Cyclic GMP Release by Acute Enhanced External Counterpulsation

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Background: Enhanced external counterpulsation (EECP) is a noninvasive, pneumatic technique that provides favorable effects in patients with coronary artery disease and heart failure. The mechanisms by which EECP exerts its beneficial effects remain poorly understood. Cyclic GMP (cGMP) regulates vascular smooth muscle tone that may improve arterial function. We investigated the effect of a single session of EECP on plasma and platelet cGMP in asymptomatic subjects with cardiovascular risk factors (HCVR) and in patients with coronary artery disease (CAD).

Methods: Fifty-five subjects were included (25 HCVR and 30 CAD) and randomized into two groups to receive either sham (control) or active EECP during 1 h. Plasma and platelet cGMP were measured immediately before and after EECP by radioimmunoassay.

Results: One hour of EECP increased cGMP plasma concentration by 52% ± 66% (SD) ($P < .001$) and platelet content by 19% ± 28% ($P < .01$). The increase in plasma cGMP was particularly marked in CAD patients receiving active EECP ($P < .01$), mainly in those with low LDL-cholesterol. Platelets, inhibition of nitric oxide synthesis by N^G-monomethyl-L-arginine (L-NMMA) reduced cGMP by 23% ± 31% ($P < .001$), whereas presence of superoxide dismutase and inhibition of phosphodiesterase-5 increased cGMP by 46% ± 49% and 70% ± 77%, respectively ($P < .001$). In all of the cases EECP increased additional platelet cGMP content, which suggests nitric oxide synthase activation.

Conclusions: Acute external counterpulsation showed that very early treatment increases the cGMP production that may participate in the mechanism by which EECP exerts its clinical benefit. Analysis of the modulation of platelet cGMP content suggests that EECP activated the nitric oxide-dependent pathways. Am J Hypertens 2006; 19:867–872 © 2006 American Journal of Hypertension, Ltd.

Key Words: Enhanced external counterpulsation, coronary disease, risk factors, cyclic GMP, nitric oxide.

Enhanced external counterpulsation (EECP) is a noninvasive technique that improves anginal symptoms in the majority of patients with coronary artery disease (CAD).[1–3] The EECP involves sequential inflation at the onset of diastole and rapid deflation at the beginning of systole of compressive air cuffs wrapped around the lower extremities. As a result, diastolic augmentation increases coronary artery blood flow, the decrease in systolic pressure reduces cardiac afterload, and increased venous return raises cardiac output.[4,5]

Despite these clearly observed hemodynamic effects, the mechanisms by which EECP alleviates angina pectoris remain poorly understood. It has been suggested that EECP promotes coronary collateral development and recruitment,[6] enhances ventricular function, and exerts peripheral effects similar to those of physical exercise.[7–9] Furthermore, EECP may improve endothelial function by increasing arterial blood flow and vascular shear stress. Intra-aortic balloon pumping in a canine model has been reported to augment coronary artery diameter and blood flow, and inhibition of nitric oxide (NO) synthesis by N^G-monomethyl-L-arginine (L-NMMA) administration markedly attenuated these effects.[10] Recently it was observed that EECP induces a significant acute increase in tonometrically assessed finger reactive hyperemia[11] or in flow-mediated dilation of the brachial artery[12] in patients with symptomatic CAD, suggesting an improvement in endothelial function.

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Our study investigates, in subjects with and without CAD, the acute effect of EECP on cyclic GMP (cGMP), the second messenger of NO/natriuretic peptides (NPs), based on the knowledge that NO/NPs regulate vascular smooth muscle tone through the cGMP-dependent protein kinase signaling pathways.[13]

