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Biological Effects of a Software-controlled Voltage Pulse Generator (PhyBack PBK-2C) on the Release of Vascular Endothelial Growth Factor (VEGF)

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Abstract. *Background: Electrical stimulation (ES) may induce vascular permeability and physiological angiogenesis. ES of rat muscles significantly increases the microvessel density and vascular endothelial growth factor (VEGF) protein levels. Thus, a pilot study was designed to analyze the effects of low-voltage electric impulses on VEGF levels in patients with dystrophic ulcers. Materials and Methods: Circulating VEGF levels were analyzed in 9 patients undergoing an ES session with low voltage software-controlled impulses applied through topical transducers (1-9 μs width, 1-420-Hz frequency and 30-120 V strength-100 μA max). Results: The session was accompanied by a peak of circulating VEGF (3-10 min from start) in all 9 patients, which was preceded by a rise of TNF-α (2-min) and was independently associated with soluble E-selectin levels. Nitric oxide generation was significantly improved on the day after treatment. No hemostatic activation or sustained inflammatory reaction were observed. Conclusion: ES may represent a safe method for augmenting VEGF-mediated vascular protection, either directly or by induction of NO.*

The homeostasis of the vascular wall is complex and involves a growing number of humoral, neuronal and endothelial factors (1), among which vascular endothelial growth factor (VEGF) seems to play a pivotal role. VEGF was initially recognized as a factor that increased vascular permeability (2, 3), and it is now apparent that it can enhance several protective functions of essentially intact endothelia independently of significant mitogenic or angiogenic effects (4). VEGF, in fact, is capable of regulating multiple biological functions in endothelial cells, among which are the enhanced production of vasoactive mediators, increased expression of components of the thrombolytic and coagulation pathways, hypotension and vasorelaxation (5, 6).

Thus, the use of VEGF as a therapeutic cytokine in human diseases has attracted much interest. For instance, therapeutic angiogenesis is thought to be beneficial for arterial occlusive diseases (7), since local administration of recombinant VEGF salvaged ischaemic areas of the myocardium and hindlimb in animal models (8-10). However, concerns about possible pro-inflammatory adverse effects that might be associated with therapeutic attempts to deliver VEGF have latterly been highlighted (11), and a recent study in an animal model suggested that overexpressed VEGF in ischemic limbs might result in major complications (12).

The use of targeted low-level VEGF delivery to augment vascular protective effects locally in the absence of angiogenesis may be another way in which the therapeutic potential of VEGF can be harnessed more effectively and possibly more safely (13). In this respect, the use of electrical stimulation (ES) to locally up-regulate VEGF production is intriguing. It is well known that ES may induce important changes to vascular cells in the microcirculation, modulating the cAMP signal transduction

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mechanisms (14), the influx of extracellular Ca^{2+} (15), or the endothelial nitric oxide (NO) release (16). A number of reports have been published on investigations of the physiological and pharmacological behavior of different vessels by means of ES both in animal (17-19) and human models (15). Physiological angiogenesis occurs in electrically-stimulated skeletal muscles, probably due to increased shear stress and/or capillary wall tension (20, 21), which may be responsible for the proliferation of endothelial cells either directly, or by release of various humoral factors (22), mainly VEGF (23).

In this respect, the findings by Kanno *et al*. (24) are of particular interest. Using a rat model of hindlimb ischemia, these authors demonstrated that a 5-day ES (50 Hz, 0.1 V, which was far below the threshold for muscle contraction) of the tibialis anterior (TA) muscle was followed by a significant increase of VEGF synthesis and capillary density, without any muscle injury (24). Similar results were obtained in subsequent studies by Amaral *et al*., who showed that chronic ES of the TA and extensor digitorum longus (EDL) muscles of rats led to significant increases in vessel density and increased VEGF protein levels within 7 days (25, 26).

Based on these considerations, a pilot study was designed aimed at verifying whether a newly-developed medical device, that allows the application of software-controlled electric impulses, may result in the release of circulating VEGF in patients with peripheral vascular disease. NO production and the release of inflammatory cytokines and soluble selectins were also determined to assess the impact of low voltage pulses on the endothelial-induced pro-inflammatory, pro-thrombotic responses.

