seguro Social
Lourdes Arriaga-Pizano
Instituto Mexicano del Seguro Social
Miguel Lozano-Ramírez
Instituto Mexicano del Seguro Social
Juan José Pérez-Barragan
Instituto Mexicano del Seguro Social
Dulce María López-Sánchez
Instituto Mexicano del Seguro Social
Minerva Mata-Rocha
Instituto Mexicano del Seguro Social
Guadalupe Carrillo-Montes
Instituto Mexicano del Seguro Social
Horacio Márquez-González
Instituto Mexicano del Seguro Social
Ramón Paniagua-Sierra
Instituto Mexicano del Seguro Social
Juan Manuel Mejía-Aranguré
Instituto Mexicano del Seguro Social

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Abstract

There are very few data on analytical biomarker levels in children with congenital heart disease (CHD) before and after cardiopulmonary bypass surgery. We conducted a preliminary study in the Pediatric Hospital of Medical Center IMSS Mexico, to evaluate plasma levels of endothelin-1 (ET-1) and circulating endothelial progenitor cells (CPEC) before and after correction surgery. 56 patients were included: 41 with CHD that required CPB and 15 controls with surgery other than CPB, which were stratified by age (infants, preschool-age-children, and school-age-children). Samples of peripheral blood were taken 24h before and 48h after surgery, cytokine levels (TNF/IL-1β/IL-6/IL-8/IL-10 and IL-12p70) and brachial-artery-flow-mediated dilation (BAFMD) were explored too. Demographic and clinical data were collected. BAFMD was evaluated by-Doppler-ultrasound, the percentage (%) and absolute number (N) of CPEC [CEC and EPC], ET-1 and cytokines concentrations were measured by flow-cytometry, radioimmunoassay and a Bead-Array-cytometric-system, respectively. The% and N of $CEC^{[CD34 + / CD146 + / VEGFR2 + / CD133-]}$ increased in infants versus their control group; and $EPC^{[CD34 + / CD146 + / VEGFR2 + / CD133 +]}$ were higher in the control group of infants versus patients. ET-1 levels were higher in patients than in controls ($p = 0.002$). N-CEC showed differences between 24 and 48 hours; BAFMD in CPB-preschool at 24H and 48H showed a significant difference. Higher levels of IL-6 and IL-8 were detected in CPB-school-age at 48H. This pilot study observed a significant increase in the levels of CEC and ET-1. To validate these data, subsequent studies should increase the sample size.

1. Introduction

Congenital heart disease (CHD) is the most common abnormality diagnosed in newborns worldwide [1]. In Mexico, ventricular septal defect is the second most common cause of coronary disease [2, 3]. The Pediatric Hospital of the Centro Médico Nacional Siglo XXI of the Instituto Mexicano del Seguro Social attends approximately 100 children with CHD each year, and most of them are cases of intraventricular communication and tetralogy of Fallot, in which cardiac surgery by cardiopulmonary bypass is used (CEC). In these alterations, surgical mortality is around 3% in children older than one year, and is slightly higher in infants and children with multiple defects, with high morbidity in these patients [4]. CPB activates a kind of inflammatory response that resembles the systemic inflammatory response syndrome, characterized by alterations in cardiovascular and pulmonary functions [5]. And it is hypothesized that this post-CPB inflammatory response contributes to postoperative morbidity and mortality, particularly in newborns [6]. The cellular and molecular mechanisms involved in this inflammatory response in CPB have not been elucidated, but it has been hypothesized that a release of cytokines increases the level of Endothelin-1 and activates circulating endothelial progenitor cells (CEPC), which leads to possible myocardial diseases and vascular lesions [7]. Contact of blood with artificial surfaces in the CPB machine activates leukocytes, platelets, the complement system and CEPCs that are released into the blood, thus the differential expression of adhesion molecules detected in peripheral blood could reflect a regulatory mechanism in these processes [8]. Currently, in our institution we do not have specific blood biomarkers of endothelial injury available in practice and only the determination of endothelial function is used by means of flow-mediated dilation of the diameter of the brachial artery (BAFMD), as a measure of function microcirculatory for pediatric patients requiring surgical interventions [9].