Methods

Subjects

Fifty-five subjects were included in the study, divided into two groups. One was comprised of 25 asymptomatic subjects with high cardiovascular risk (HCVR) presenting to our Center of Cardiovascular Prevention[14] who met the following criteria: 21 to 85 years of age, presence of either hypertension (>160 mm Hg systolic or 100 mm Hg diastolic), hypercholesterolemia (total cholesterol >2.80 g/L), diabetes, or multiple risk factors (Framingham score >20% at 10 years) or intermediate risk factors (Framingham >10% at 10 years), together with diffuse subclinical atherosclerosis constituted of echographic plaques at two or three extracoronary sites (carotid arteries, abdominal aorta, or femoral arteries).[14] These subjects were free of any treatment (for hypertension, hypercholesterolemia, or diabetes) and of any cardiovascular disease (stroke, coronary heart disease, or peripheral vascular disease). The other group was comprised of 30 patients with stable CAD demonstrated by angiographically proven stenoses more than 50% in at least one major coronary artery or a documented history of myocardial infarction. These patients were treated according to secondary prevention guidelines,[15] exclusive of nitrate derivatives that could interfere with NO pathway assessment. Patients were excluded if they had congestive heart failure, significant valvular heart disease, myocardial infarction or revascularization in the preceding 3 months, left ventricular ejection fraction lower than 35%, blood pressure (BP) more than 180/110 mm Hg, permanent pacemaker, atrial fibrillation or ventricular premature beats that would interfere with EECP triggering, peripheral vascular occlusive disease, phlebitis, deep vein thrombosis, hemorrhagic diathesis, severe renal failure, were pregnant, or were enrolled in another research program. The study was placebo controlled, consisting of assigning HRCV subjects and CAD patients to receive at random either sham or active counterpulsation. Local ethics committee approval was granted for this study and informed consent was obtained from all subjects after they were given a detailed description of the protocol.

Clinical Parameters

Body mass index (BMI) was calculated as the ratio of weight to height squared. Resting brachial BP was measured by an automated recorder (Omron HEM 705CP, Tokyo, Japan). Fasting blood lipids (after precipitation of LDL and very low density lipoprotein (VLDL) for HDL measurement) and fasting glucose were measured by enzymatic methods. Current smoking was defined as daily consumption of at least one cigarette for at least 3 months.

EECP and Study Design

The EECP equipment (Vasomedical, Westbury, NY) consisted of an air compressor, a control console, a treatment table, and an integrated set of air cuffs designed to be wrapped around the patient's lower extremities. The cuffs are sequentially inflated from the calf to the lower thigh to the upper thigh and buttock at the onset of diastole, followed by a rapid deflation at the beginning of systole. Pressures applied to the cuffs range from 0 to 300 mm Hg. Pressure applied to the cuffs in this study were 75 mm Hg in the sham control group ($n = 28$) and about 300 mm Hg in the active group ($n = 27$). All of the subjects underwent 1 h of either sham or active counterpulsation. In the latter group, the pressure applied to the cuffs was increased until a diastolic augmentation ratio more than 1.0, indicated by a peak diastolic pressure greater than peak systolic pressure. Blood pressure changes were monitored by finger plethysmography.

Circulating cGMP Concentration

Blood samples were collected before and after 1 h of EECP in citrate anticoagulated tubes and immediately centrifuged. Plasma was deproteinized by the addition of 2.5 volumes of absolute ethanol and boiling before centrifugation. After evaporation of the supernatant in a Speedvac concentrator, the sample was dissolved in 50 mmol/L Na-acetate buffer at pH 6.2. Cyclic GMP was acetylated and measured by radioimmunoassay (Perkin-Elmer, Boston, MA). Fifty percent displacement was obtained at 0.158 ± 0.006 pmol of cGMP. The reproducibility of measures defined by the coefficient of variation averaged 6.4% between repeated measures in the same blood sample and 10% between two repeated examinations in the same subject.[16]

Platelet cGMP Content

Platelets were isolated from platelet-rich plasma by centrifugation at 350 *g* for 15 min at 20°C. They were resuspended in a medium containing 145 mmol/L NaCl, 5 mmol/L KCl, 0.5 mmol/L $MgCl_2$, 5 mmol/L glucose, and 25 mmol/L HEPES pH 7.4 at 37°C, at a density of 2.5×10^7 platelets per milliliter. Platelets were allowed to equilibrate at 37°C for 5 min. One mmol/L $Ca(NO_3)_2$ was then added and cGMP content was determined in 200 μL of platelet suspension 2 min later. To investigate the role of NO in platelet cGMP, platelets were preincubated for 5 min at 37°C with 1 mmol/L L-NMMA (Alexis, Carlsbard, CA), a L-arginine analog known to reduce NO synthase activity, or for 1 min with 200 U/mL bovine copper–zinc superoxide dismutase (SOD) (Alexis) in the presence of 100 U/mL catalase (Calbiochem, Darmstadt, Germany) to suppress a possible influence of superoxide anion, the main in vivo NO inactivating compound formed. To eval-