Materials and Methods

Patients' recruitment and sample collection. Nine patients (7 males, 2 females, mean age 67±12 years), affected by dystrophic ulcers due to peripheral arterial disease, were recruited on the basis of their willingness to participate in the study. Type 2 diabetes mellitus (fasting blood glucose level >110 mg/dL or treatment with a hypoglycemic agent), history of alcohol or drug abuse, body mass index >25, concomitant inflammatory diseases, administration of non-steroidal anti-inflammatory drugs (NSAID) in the 2 weeks preceding the study, disorders of coagulation and/or platelets, unwillingness to participate in the study for any reason or incompetence to give informed consent were considered as exclusion criteria. All patients refrained from smoking for at least 6 hours before treatment. A chart with the demographic, clinical and laboratory data of interest for the study was completed by an independent investigator for each recruited patient. The clinical and laboratory characteristics of the patients are summarized in Table I.

Table I. *Clinical and laboratory characteristics of the recruited patients.*

	Baseline values	Post-stimulation	*P*-value
Age (y)	67±12	NA	
Sex (male/female)	7/2	NA	
Leukocytes (10^3/μL)	6.4±1.5	6.5±1.6	0.91
Neutrophils (10^3/μL)	3.7±1.1	4.0±1.5	0.72
Lymphocytes (10^3/μL)	1.9±0.9	1.8±0.8	0.44
Platelets (10^3/μL)	192±37	171±59	0.28
Mean platelet volume (fL)	8.8±0.7	8.7±0.5	0.47
aPTT (sec)	37.4±12.4	41.4±18.7	0.14
INR	1.14±0.22	1.19±0.31	0.13
Anti-thrombin III (%)	95±11	96±14	0.63
D-dimer (μg/ml)	0.97±1.18	0.94±1.02	0.87
Fibrinogen (mg/dl)	409±45	398±45	0.21
Myeloperoxidase (ng/ml)	127±62	123±65	0.86
sL-selectin (ng/ml)	662±120	664±122	0.77
sP-selectin (ng/ml)	53.5±25.8	63.6±43.8	0.11

aPTT: activated partial thromboplastin time;
INR: international normalized ratio;
NA: not applicable.
Data are expressed as mean±standard deviation.

The patients underwent an ES session of 20 minutes, using a newly-developed biomedical device that allows the application of software-controlled electric impulses of variable tension, frequency and duration (PhyBack PBK-2C, Lawrence Medical Device). The PhyBack device consists of a PBK-2C software-controlled voltage pulse generator with two independent channels, which varies the "pulse patterns", according to the pathology being treated and patient response (Figure 1). The technical characteristics of the device are: a frequency ranging from 0.1 to 999 Hz, with a resolution of 0.1 Hz; a phase duration from 0.1 μs to 499 μs with a resolution of 0.1 μs; an amplitude of 1 to 215 V, with a resolution of 1 V; and a rise time lower than 25 ns. Safety devices built into the system ensure that no possible harmful voltages or currents can ever be delivered to the patient. The system complies with the European Medical Directive MD 93/42 as a Class IIa device. It also complies with IEC 947-1: 1988 and CE 0476 - N.MED 21001. For this pilot study, Phyback sessions were started at time 0 and low voltage impulses were applied through topical transducers with a 1-9 μs stimulus width, 1-420-Hz stimulus frequency and 30-120 V stimulus strength (100 μA max) over a 10-minute session in each recruited patient. The impulses were managed by specific software, which varied the pulse patterns according to the patient response.

Sample collection and laboratory measurement. Samples of peripheral venous blood were withdrawn from each consenting patient, according to the algorithm reported in Figure 2. Complete and differential blood cell counts and routine coagulation studies were performed at baseline and after treatment. The latter included prothrombin time (PT), activated partial thromboplastin time (PTT), antithrombin III activity, fibrinogen and D-dimer levels, all measured by an automated coagulometer (STA analyzer, Roche Diagnostics, Mannheim, Germany).

Citrated blood samples were processed within 30 minutes from withdrawal; the plasma was then aliquoted and immediately frozen at –40˚C. In order to standardize the serum collection, blood samples without additives were kept at 37˚C for 2 hours to allow maximal clot, then the samples were centrifuged, aliquoted and stored at –40˚C. All samples were kept frozen until assayed.