Endothelin-1 (ET-1) is a polypeptide that is released from endothelial cells and cardiomyocytes among other cells, and is considered the most powerful endogenous vasoconstrictor involved in different disorders; its functions include promoting cell growth and thickening the arterial wall 10]. ET-1 has been identified in children during operations requiring CPB [11], showing overexpression in both early and late stages [12, 13], its plasma concentration has also been reported to increase progressively during CPB [14, 15]. On the other hand, CEPCs are considered a specific and sensitive marker of endothelial activation and damage in different vascular disorders [16]. They are a rare population of mononuclear cells that circulate in the peripheral blood and are involved in vascular repair of damaged tissues [17]. Since its discovery [18] its definition is still unclear, as this population includes myeloid angiogenic cells of hematopoietic origin that express CD45, and endothelial colony-forming cells of mesenchymal origin that do not express CD45 that are late-growing and are defined as endothelial progenitor cells (EPC) [19]. Both cells of mesenchymal and hematopoietic origin are involved in the formation and repair of blood vessels, but mesenchymal cells form vessels unlike cells of myeloid origin, which are called circulating endothelial cells (CEC) and which only support this process through the production of growth factors [20]. There are few studies related to the quantification of CPE levels in the pediatric population after surgery using CPB [21–23]. And it is important to mention that so far there is no consensus for its identification despite the fact that around 20 phenotypes of human EPC cells used by different researchers have been described [24], the lack of a specific marker and the low number of these cells in organs and peripheral blood they give rise to multiple problems of identification, isolation and application [25, 26].

To date, in our Institute there is no clinically accessible standardized protocol for the reproducible enumeration of CEPC, especially for EPC, so in this pilot study we consider it important to carry out its identification by multiparametric Flow Cytometry with immunomagnetic

separation of CPECs, which makes it possible to assess cell viability by counting EC in absolute numbers [23] and to analyze its relationship with determining the levels of ET-1 and circulating cytokines in this pediatric population.

2. Methods

Patients and controls

The study protocol was approved by the local ethical committee (CNIC-R-2010-785-038) according to the principles outlined in the 1975 Helsinki Declaration (Anonymous, 2002), and an informed consent was signed, obtained from the parents of all patients and control subjects. In total, we recruited sixty-two patients, of which 47 had CHD and 15 were patients of the control group. Of the 47 patients six cases were eliminated since they died after surgery, so in the end, the group of patients with CHD remained in 41 cases, see Table 1. Forty-one paediatric patients (21 males and 20 females, ages ranged from 0 to 108 months, mean ± 22.6 months) with CHD underwent surgery for partial or total correction of heart defects at birth, and for heart surgery for CPB were admitted to the Pediatric Intensive Care Unit in the Pediatric Hospital National Medical Center XXI Century, IMSS. Their diagnoses were: ventricular septal defect in twenty patients, tetralogy of Fallot in nineteen patients, transposition of the great arteries in two. The patients were divided into infants (from 0 to 12 months of age, n = 5), preschoolers (from 12.1 to 60 months of age, n = 30) and school-aged (> 60.1 months of age, n = 6). We also studied 15 children (9 males and 6 females; age 2 to 180 months, mean 72.3 ± 63.7 months), who underwent surgery other than CHD (colongitis in one patient, choledochal cyst in seven patients, fistula retro vestibular in one patient, portal hypertension in one patient, gastroesophageal reflux in one patient, empyema in one patient, pituitarism in one patient, craniotomy in one patient and gastrocutaneous fistula in one patient). These patients were also divided into infants (from 0 to 12 months of age, n = 5), preschoolers (from 12.1 to 60 months of age, n = 4) and school-aged (> 60.1 months of age, n = 6) see **Table 2.**

Table 1
Patients and controls demographic data.

	Patients	**Controls**
Variable	Mean ± SD	Mean ± SD
Age (months)	34.5 ± 22.6	72.3 ± 63.7
Sex	21 M / 20 F	9 M / 6 F
CPB (cardiopulmonary bypass)	107	-
ACT (aortic clamping time in minutes)	66	-
Died	6	-

