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Endothelial Activation Response to Oral Micronised Flavonoid Therapy in Patients with Chronic Venous Disease – a Prospective Study

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Background: *endothelial activation is important in the pathogenesis of skin changes due to chronic venous disease (CVD). Purified micronised flavonoid fraction has been used for symptomatic treatment of CVD for a considerable period of time. The exact mode of action of these compounds remains unknown.*
Aim: *to study the effects of micronised purified flavonoidic fraction (Daflon® 500 mg, Servier, France) treatment on plasma markers of endothelial activation.*
Materials and methods: *twenty patients with chronic venous disease were treated for 60 days with DAFLON® 500 mg twice daily. Duplex ultrasonography and PPG was used to assess the venous disease. Blood was collected from a foot vein immediately before starting treatment and within 1 week of stopping treatment. Plasma markers of endothelial activation were measured using commercial ELISA kits.*
Results: *reduction in the level of ICAM-1, 32% (141 ng/ml: 73 ng/ml) and VCAM 29% (1292 ng/ml: 717 ng/ml) was seen. Reduction in plasma lactoferrin (36% decrease, 760 ng/ml: 560 ng/ml) and VW factor occurred in the C4 group only.*
Conclusions: *micronised purified flavonoidic fraction treatment for 60 days seems to decrease the levels of some plasma markers of endothelial activation. This could ameliorate the dermatological effects of (CVD). This could also explain some of the pharmacological actions of these compounds. Our study demonstrates the feasibility of using soluble endothelial adhesion molecules as markers for treatment.*

Introduction

Chronic venous disease (CVD) and its sequelae are a major cause of morbidity in the Western world.[1,2] The inflammatory response seen in CVD involves leucocyte and endothelial adherence, cell activation and cell migration. This involves adhesion molecule expression by endothelial cells, leucocyte activation and local cytokine activity. Currently there are no studies regarding the changes in these endothelial markers following therapy for CVD.

Endothelial cell activation by cytokines or thrombin leads to increased adhesion molecule expression. These include ICAM-1 (binds neutrophil/lymphocytes) and VCAM-1 (binds lymphocytes/monocytes). This can lead to MHC-II (T-cell response) and GMP-140 (binds platelets) expression. Binding of platelets increases the availability of platelet-activating factor and further accelerates the expression of adhesion molecules. The circulating levels of many of these molecules are known to be increased in patients with CVD.[3]

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Pharmacological treatment is widely used to ameliorate the symptoms of venous disease in many European countries. It is hardly used in other places. One reason for these discrepancies is the lack of controlled scientific trials establishing the role of these compounds. At least one recent study shows increased ulcer healing rates in response to flavonoid treatment.[4] Unfortunately, no "phlebotonic" drugs exist that claim to correct venous valvular incompetence. However, many of these do affect the inflammatory changes responsible for the skin damage.

The response of the increased endothelial activation to any form of therapy remains unknown. The medical compounds that have been used for treating venous disease include flavonoids (diosmin/hydoxyethylrutosides), methyl-xanthines, prostaglandin and fibrinolytics amongst others. A purified micronised flavonoid fraction (Daflon®500, Servier Laboratories, France) has been used in Europe for the treatment of CVD for some time. Daflon consists of 90% Diosmin (3′, 5,7 trihydroxy-4′-methoxyflavone 7 rhamnoglucoside; $C_{28}H_{32}O_{15}$) and 10% hesperidin flavonoids (3′,5,7 trihydroxy-4′-methoxylflavone 7 rhamnoglucoside, $C_{28}H_{34}O_{15}$). The use of pharmacological treatment is

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Table 1. Distribution of the patients according to their CEAP stage and anatomical localisation of CVD. There are equal numbers of patients with and without skin changes ($n = 10$). Two patients had reflux localised to "minor" tributaries that did not relate to either of the two major superficial systems. Perforator incompetence is not listed separately because none of the patients had isolated perforator incompetence.

	SVI	DVI	SVI/DVI	LSV	SSV	LSV/SSV	Other
C2	3	—	—	2	1	1	1
C3	4	—	3	2	—	2	1
C4	7	1	2	5	—	4	—
Median age 58 (range 39–82)	14 (2m:12f)	1 (m)	5 (3m:f)	9 (3m:f)	1 (f)	7 (3m:4f)	2 (f)