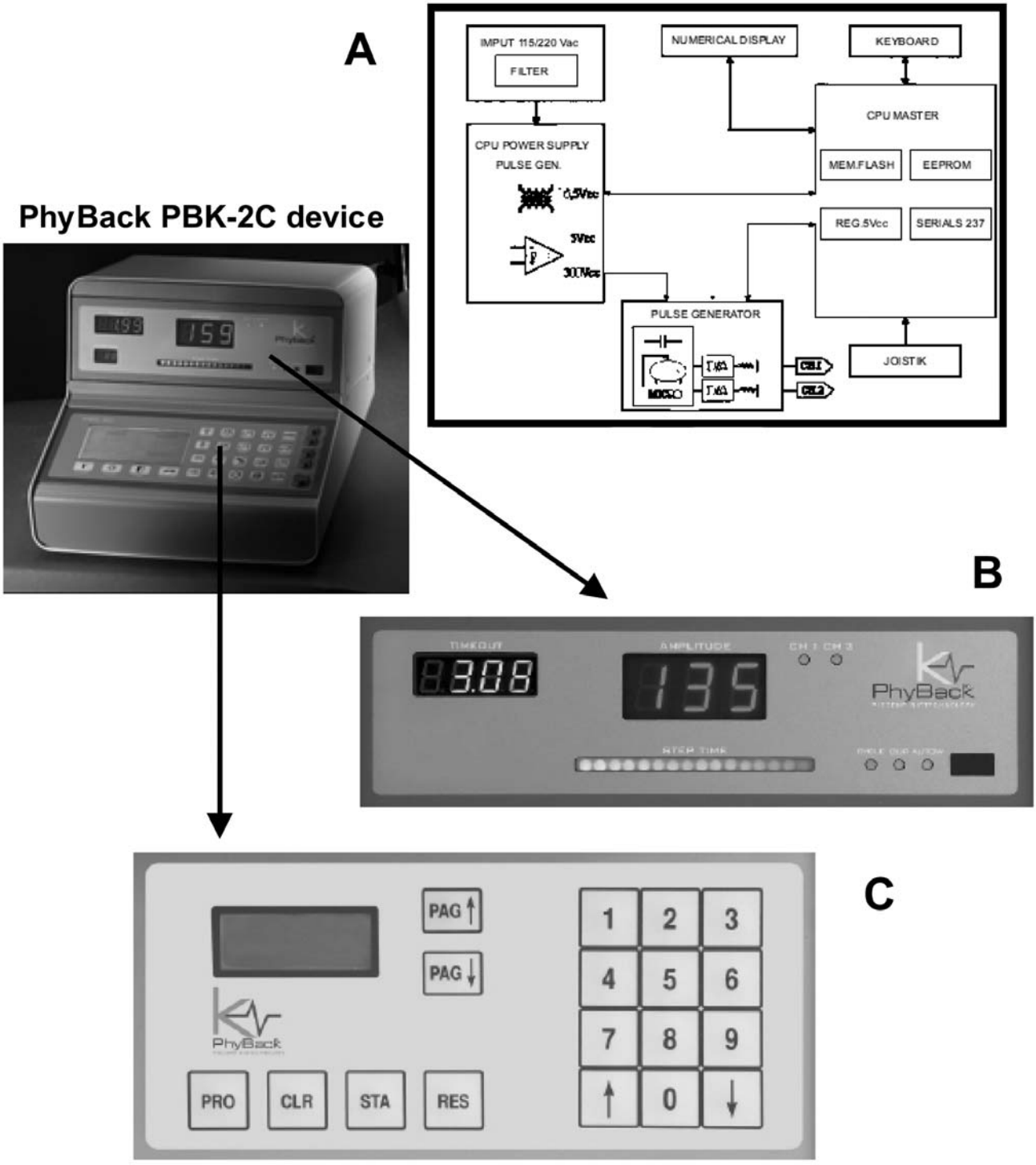

Figure 1. *PhyBack PBK-2C device. Panel A: Block diagram. Panel B: USER INTERFACE. This interface allows the patient the following controls: total duration of the session; variations in the activity of channels 1 and 2; adjustment of the pulse voltage; duration of the therapy phases with information on the active signal emission times for the physiological response. Panel C: OPERATOR INTERFACE. This interface not only allows precise control of PhyBack operation during therapy, but also the instant monitoring of patient reactions, selection of treatment and setting of personalized pulse sequences that can be stored in an archive.*