Table 2. Clinical and analytical data for CPB patients and controls

Variables	CONTROL GROUP						CPB PATIENTS					
	Infants		Preschoolers		School-aged effects		Infants		Preschoolers		School-age	
n	5		4		6		6		30		5	
Age (months)	0–12		12.1–60		> 60.1		0–12		12.1–60		> 60.1	
Gender (male/female)	3M/2F		2M/2F		4M/2F		4M/2F		13M/17F		4M/1F	
Time: Before/After	24	48	24	48	24	48	24	48	24	48	24	48
BAFMD (%)	1.4 (1.4-2)	1.0 (1–2)	1.3 (1.1-2)	1.2 (1-1.4)	1.3 (1–2)	1.4 (1–2)	1.0 (1–2)	1.3 (1–2)	1.3 (1–2)[b]	2.0 (1–4)[b]	1.3 (1.2-2)	1.3 (1.2–1.4)
Endothelin-1 (pg/ml)	2.3 (1.4–20.3)[f]	5.0 (3–17)[i]	16.3 (2-152.2)	18.4 (5–73)[i]	335 (2.1–457)	156 (4-313.4)	15 (0.4–32)[f,l,n]	20 (1-44.1)[i]	95.1 (58.2–814)[a,l]	154 (81-583.2)[b,i]	111 (80–696)[n]	133 (81.4–600)
EPC (%)	0.2 (0-0.2)[g]	1.0 (1–4)	1.0 (0.2-2)[h]	1.4 (0.2-2)	0.3 (0.1–0.4)	0.2 (0.2–0.2)	0 (0–8)	1.0 (0.1–17)	0.1 (0–9)	0.1 (0-12.3)	0.3 (0-0.4)	1.0 (0.2-1)
EPC (N)	36 (5-196)	27 (8-108)	13 (13 a 24)	52 (19–91)[j]	20.2 (3.3–37)	11 (10–11)	1.2 (0-250)	12.1 (1-204)	8 (0-255)[c]	2 (0-233)[c,j]	9 (4.2–14)	17 (3–58)[n]
CEC (%)	0.02 (0-0.2)	3.0 (0–3)	0.0 (0-0.1)	0.0 (0-0.1)	0.0 (0–0)	0.0 (0–0)	2 (0-3.4)[g]	7 (1–14)[m]	1 (0–28)[h]	0.4 (0–26)[m]	1 (0.1-5)	0.4 (0.1–14.2)
CEC (N)	1.2 (0.3–14)	2 (0–7)	0.1 (0.1-1)	4 (2–4)	0.4 (0.1-1)	0.3 (0.2-1)	76 (0-173)	78 (10–125)[m,n]	36 (0-2438)	7.4 (0-1053)[m]	18 (4–70)	6 (5.2–582)
Inflammation markers (pg/ml)												
TNF-α	2.7	2.7	2.7	2.7	2.7	2.7	2.7	2.7	2.7	2.7	2.7	2.7
IL-1β	6.2	6.2	6.2	6.2	6.2	7.0	6.2	6.2	6.2	6.2	6.2	6.8
IL-8	26.4 (8-49.2)	8.4 (5–67)	16.3 (6–24)	108.0 (8-317.2)[k]	19.2 (7-767)	15.0 (3-647.1)	5.0 (4-38.1)	16.1 (5.2–53)	8.0 (3–97)	11.0 (3–48)[k]	9.0 (3–17)	210.4 (21.2–674)
IL-6	34.2 (2–72)	29 .0 (2-147.3)	3.0 (2–58)	135.0 (5-209.3)	11.0 (4-424.1)	7.0 (1.5–182)	4.1 (2–58)	32 .0 (2-298)	5.0 (2-233)	32.9 (2-241.4)	3.6 (2–44)	129.0 (85–401)
IL-10	2.3 (2.3–2.3)	2.3 (2.3–11)	2.3 (2.3–3.3)	6.2 (2.3–15)	3.1 (2.3–31)	3.1 (2.3–10.3)	2.3 (2.3–11)	5 (2.3–21.4)	2.3 (2.3–6.1)	2.3 (2.3–16.4)	2.3 (2.3-4)	22 (4-41.3)
IL-12	3.0 (1-3.4)	2.3 (1-5.2)	4.0 (1-6.4)	4.0 (2.3-5)	4.1 (1–8)	3.0 (1–6)	2.3 (1-6.4)	3.4 (1–6)	3.0 (1–5)	2.3 (1–5)	4.0 (2.3–5.2)	4 .0 (2.3–4.1)