Table 1. Clinical characteristics

Parameters	CAD	HCVR	EECP	
			Control	Active
Number	30	25	28	27
CAD/HCVR			15/13	15/12
Age (y)	62 ± 9	53 ± 7‡	58 ± 10	57 ± 9
Gender (M/F)	28/2	23/2	25/3	26/1
Current smokers (*n,* %)	1 (3)	7 (28)*	4 (14)	4 (15)
Body mass index (kg/m^2)	26.4 ± 3.1	26 ± 3.7	27.1 ± 3.6	25.3 ± 2.9
Systolic blood pressure (mm Hg)	135 ± 16	142 ± 17	142 ± 17	135 ± 17
Diastolic blood pressure (mm Hg)	77 ± 9	85 ± 12†	80 ± 11	81 ± 12
Total cholesterol (mmol/L)	4.16 ± 0.86	6.54 ± 1.07‡	5.29 ± 1.63	5.20 ± 1.45
LDL-cholesterol (mmol/L)	2.46 ± 0.67	4.56 ± 0.91‡	3.47 ± 1.35	3.36 ± 1.31
HDL-cholesterol (mmol/L)	1.16 ± 0.29	1.26 ± 0.35	1.20 ± 0.3	1.21 ± 0.34
Triglycerides (mmol/L)	1.43 ± 0.87	1.57 ± 1.04	1.58 ± 1.16	1.41 ± 0.66
Blood glucose (mmol/L)	6.23 ± 2.39	5.49 ± 0.84	6.13 ± 2.33	5.62 ± 1.18

CAD = coronary artery disease; EECP = enhanced external counterpulsation; HCVR = asymptomatic high risk patients.
Data are given as number as mean ± SD.
* , † , and ‡ $P < .05$, $P < .01$, and $P < .001$ when compared to CAD.

uate the degradation of cGMP by phosphodiesterase 5, the latter determination of platelet cGMP was also performed after incubation for 3 min in the presence of 100 μmol/L Zaprinast (Sigma-Aldrich, St. Louis, MO).

Measurement of Blood Nitrites

Blood nitrites were measured in a subset of nine patients (5 HCVR and 4 CAD). Samples were prepared according to Schulz et al.[17] Briefly, blood was sampled on the same volume of 100 mmol/L NaOH. The pH value was restored to 7.0 with HCl. The sample was centrifuged for 5 min at 10,000 *g*. The supernatant was transferred to washed ultrafiltration tubes (Ultrafree Millipore, Sigma Aldrich) with a cutoff of 10,000 Da and centrifuged for 30 min at 3000 *g* at 4°C. Nitrite concentration in the ultrafiltrate was determined by the Griess reaction.

Statistical Analysis

Results are expressed as mean ± SD unless otherwise specified. Between group differences were assessed by Student *t* test. Pre- and post-EECP values, as well as the effects of in vitro platelet treatments, were analyzed by the paired Student *t* test. Univariate regression analyses were performed using the least squares method. Multivariate regressions were performed by general linear model using JMP (SAS Institute, Cary, NC) software. Statistical significance was set at $P < .05$.

Results

Characteristics and Randomization of Patients

Table 1 gives the characteristics of the subjects. The HCVR subjects were younger ($P < .001$), had higher diastolic BP ($P < .01$), total and LDL-cholesterol ($P < .001$), and more smokers ($P < .05$) than the CAD patients. The distribution of HCVR-to-CAD subjects between control and active EECP was similar. No difference was

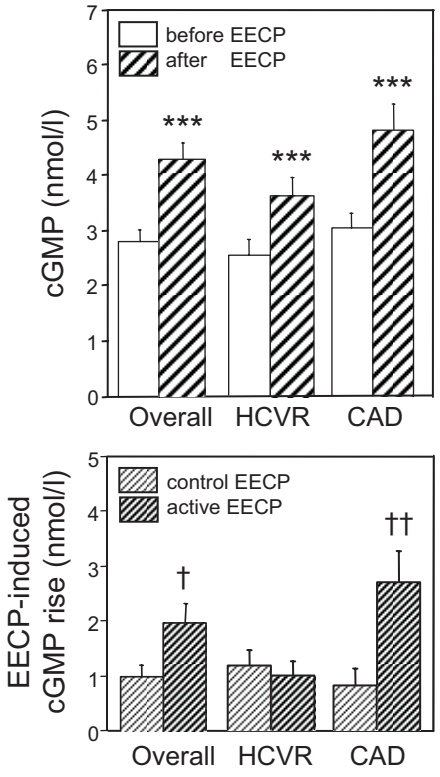

FIG. 1. (**Top**) Acute effects of enhanced external counterpulsation (EECP) on cyclic GMP (cGMP) in the overall population and in both, high cardiovascular risk (HCVR) and coronary artery disease (CAD) groups. (**Bottom**) Absolute changes in plasma cGMP in patients receiving sham (controls) compared to active counterpulsation. Data are mean ± SEM. ***$P < .001$ when compared to pre-EECP values; † and ††$P < .05$ and .01 when compared to controls.