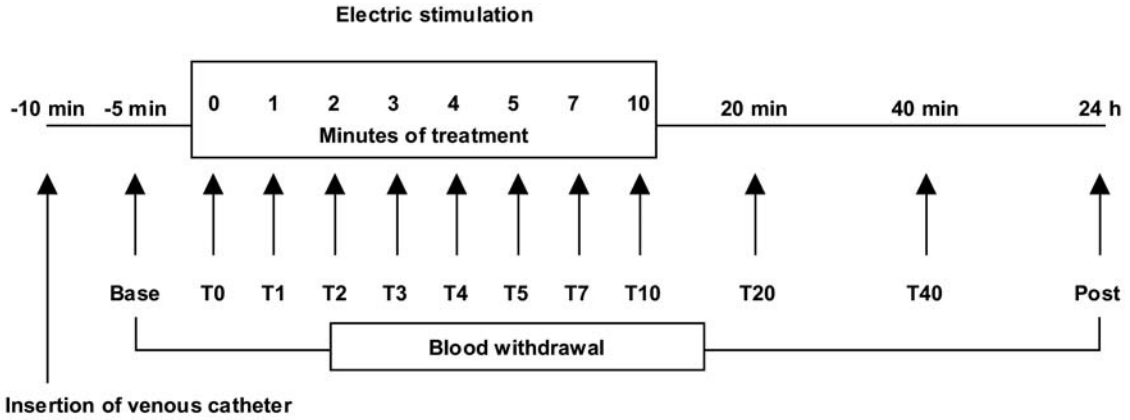

Figure 2. *Algorithm of blood sampling during a PhyBack session.*

Serum and plasma VEGF levels were measured by an enzyme-immunometric assay (R&D Systems, Minneapolis, MN, USA), according to the manufacturer's instructions. Plasma soluble (s) P-selectin (marker of *in vivo* platelet activation) and serum sE-selectin (marker of *in vivo* endothelial activation) and sL-selectin (marker of *in vivo* leukocyte activation) levels were measured by enzyme-immunometric assays (all by R&D Systems), according to the manufacturer's instructions. Serum IL-6, TNF-α and IL-1β levels (all by R&D Systems) were measured by enzyme-immunometric assays, according to the manufacturer's instructions.

NO generation was evaluated by measurements of serum nitrite (NO_2^-) and nitrate (NO_3^-) using a colorimetric, non-enzymatic assay based on the Griess reaction (R&D Systems).

Plasma myeloperoxidase (MPO) (a marker of neutrophil activation) levels were measured by an enzyme immunoassay (Bioxytech MPO-EIA, Oxis International Inc., Portland, OR, USA), according to the manufacturer's instructions.

Measurements were done blinded. All samples were assayed in duplicate and those showing values above the standard curve were re-tested with appropriate dilutions.

Statistical analysis. Statistical analysis was performed by the paired *t*-test and Pearson's correlation coefficient. When necessary, log transformation was used to normalize the data, or appropriate non-parametric tests were employed (Spearman's correlation coefficient and Wilcoxon signed rank test). Multivariate regression analysis was used to assess relationships among the variables. The data are presented as mean±standard deviations unless otherwise indicated. Only *p*-values lower than 0.05 were regarded as statistically significant. All calculations were made using a computer software package (Statistica, StatSoft Inc., Tulsa, OK, USA).

Results

Blood samples were obtained at different time-points before, during and after treatment, as outlined in Figure 2. Complete and differential blood cell counts, sP-selectin, fibrinogen and D-dimer levels, and clotting activity were determined at baseline and 24 hours post-treatment (Table I). As shown, the mean leukocyte counts of the recruited subjects, as well as their serum sL-selectin levels (a marker of leukocyte activation), did not significantly change in response to treatment. Furthermore, the plasma MPO levels (a reliable index of neutrophil activation) did not differ before and after treatment. Platelet counts, as well as the mean platelet volume, did not show any difference between baseline and the values obtained 24 hours after treatment. Also, no significant changes were observed in the sP-selectin levels (a marker of *in vivo* platelet activation) and coagulation indices, suggesting that treatment did not cause any activation of the hemostatic pathway. No adverse effects were observed in any of the recruited patients.

Serum and plasma VEGF levels were determined on all samples obtained before, during and after treatment. The mean levels obtained in all 9 patients at the different time-points are reported in Figure 3. As shown, treatment resulted in maximum VEGF concentration during the first 10 minutes, followed by a rapid decrease of VEGF levels toward the baseline value. Indeed, an immediate rise of the mean serum VEGF level was observed in samples obtained during treatment, reaching a peak at 7 minutes from its start (245±147 pg/ml *vs.*, 260±149 pg/ml, $p=0.17$) (Figure 3A). The concomitant determination of plasma VEGF levels confirmed the occurrence of VEGF release during treatment, with two separate peaks after 3 (from 15.8±6.8 pg/ml at baseline to 30.8±19.9 pg/ml, $p<0.05$) and 10 (25.9±9.8 pg/ml, $p<0.05$) minutes from the start (Figure 3B). These differences observed in response to treatment were of the same order of magnitude in both the plasma and serum samples. Furthermore, the change in serum VEGF correlated significantly with the change in plasma VEGF at the various time-points (Rho=0.48, $p<0.001$).