Table 2. Clinical and analytical data for CPB patients and controls

Time surgery: Before = 24H-/**After** = 48H+. **CPB**, cardiopulmonary bypass; **BAFMD**, brachial artery flow-mediated dilation; **CEC**, circulating endothelial cells [CD34+/146+/VEGF+/133-]; **EPC**, circulating endothelial progenitor cells [CD34+/146+/VEGF+/133+]; **IL**, interleukin; **TNF-α**, tumour necrosis factor-alpha; *P* values are based on Student's t-test or chi-square according to the variable characteristics; a$P < 0.05$ versus C; b$P < 0.001$ versus C. Values are median (intervals). Before vs after: a$P = 0.014$, b$P = 0.012$, c$P = 0.021$. Cases versus Controls: f$P = 0.005$, g$P = 0.017/0.037$, h$P = 0.031/0.040$, i$P = 0.002$, j$P = 0.05$, k$P = 0.047$. Infant versus Preschoolers: l$P = 0.15$, m$P = 0.033/0.011$. Infants versus Schoolers: n$P = 0.035$.

The preliminary criterion included children between 0 days to 198 months requiring CPB surgery. Patients with associated comorbidities (previously infected with kidney failure before surgery, oncology or prior evidence of ischemia) or trans-surgical death were excluded. The markers were determined in both groups 24 hours before surgery (24H-) and 48 hours after surgery (48H+), once their general condition was stabilized with a maximum initial period of inflammatory response secondary to the surgical procedure, according to recommended guidelines of the International Brachial Artery Reactivity Task Force (IBARFF) [27].

Assessment of endothelial function

Endothelium-dependent responses of the BAFMD were measured for each patient subject according to the guidelines of the IBARFF, using a 10-MHz Ultrasound Doppler probe (Toshiba Doppler Color (Spectral Advanced Technology Laboratories, Hamburg, Germany). Briefly, a baseline internal diameter was measured in supine position. Thereafter, a blood pressure cuff, placed distal to the elbow, was inflated to 200 mmHg or at least 50 mmHg above the peak systolic pressure for 4 min. Post-occlusion measurements were taken every 30s over the following 270s and analyzed using an automatic edge tracking method by an independent, blinded investigator. BAFMD was assessed by measuring the maximal increase in the diameter of the brachial artery to maximal post-occlusion value, expressed by mean differences in percentage. Images were stored on a magnet-optical disk and analyzed after the procedure [28].

For preparation of blood samples, Antibodies and reagents Immunomagnetic separation and Cytokine quantification: **See supplemental Material 1, 2 Methods.**

Plasma levels of ET-1

For ET-1 assay immediately after collection of blood for anti-coagulation, the blood was transferred from the vacutainer tube to chilled, siliconized disposable glass tubes containing aprotinin (1,000 kallikrein inactivator units/ml) and gently rocked several times, then centrifuged at 4ºC and the plasma was collected. Subsequently, peptide extraction was performed using HPLC cartridges, C-18 SEP-COLUMN (Waters Inc. Milford, Mass) as the manufacturers recommended, and then a concentrator (Vacufuge™, Eppendorf, USA) was used to dry samples, subsequently to be freeze-dry overnight using a lyophilizer and storaged at-70ºC until radioimmunoassay (RIA) was made. Plasma ET-I concentration was measured with a RIA kit (Phoenix Pharmaceuticals, Inc. Burlingame CA, USA) according to the manufacturer ´s protocols. Briefly, the samples were reconstituted in RIA buffer. ET-1labeled with I^{125} was used as tracer and porcine ET-1 as a standard. The assay was incubated at 4^{o} C in 0.1mol/L phosphate buffer, pH 7.4, containing 0.1% BSA and 0.1% Triton-X-100. Bound and free fractions were separated using a secondary antibody. Recovery from the extraction procedure was 85% ± 5% on the basis of spiked plasma standards (4–20 fmol/mL). For repeated measurements of plasma samples and the inclusion of plasma samples with a known concentration of exogenous ET-1 added to the sample, the coefficient of variation was less than 12%. By using an optimized extraction procedure, the sensitivity of this assay system was 0.75 fmol/mL (1 fmol/ml = 2.5 pg/ml). The within-assay precision for duplicate determinations was determined for 20 plasma samples, and the coefficient of variation was 4.4%.