observed between the clinical characteristics of the randomized groups (Table 1).

Plasma cGMP Levels at Baseline and EECP-Induced Changes

Baseline plasma cGMP levels did not differ either between patients with CAD and HCVR subjects (3.03 ± 1.51 *v* 2.54 ± 1.39 nmol/L, respectively, $P > .05$) or between control and active groups (2.72 ± 1.30 *v* 2.89 ± 1.64 nmol/L, respectively). Acute application of EECP for 1 h increased plasma cGMP concentration significantly in the overall population (+52% ± 66%; $P < .001$), as well as in HCVR (+43% ± 48%; $P < .001$) and CAD (+58% ± 70%; $P < .001$) groups individually (Fig. 1).

The increase in cGMP effectively doubled in subjects undergoing active counterpulsation compared with controls ($P < .05$). Active counterpulsation caused a significant difference in the EECP-induced increase in cGMP in the group of patients with CAD (+235% ± 248%; $P < .01$), but not in the group with HCVR (Fig. 1).

Basal values of cGMP were associated negatively with LDL-cholesterol ($P < .05$) in the overall population (Fig. 2). The values of cGMP reached after 1 h of counterpulsation showed significant negative association with LDL-cholesterol only in the group receiving active EECP ($P < .001$) (Fig. 2). The EECP-dependent increase in cGMP remained significant after adjustment for age, gender, smoking, BMI, LDL-cholesterol, systolic BP, blood glucose, and treatment in multivariate analysis of the overall population ($P < .05$) and the CAD group ($P < .05$), but not in the HCVR subjects.

Platelet cGMP Levels at Baseline and EECP-Induced Changes

Basal platelet cGMP content did not differ between patients with CAD and HCVR (0.36 ± 0.30 *v* 0.39 ± 0.20 pmol/10^8 platelets, respectively). Application of EECP for 1 h significantly increased platelet cGMP content in the overall population (+19% ± 28%; $P < .01$) (Fig. 3). To evaluate NO involvement in the EECP-induced cGMP increase, cGMP levels were determined in the presence of L-NMMA or SOD with and without inhibition of phosphodiesterase 5. As expected, before EECP, platelet cGMP content was reduced by L-NMMA (−23% ± 31%; $P < .001$), increased by SOD (+46% ± 49%; $P < .001$) and by inhibition of phosphodiesterase 5 (+70% ± 77%; $P < .001$) (Fig. 3). In all of the cases, EECP significantly increased additional platelet cGMP content, compared to their respective basal values (by 22% ± 36%; $P < .01$ for L-NMMA, by 21%; $P < .01$ for SOD-treated platelets, and by 63% ± 76%; $P < .001$ after inhibition of phosphodiesterase 5) (Fig. 3).