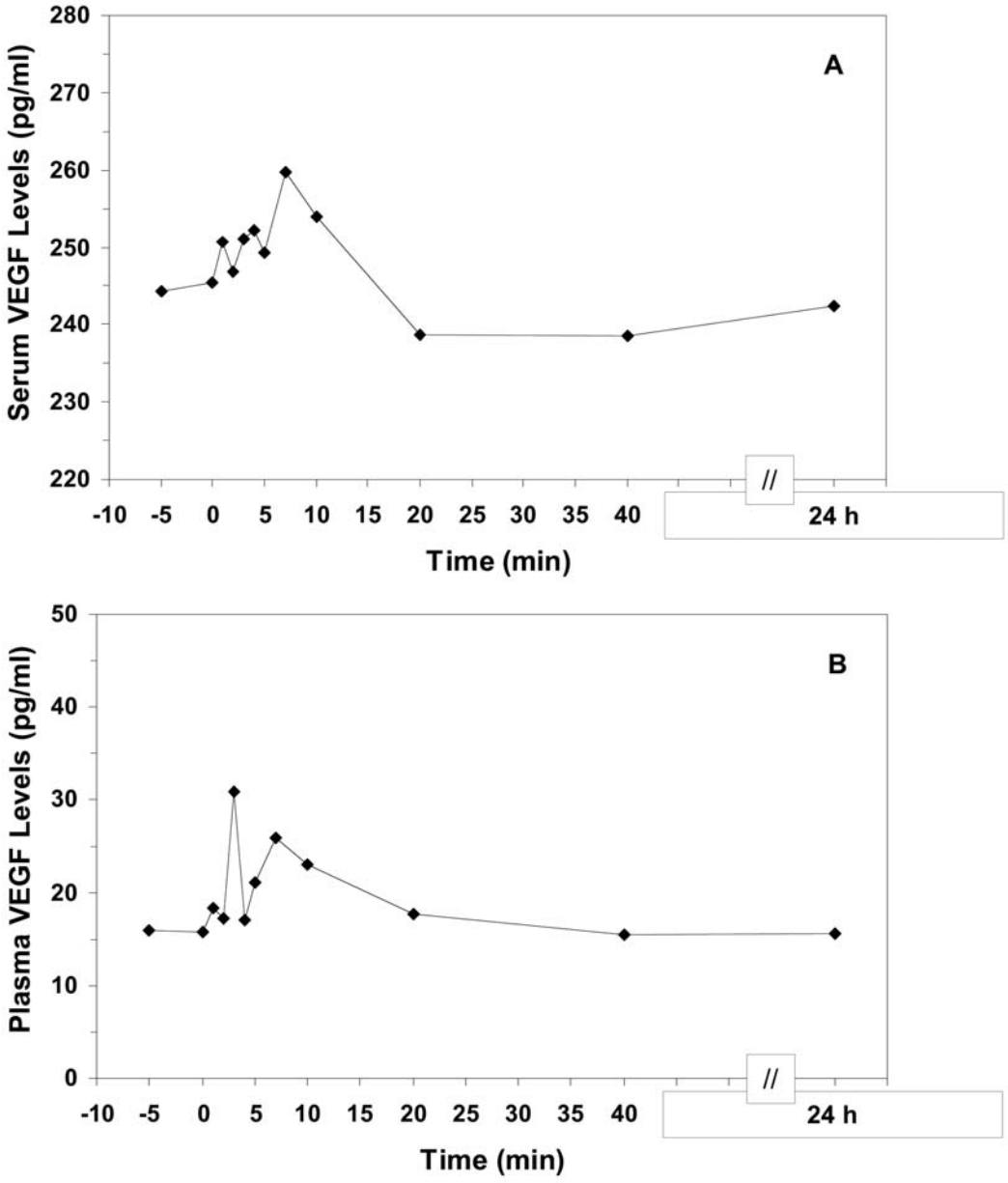

Figure 3. *Mean levels of serum (Panel A) and plasma (Panel B) VEGF levels determined on samples obtained from 9 patients with dystrophic ulcers before, during and after treatment.*

This rise in VEGF levels was accompanied by significant increases of both serum TNF-α (Figure 4A) and IL-1β (Figure 4B), whereas IL-6 did not show any substantial change during or after treatment (Figure 4C). In particular, there was an immediate rise of mean TNF-α levels at 2 minutes from the start (from 4.3±3.2 pg/ml to 6.8±3.5 pg/ml, $p<0.05$), followed by a second peak after another 2 minutes (up to 9.6±7.5 pg/ml, $p<0.05$) (Figure 4A), and a rapid decline thereafter. Changes of serum TNF-α levels in the systemic circulation were accompanied by a transient rise of the mean IL-1β levels, which peaked at 5 minutes from treatment start (from 0.5±0.5 pg/ml to 2.3±2.1 pg/ml, $p<0.01$)(Figure 4B).