Circulating Endothelial cells: CEC $^{[CD34+CD146+VEGFR2+CD133-]}$ and Circulating Endothelial Progenitor cells: CEPC $^{[CD34+CD146+VEGFR2+CD133+]}$ Identification by FC

PBMCs isolated by density gradient from blood drained from patients or control subjects were incubated for 30 min at room temperature with fluorochrome conjugated antibodies: αCD34/APC, αCD133/APC, αVEGF/FITC and αCD146/PE. Fixing and Lysing solution (FLS, Becton Dickinson, USA) was added (100 µL) and incubated for 10 minutes. PBS was added (2 mL) and tubes centrifugated at 1200 g/5min. Cell pellet was resuspended with PBS and analyzed using a FACS Aria Flow Cytometer with DIVA Software v 6.1. Algorithm for endothelial identification was: cells were first selected based on forward vs side scatter pattern. From $CD146^{+}$ cells gates were made for $VEGF^{+}/133^{+}$ or $VEGF^{+}/133^{-}$and $VEGF^{+}/CD34^{+}$ or $CD34^{-}$. Percentage values were obtained and cell quantification calculated based on total PMCs and total leucocyte number. The absolute CEC and CEPC number were derived from the absolute number of white blood cells

provided by hematology analyzer and the percentage of CEC and CEPC was determined by FC, using the following formula [29]: *percentage of cells x white blood cell (WBC) count/100.*

Statistical Analysis

The mean ± standard deviation (SD) of the percentage, for the absolute number of CEC, and CEPC were calculated together with their range distribution. The distribution's continuous variables were assessed with a Shapiro test but for the ones that did not have a normal distribution, Mann-Whitney test was employed to compare the groups. A correlation analysis was done to assess if ET-1 was associated with precursor and endothelial cells, as well as to make associations between that cell and other clinical factors like age, their CHD, or mortality risk, etc. A p-value < 0.05 was considered significant. The patients were divided into infants (from 0 to 12 months of age), preschoolers (from 12.1 to 60 months of age) and school-aged (≥ 60.1 months of age). All calculations were performed using SPSSw-v19 software for Windows (SPSS Inc., Chicago, Illinois, US).

3. Results

Forty-one patients and fifteen controls were included in the study. Their demographic and operative data are presented in **Table 1**, and **Table 2**. Some of the controls had a higher age than the CPB patients, however only non-significant differences were found.

Assessment of endothelial function

Measurements of BAFMD in the 24H-group were lower than those obtained in the control group, observing opposite data behavior obtained in the group 48H+. This increase was statistically significant in the subgroup of preschoolers. (CPB-H24: 1.27±2.1% vs C-H24:1.33±0.8%, p<0.05; and CPB-H48:1.45± 0.9%vsC H48 1.18 ± 0.8%, p< 0.05, respectively) (See **Table 2)**.

Plasma levels of ET-1

Plasma levels of ET-1 were significantly higher in patients than in controls. ET-1 decreased not only significantly after CPB, but it also showed a little increase in control group after a different surgery. When CPB group was stratified by age we observed in school-age group a statically significant increase of ET-1 in the preschool group (CPB-H24: 95.1pg/ml vs C-H24: 16.3 pg/ml, p<0.05; and CPB-H48: 154± 99.7pg/ml vs C H48 18.4 pg/ml, p< 0.002, respectively) (**Table 2, and Fig. 1**). Endothelin-1, circulating endothelial cells were different in the basal level among the groups, however just the percentage of circulating endothelial cells showed difference between basal and postsurgical period; this difference was shown in both groups. No correlation was found among endotelin-1 and CEC%, CEC number or CPC% or CPC number (data not shown).

CEC and circulating EPC counting by flow cytometry

Using this protocol, two populations were detectable by FC. One population consisted of [$CD34^+CD146^+VEGFR2^+CD133^-$]−cells (referred to as viable CEC) and the other was represented by[$CD34^+CD146^+VEGFR2^+CD133^+$] (referred to as viable EPC). Levels of CEC after immune magnetic separation and detected by FC were different by age (**Table 2**). FC was used to identify circulating endothelial cells. As c seen in **Figure 2**, once cells were selected according to size and granularity, the cells totally positive to CD146 were selected. From this CD146+ population the value of median fluorescence intensity (MFI) was determined by the relative expression of CD133. All cells were also positive to VEGF (data not shown). A greater proportion of circulating endothelial cells was observed in particularly in patients than in healthy subjects. In general, the CPB group had elevated CEC counts in absolute number (>3 cells/ml), as well as a percentage in relation to control group in 24H-and 48H+ times.