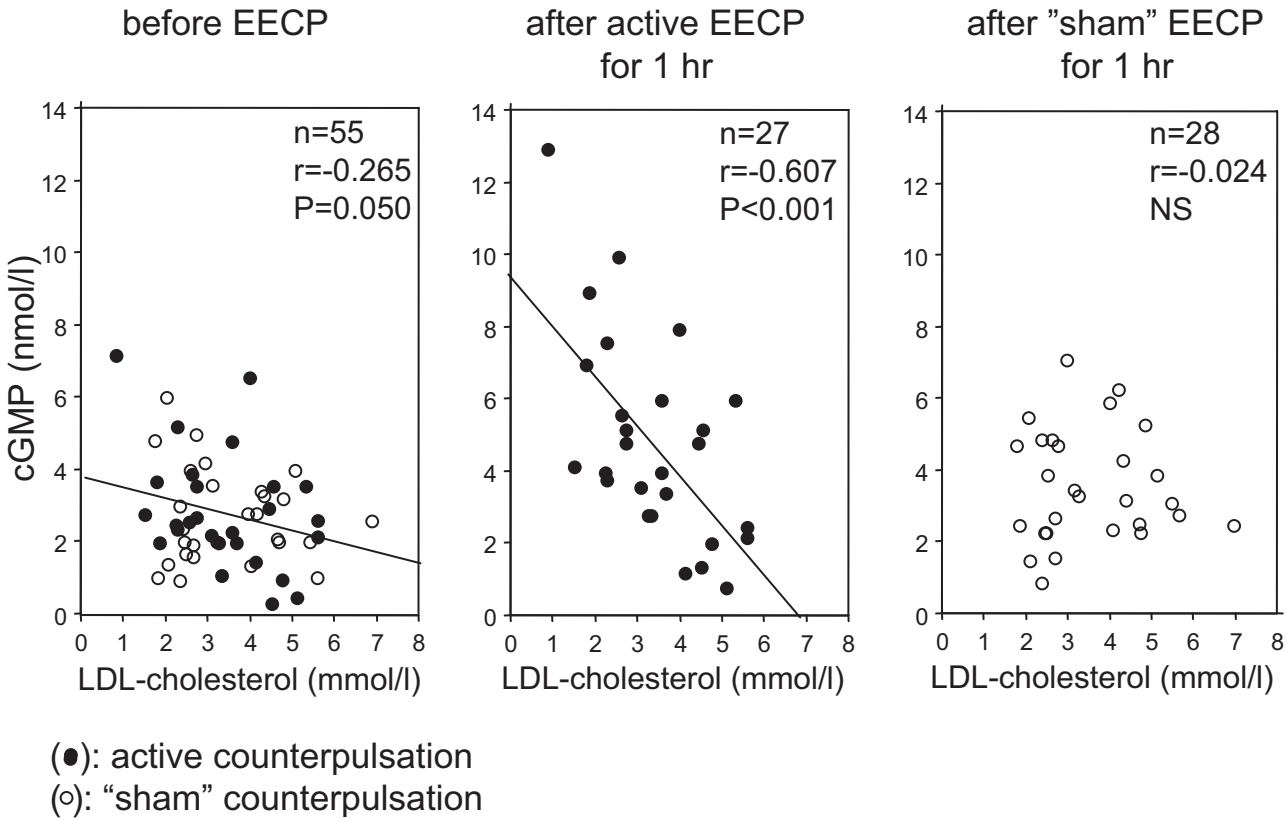

FIG. 2. Relationship of plasma cyclic GMP (cGMP) with LDL-cholesterol before enhanced external counterpulsation (EECP) and after active or sham counterpulsation.

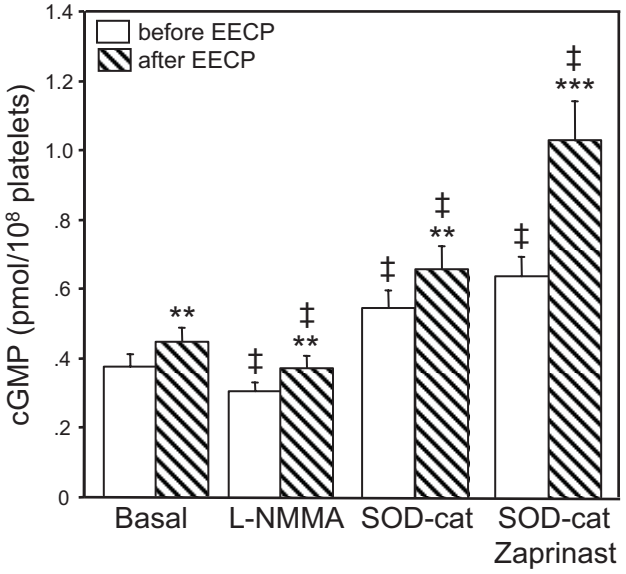

FIG. 3. Effect of enhanced external counterpulsation (EECP) on platelets cyclic GMP (cGMP) content before and after inhibition of nitric oxide synthase by N^G-monomethyl-L-arginine (L-NMMA), destruction of superoxide anion by superoxide dismutase (SOD) in the presence of catalase (cat), with and without inhibition of phosphodiesterase 5 by Zaprinast. Data are mean ± SEM. ** and ***$P < .01$ and .001 when compared to values before EECP; ‡$P < .001$ when compared to their respective basal values.