Circulating levels of serum sE-selectin and NO (both markers of endothelial activation) were determined at the same time-points (Figures 5 and 6). Overall, a modest elevation of the mean sE-selectin levels (from 35±16 ng/ml to 39±16 ng/ml, $p<0.05$) after a 10-minute

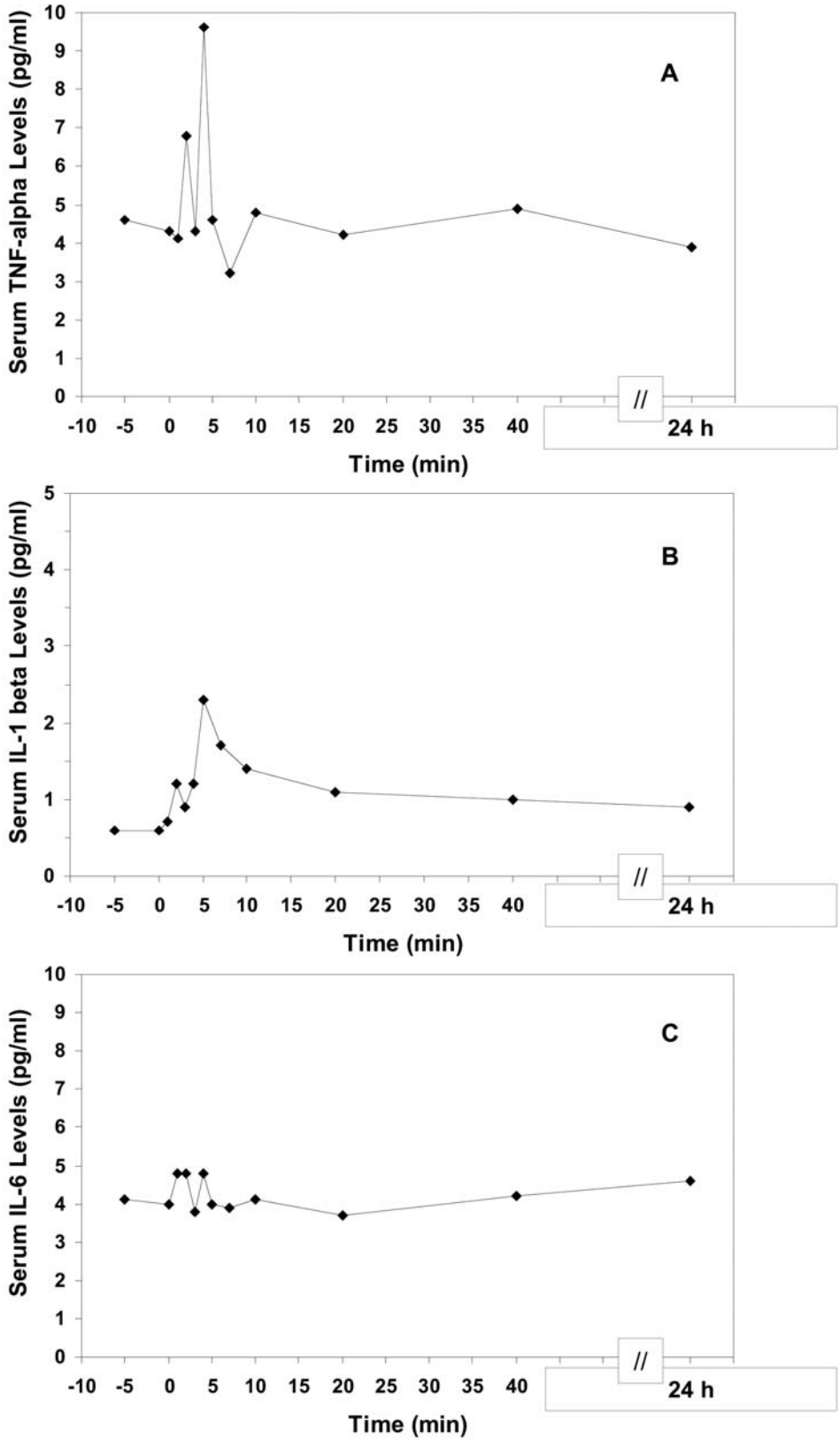

Figure 4. *Mean levels of TNF-α (Panel A), IL-1β (Panel B) and IL-6 (Panel C) levels determined on serum samples obtained from 9 patients with dystrophic ulcers before, during and after treatment.*