When CPB group was stratified by age a statistically significant difference was observed in the CEC counts before and after surgery at the subgroups of infants (CPB_24H-:2.2±1.7% vs C_24H-:0.02±0.1%, p<0.05; 76±328.2 cells/ml vs 1.2±103.3 cells/ml, p<0.05 and CPB_48H: 7% vs 3%, p<0.05, respectively); and $CEPC^{[CD34+/CD146+/VEGFR2+/CD133+]}$ were increased in younger control group over patients (CPB_24H-:0.022±2.2% vs C_24H-: 0.22±0.6%, p<0.05; 1.2±283.2 cells/ml vs 36±101cells/ml, p<0.05 and CPB_48H+: 0.31± 0.8 % vs 1 ± 0.3%, p< 0.05; 12.1 ±283.2 cells/ml, p<0.05 vs 27±101 cells/ml, p< 0.05, respectively) (**Table2**). During the patients' follow-up just six died; CEC%, CEC number or CPC% and CEPC, thus this number was not correlated with the death. When the age groups were assessed and the infants were observed, the most important differences over all in the levels of CEC number, with ET-1, CEPC number and BAFMD the differences were in the limit of the significance (**Table 2**). Higher after-surgery IL-6 and IL-8 levels was detected. Levels of cytokines in general showed a constant variation in the two measurements, which probably would be improved if the same measurement times were extended, see **Table 2**.

Table 3 describes some information about the six patients of the group who died during the study, mentioning the Aristotle score, age, CPB and aortic clamping times, and the CEC number and CEPC%. All of these patients were classified in high risk mortality group, and showed higher CPB and aortic clamping times than the median group, and were also a higher CEC number or CPC% than the median group.

Table 3. Clinical and laboratory dates of the six patients who died

Cardiopathy Diagnosis	Age (Month)	CBP* time (min)	AoC** time (min)	Aristotle Score	CEC#$^{+}$	CEC %$^{++}$
Ventricular septal defect	9	80	65	2	70	5
Ventricular septal defect	49	90	55	2	805	10
Ventricular septal defect	29	103	45	2	65	2
Fallot Tetralogy	24	124	82	3	200	4
Right pulmonar artery agenesia	45	70	45	3	70	7
Total anomalous venous return	2	120	90	3	60	2

* Cardiopulmonary bypass; ** Aortic clamping; $^{+}$ Number of endothelial cells (median for group of children with CHD = 34); $^{++}$Percent of endothelial cells (median for group of children with CHD=0.9)

4. Discussion

To our knowledge, this is the first exploratory study in our Institute that suggests that the determination of these laboratory biomarkers could help in the evaluation of pediatric patients who are candidates for cardiac surgery with CPB, where contact activation of the blood cells with artificial surfaces, air, surgical trauma etc., trigger an inflammatory process with cell activation and endothelial dysfunction [30, 31]. The bioactive peptide ET-1 has been shown to mediate vasoconstriction of the systemic circulation and influence myocardial contractility [15]. The results of the present study demonstrated that ET-1 levels were significantly elevated in the post-operative period (24H) and were significantly lowest in the pre-operative period (48H) in patients undergoing CPB. These results are consistent with some previously published studies in which elevated ET-1 levels were detected during the perioperative period of cardiac operations in children [32, 33].

We measured higher baseline values of ET-1 than did Komai et al [32] and Xia et al [33]; this discrepancy cannot be completely explained by variation in specificity of the monoclonal antibodies used in each study. Despite the variations that ET-1 concentrations detected in the plasma of healthy subjects by different researchers may be between 0.1 and 48 fmol/mL (0.25-120 pg/mL) [32], it may reflect patient differences given to age or ethnic origin. The mean age of the patients in Komai's study was 1.6 years, the patients in Xia's study were older, and our patients had an intermediary age, so it is possible that ET-1 levels increase in patients with congenital heart defects at different ages. In our study, the concentrations of ET-1 in pediatric patients after CPB were much higher than the documented plasma levels, which can be explained by the use of aprotinin for inactivity of kallikreins on the plasma samples, that limits the peptide detection. The elevated number of CEC in preoperative patients provides evidence that in CPB there is a pronounced endothelial injury and damage [34, 35]. We also assume that the detection of elevated numbers of CEC may be the most direct marker of endothelial activation or injury and, perhaps, may enable the quantification of the inflammatory response in conjunction with CPB. Since these cells are found very rarely in healthy people's blood; the increased number may reflect the degree of endothelial activation or damage, and even represent a prognostic indicator in patients developing an overwhelming inflammatory response after a period of CPB [35, 36].