Blood Nitrites EECP-Induced Changes

At baseline the nitrite concentration in blood was on average 415 nmol/L in HCVR and 1103 nmol/L in CAD patients and it increased by 66% ± 21% after EECP ($P = .01$). A positive and significant relationship existed between blood nitrite concentrations and cGMP values before and after EECP ($r = 0.62$, $P < .01$).

Discussion

These results suggest that in very early EECP treatment, there is an increase in cGMP that may be significant for its cardiovascular effects. This possibility is supported by the observation that even the first hour of EECP treatment enhances peripheral endothelial function as evaluated by measuring reactive hyperemic response in the finger.[11] The fact that cGMP elevation was observed in the overall population (with and without CAD) seems to indicate that EECP acts directly on the response, independently of the subject's clinical status. A noteworthy strength of our study is that it was randomized comparing classic EECP therapy to a sham control group. Such a randomization was considered to compare an active to an inactive counterpulsation process in the controlled Multicenter Study of Enhanced External Counterpulsation (MUST-EECP).[2] In the present study we observed some differences in the overall population according to the clinical status when active EECP was applied. The counterpulsation-induced cGMP increase was twice as large in subjects receiving active EECP versus those serving as sham control. In addition, active CAD patients showed a greater counterpulsation-induced increase in cGMP than in active HCVR subjects. A possible explanation for this difference could be due to the higher frequency of uncorrected risk factors in untreated subjects with HCVR compared to treated CAD patients. Although a counterpulsation-induced cGMP increase remains significant in CAD patients after adjustment for all the measured risk factors as well as medication, the higher LDL-cholesterol values may have blunted this response in HCVR subjects. In accordance with this proposal, as shown in Fig. 2, a greater increase in cGMP was observed in subjects with lower values of LDL-cholesterol when submitted to active counterpulsation.

It is unlikely that the observed acute increase in cGMP after counterpulsation was generated by the family of natriuretic peptide, based on previous studies showing a significant reduction of atrial natriuretic peptide (ANP) and brain natriuretic peptide (BNP) plasma levels in response to EECP therapy.[3,18] On the other hand, the observation of small EECP-induced increases in cGMP at low applied cuff pressure may be the consequence of atrial wall stretch due to increased venous return. The randomized group receiving sham counterpulsation at low applied pressure (75 mm Hg) is largely above the venous occluding pressure inducing venous return. It is well known that an increase in right atrial filling pressure is associated with a rapid increase in ANP release from the heart.[19] This possibility deserves further investigation because our study is limited by the lack of determination of natriuretic factors.

One hour of EECP treatment also significantly increased cGMP content of platelets. These cells are devoid of natriuretic receptors coupled to particulate guanylate cyclase,[13] but express functional NO synthase. To evaluate NO involvement in the EECP-induced cGMP increase, we modulated NO bioavailability by reducing its synthesis or its degradation. To this end, cGMP levels were first determined in L-NMMA-treated isolated platelets, a L-arginine analog known to reduce NO synthase activity. The EECP application also significantly increased platelet cGMP content, which we demonstrated to be reduced by inhibition of NO synthase and increased by enhanced NO availability due to the reduction of its degradation by superoxide anion. The potentiation of platelet cGMP in the presence of SOD was similar to that induced by a preferential inhibitor of phosphodiesterase 5, the main cGMP degrading enzyme in platelets. The observation that the EECP-induced stimulation remains proportionally the same in the presence or absence of an L-arginine antagonist, SOD, or Zaprinast demonstrate that neither altered endogenous NO, nor superoxide-dependent reduction in NO availability or cGMP metabolism were involved in the cGMP increase. This suggests that NO synthesis could be modified by EECP. An additional argument in favor of EECP effects on the NO pathway is provided by the measurement of NO metabolites in a subset of patients

showing that blood nitrite concentration increased after the EECP session.

In addition to the well-known vascular smooth muscle relaxing effect of cGMP, the effect of EECP on platelet cGMP content may play a protective role against platelet activation. Finally it is possible that cGMP may have increased earlier than 1 h because its synthesis is a fast process, as shown in in vitro conditions. Unfortunately, our study design did not allow assessing the time dependence of cGMP change during EECP and further study should examine this interesting question.

In conclusion, the relevant finding of our study showed that very early external counterpulsation increase cGMP production, which may be important for its clinical effects in the mechanisms by which EECP alleviates angina pectoris.

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