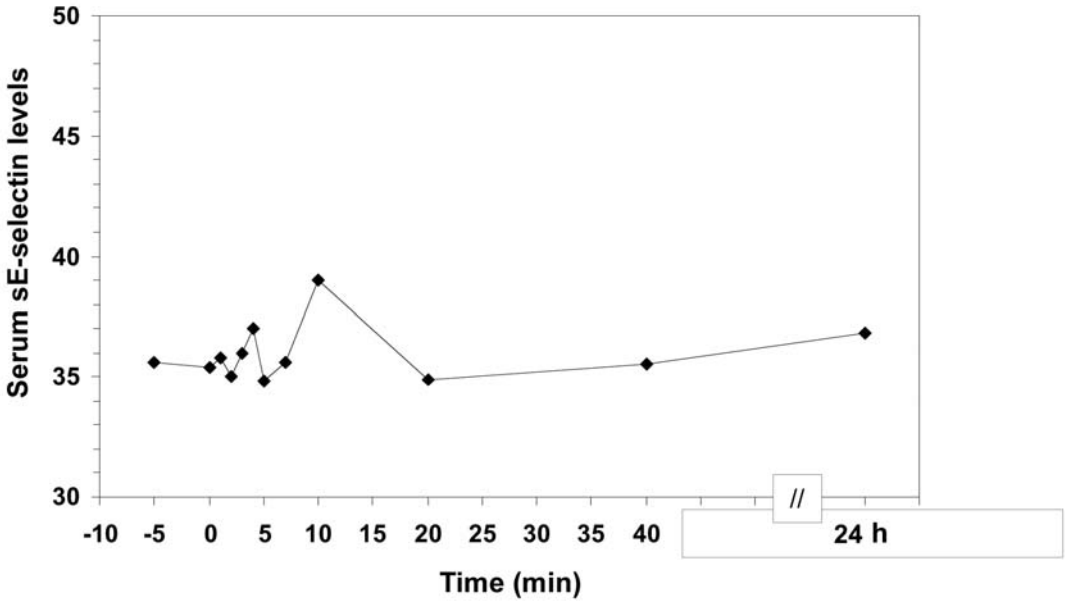

Figure 5. *Mean serum sE-selectin levels determined on serum samples obtained from 9 patients with dystrophic ulcers before, during and after treatment.*

treatment was observed (Figure 5). On the other hand, circulating NO levels significantly rose from 30±28 μmol/L to 39±28 μmol/L ($p<0.02$) only on the day after treatment (Figure 6).

A correlation analysis of the percent changes obtained for all the variables at the various time-points, followed by forward stepping multivariate analysis, showed that VEGF was significantly associated to sE-selectin (regression coefficient=0.59, $p<0.005$), whereas the latter was independently related to TNF-α levels (regression coefficient=0.32, $p<0.01$).

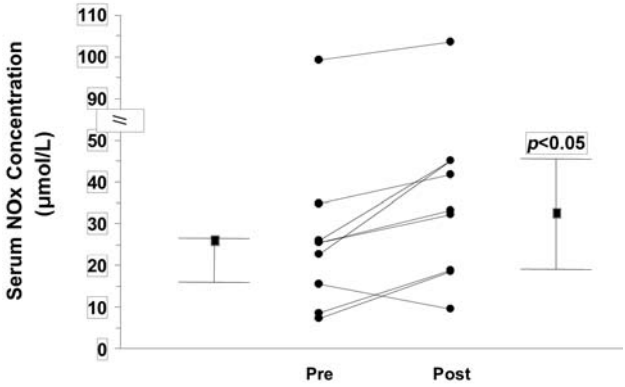

Figure 6. *Serum NO levels before and after a PhyBack session in each recruited patient. Closed squares represent means; whiskers represent standard deviations.*

Discussion

The evolving development of medical and surgical therapies has significantly improved the physician's ability to manage patients with peripheral atherosclerotic diseases, yet many continue to suffer debilitating symptoms from their disease and remain at risk of limb loss. This clinical imperative has led to the exploration of therapeutic angiogenesis strategies. The preliminary data have been quite promising and promote the general concept that VEGF therapy may be beneficial in vascular disease (27, 28). However, practical and potential obstacles exist when VEGF is delivered either as a protein or through gene-therapy approaches, which may, to a certain extent, temper the enthusiasm generated by these preliminary studies (11, 12, 29).

In 1999, Kanno *et al*. examined the effect of subcontractile ES on a rabbit ischemic hindlimb model and demonstrated that low-intensity ES is capable of increasing blood flow, capillary density and VEGF expression *in vivo* (24). Modern ES therapies are used to treat chronic pain syndromes and may facilitate the healing of fractures and soft-tissue wounds (30,31). Thus, it is tempting to speculate that the induction of VEGF may contribute to the salutary effects of ES in these settings.