Previous studies have used different protocols for the measurement of CEC and CEPC. Up to now, several assays enabling the detection of CEC and CEPC have been described and others are likely to be published in the near future. In addition to establishing the true value of CEC and CEPC enumeration, general consensus on the best way to enumerate these cells is now definitely required [37, 38].

Using this protocol, two populations were detectable by FC: *[CD34^{+}CD146^{+}VEGFR2^{+}CD133^{-}]* and *[CD34^{+}CD146^{+}VEGFR2^{+}CD133^{+}],* which have been reported, ranging typically from 0.1% to 6.0% of blood mononuclear cells from CEC and 0.01–0.20% of blood mononuclear cells for CPC [39].

CEC were easily quantified in this pediatric population, and counts in the 15 pediatric control subjects were close to those described in adults by the consensus network [38]. Besides, CEC counts in peripheral blood were similar in control subjects and patients with reversible CBP and were consistent with normal values defined by consensus (<10 CEC/mL), similar to the number found in normal subjects, as defined with the consensus protocol [38].

The CEC%, CEC number or CEPC% and CEPC number were not statistically significant and apparently did not correlate with the patient´s course. However, our report had few patients, and a type II error is possible, and it may be that higher number of patients would have shown a clinical and statistical significance, especially in infants, where the changes were more important. Sun et al [22], reported than the CEPC were higher in infants than in older children; they included children from a month to 3 years old. In our study, one advantage was that some children were from 0 months to 108 months, so this is the first observational study to report this CHD age range. At the first analysis, both groups had the same behavior, among these, differences were not found before and after of surgery. However, the age was the main doubtful variable. When the infants were analyzed separately, the most important differences were showed before and after surgery. According to Sun et al [22], this is the group where the value of endothelial cells would be representative and have more clinical significance, thus CEPC would play an important role in maintaining endothelial function and vascular repair; its higher count and percentage in infants would represent a prognostic factor in this age group [22].

We had the disadvantage that just two samples were collected from the patients (24 hours before surgery and 48hrs after of surgery). However, 24 hours before surgery has been considered a good parameter of reference of basal level of ET-1 and CEC and CEPC [22]. Schmid et al, [25], have also reported that most changes would be seen after 48 of the surgery. Finally, we intended to correlate the Aristotle Score [40] as a mortality risk, with the CPB and Aortic clamping times, and also the number of CEC and CEPC in patients who died, noting that these last biomarkers were higher in comparison with the median group of children with CHD. Thus, were correlated with the Aristotle Score of these patients, suggesting the presence of hypoxic damage secondary to CHD, leading these to be useful as an optimal biomarker for mortality in children with complex CHD, and furthermore, as aid in adult patients with coronary disease. In addition, our study's main weakness is thought to be the sample size, for it reduced to possibilities to find differences between groups. To confirm these data, prospective clinical trials need to be performed.

Limitations of the Study

The main limitation of the present pilot study was the number of patients analyzed, since its grouping process into age categories, shortened the groups even more. And although, in the methods (statistical analysis) we comment: The power of the sample size, especially for CPB and CPE, was higher than 80% when the comparisons were between at least 15 children, and the proportion between cases and controls was 1: 3; but if the ratio was 1: 1 with at least 5 cases the power was 60.9% and with 10 children the power was greater than 80% heterogeneity in the type of surgery. However, despite the difficulties given by the selection of the population and the performance of all these tests, it is important to highlight the heterogeneity of all the patients in the study with CPB in terms of the cardiac surgery performed. In general, the patients underwent a correction of septal ventricular defects, tetralogy of Fallot, transposition of the great arteries, and atrial septal defects. These different surgeries involve different surgical insults, different CPB times and, therefore, different activations of systemic inflammatory responses. Table 1 shows that the CPB time was reached an average of 107 minutes, but in the case of a broader range, from 37 to 168 minutes, different activations of the inflammatory system. If the sample size were larger, we consider that it would be important to analyze these data according to CPB time. Control group. The control group is different from the CPB group, in terms of age, and especially in terms of the different surgeries. However, due to the ethical implications in this pilot study, we were able to include only a group of older children in relation to the group of cases, with the idea stress that triggered the inflammatory response to surgical stimulation would be reflected. Therefore. Our results do not show considerable differences between the CPB and control groups or between the age groups, at least in this specific scenario after surgical procedures.