In this study, the newly-developed PhyBack PBK-2C device was chosen to verify whether the application of software-controlled electronic impulses might be

responsible for an enhancement of circulating VEGF levels in patients with peripheral vascular disease. The PhyBack device, indeed, was specifically developed on the assumption that an effective and specific reaction of the human physiological control systems can only be generated by a condition of "need to act", requested by a simulation of a state of alert and resulting in an accelerated repair mechanism based on the activation of microcirculation in the affected areas. Accordingly, the results obtained in this pilot study of 9 patients with dystrophic ulcers clearly showed the occurrence of VEGF release with a timing closely related to the voltage pulse generation.

It is well known that VEGF is stored in the α-granules of platelets and is released on activation during clotting (32, 33). Thus, sampling methods profoundly affect the VEGF assay, with the highest levels found in serum obtained from blood clotted at room temperature due to platelet activation *ex vivo* (34). Accordingly, the use of serum samples should be avoided if the objective is to measure *in vivo* platelet release of VEGF. However, serum samples may still provide useful clinical information, *i.e.*, the total pool of VEGF and releasable VEGF stored within platelets (35).

Therefore, in the present study, VEGF levels were determined on citrated plasma samples processed immediately upon venipuncture to minimize the occurrence of *in vitro* platelet activation, and concomitantly on serum samples in which maximal clotting was allowed. Of interest, the differences observed in VEGF release were of the same order of magnitude both in the plasma and serum samples, suggesting that serum sampling under the applied conditions was, indeed, able to eliminate any interference due to platelet activation during clotting. However, the calculated mean difference in VEGF was low (approximately 15 pg/ml), probably due to hemodilution of the cytokine locally released in response to treatment. This makes the use of plasma samples a more reliable index under our conditions, since the large amount found in serum might be confounding for a correct evaluation of the changes observed.

Another issue that we sought to address was the possible origin of VEGF released during treatment. Circulating VEGF, in fact, represents a pooled concentration of antigen produced in different blood compartments (*i.e.*, endothelium, leukocytes and platelets). In the present study, the sL-selectin and MPO levels were analyzed as markers of leukocyte activation, the sE-selectin levels were determined as an index of endothelial activation, whereas the sP-selectin levels were used as a platelet activation marker. The latter was chosen because, unlike other products of platelet alpha-granules (*i.e.*, VEGF), the role of which is to be exported upon activation, P-selectin is an integral component of the organelle membrane, and the particular mechanisms of P-selectin expression and cleavage following activation make this molecule resistant to *ex vivo* activation (36). The results obtained indicated that treatment did not result in sustained activation of either leukocytes or platelets. On the other hand, a modest increase of sE-selectin levels was observed immediately at the end of treatment, suggesting that endothelial cells might become activated under our experimental conditions, which is also in agreement with recent reports on the electromagnetic field (37). Finally, the possibility that activated endothelial cells may represent the source of VEGF was substantiated by the independent association found between sE-selectin and VEGF levels in a model of multiple regression analysis including all the above-mentioned variables.

As stated in the introductory section, concerns have been raised about possible pro-inflammatory adverse effects that might be associated with therapeutic attempts to deliver VEGF. In the present study, an increase of TNF-α levels was observed concomitantly with pulse generation. This release was followed by a significant rise of serum IL-1β, but not IL-6 levels. Both IL-1β and TNF-α returned to baseline values at the end of treatment, indicating that the pulse generation was transient and unable to induce a sustained inflammatory reaction. Nonetheless, the TNF-α levels were significantly associated to the sE-selectin levels by multiple regression analysis, suggesting that this cytokine could be responsible for activation of endothelial cells and subsequent release of VEGF.

A final issue that needs to be addressed is the finding of increased levels of NO at the end of treatment. This observation is in agreement with previously published observations, and it is now well established that VEGF stimulates endothelial production of NO (38-40). It is, therefore, conceivable to speculate that VEGF, released upon electrical stimulation, might be responsible for an increased bioavailability of NO, but no final conclusion can be drawn at the present. Nonetheless, the ability of VEGF to induce endothelial NO production makes it a particularly attractive candidate as an endogenous vascular protective factor.

Although VEGF-based therapy holds promise for the treatment of ischemic vascular disease, we are still in the earliest stages of testing its effectiveness and of determining the best way to enhance vascular permeability, collateral formation and new capillary growth. It will not be surprising if molecular and non-molecular strategies become alternative or even complementary therapies in human vascular disease. Taken together, the results reported here suggest that low voltage impulses may be yet another method for augmenting VEGF-mediated vascular protection, either directly or by induction of NO production. Further studies to examine its biological effects in humans with ischemic vascular disease are warranted.

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