5. Conclusions

In this preliminary study, we examined that ET-1 did not increase after CPB surgery, although it remained high at baseline in CHD patients compared to the control group. There were no significant differences in CPCs between the CPB and control groups. In addition, there was no relationship with the levels of ET-1, CEC or CPC and the degree of endothelial damage determined by the BAFMD in patients with CHD. Larger studies can be performed to determine the differences observed between the biomarkers used in this study.

We also discerned that age, by itself, is probably associated with dysregulation of ET-1 and EPC levels, which may contribute to the development of ED in childhood after CPB. Although we found that there are some interesting findings, the general state of the present study is still preliminary and to verify these data, subsequent studies with a larger number of patients will have to be carried out.

Declarations

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Figures

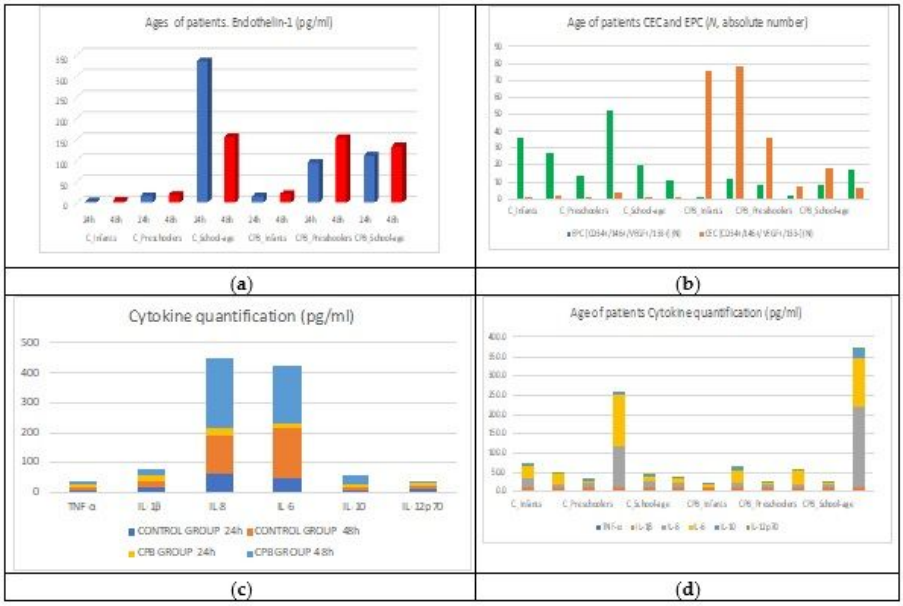

Figure 1

(a). Endothelin-1 levels in patients stratified by age; (b). Absolute numbers of CEPC in study groups; (c) and (d). Cytokine quantification in study groups.

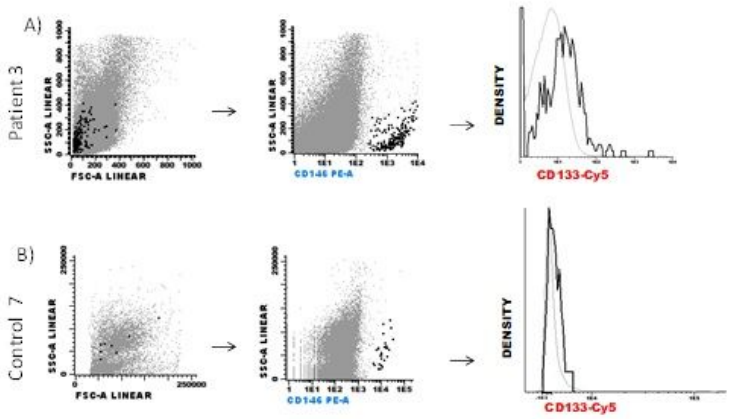

Figure 2

Flow cytometric dot panels for the identification of circulating endothelial cells. Panel (A): shows the analysis gate used to select cells according to size and granularity, the cells totally positive to CD146. From this CD146+ population the value of median fluorescence intensity (MFI) was determined, acco/rding to the relative expression of CD133. All cells were also positive to VEGF (data not shown). Panel (B): A greater proportion of circulating endothelial cells were observed in patients than in healthy subjects.

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