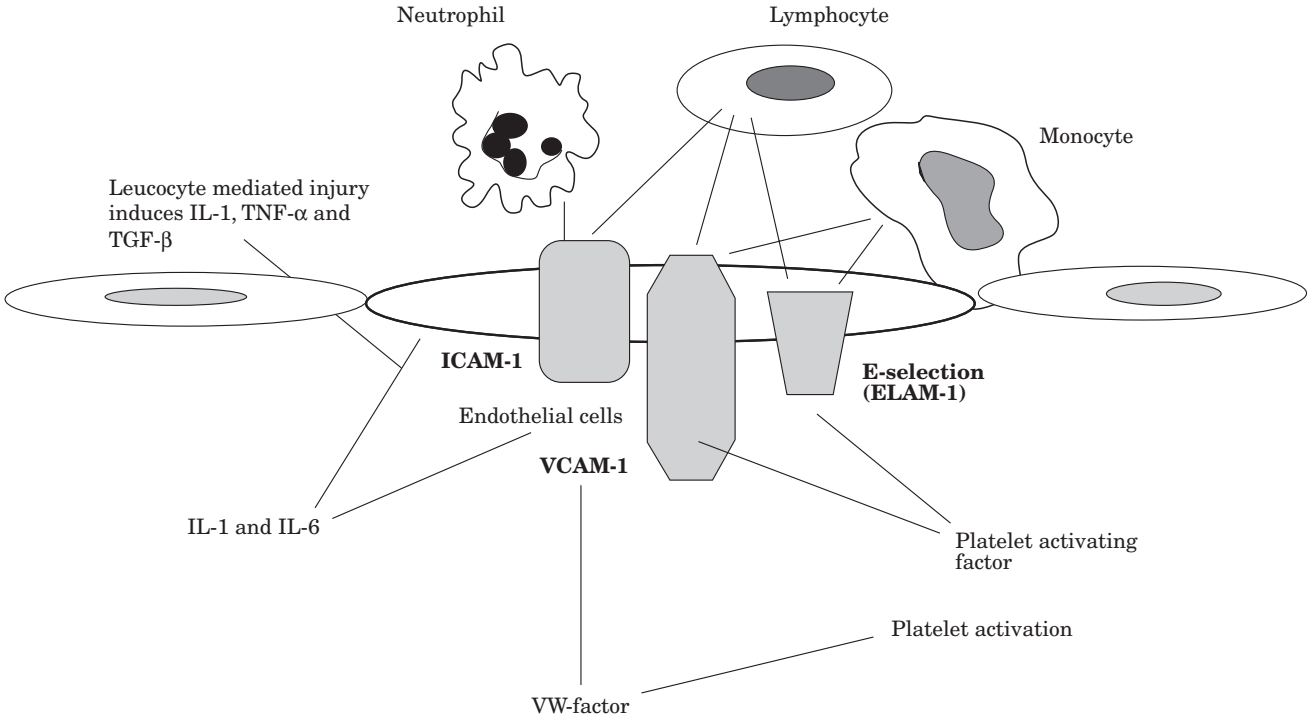

Fig. 1. Chronic venous disease leads to a microangiopathy. This involves adhesion molecule expression by endothelial cells, leucocyte activation and local cytokine activity. Endothelial cell activation by cytokines or thrombin leads to increased adhesion molecule expression. This includes ICAM-1 (binds neutrophils/lymphocytes), VCAM-1 (binds lymphocytes/monocytes) and E-selectin (ELAM-1). MHC-II (T-cell response) and GMP-140 (binds platelets) expression can occur. Binding of platelets increases the availability of platelet-activating factor and further accelerates the expression of adhesion molecules. The circulating levels of many of these molecules are known to be increased in patients with CVD.

variable, mainly because of lack of good controlled trials. There is evidence of increased healing rate of venous ulcers with Daflon.[5]

Daflon® comprises 450 mg of diosmin and 50 mg of hesperidin per tablet. The "micronisation" of the effective components increases its bio-availability. It has been reported to be a "venotonic" (increases venous tone!) and to decrease capillary "leakage" in many scenarios. A double-blind randomised trial published in 1994 had shown its efficacy in treating the clinical symptoms of CVD without any significant side-effects.[6]

The aim of our study was to study the effects of micronised purified flavonoid fraction (S5682, Daflon® 500 mg, Servier, France) treatment on expression of soluble markers of endothelial activation in CVD. In addition plasma lactoferrin levels were studied as a marker of neutrophil degranulation.[7]

Materials and Methods

Evaluation

Ethics committees' consent for the study was obtained from UCL Medical School Committee for medical ethics. Patients with chronic venous disease, clinical stage 2–4, were recruited from the vascular clinic at

Table 2. Median values of plasma levels of endothelial activation markers and plasma lactoferrin in patients with CVD. Data obtained for patients with simple CVD (C2–3) and those with dermatological changes (C4) is shown. The values are before and after a 60-d treatment with oral purified micronised fraction (parenthesis = IQR).

		ICAM-1 (μg/ml)	VCAM (ng/ml)	VW Factor % activity	SE-Selectin (ng/ml)	Sp-Selectin (ng/ml)	Lactoferrin (μg/ml)
All patients	Before treatment	141 (102–162)	1292 (950–1500)	65 (45–105)	49 (31–61)	74 (57–97)	690 (373–760)
	After treatment	73 (65–93)	717 (625–1156)	65 (50–105)	36 (28–59)	90 (55–120)	494 (350–715)
	Significance (Wilcoxon)	<0.001	<0.001	0.88	0.156	0.09	0.231
C2–C3	Before treatment	109 (91–136)	1300 (1140–1500)	50 (40–77)	41 (33–61)	78 (58–97)	420 (32–660)
	After treatment	68 (62–78)	800 (625–11250)	78 (51–109)	36 (30–47)	80 (53–1101)	420 (350–520)
	Significance (Wilcoxon)	0.001	0.001	0.012	0.1	0.74	0.59
C4	Before treatment	153 (120–186)	1075 (913–1513)	65 (51–105)	50 (27–63)	77 (58–96)	760 (635–1000)
	After treatment	78 (72–106)	880 (675–1231)	58 (50–75)	31 (25–60)	100 (72–125)	560 (380–800)
	Significance (Wilcoxon)	0.001	0.003	0.039	0.61	0.54	0.027

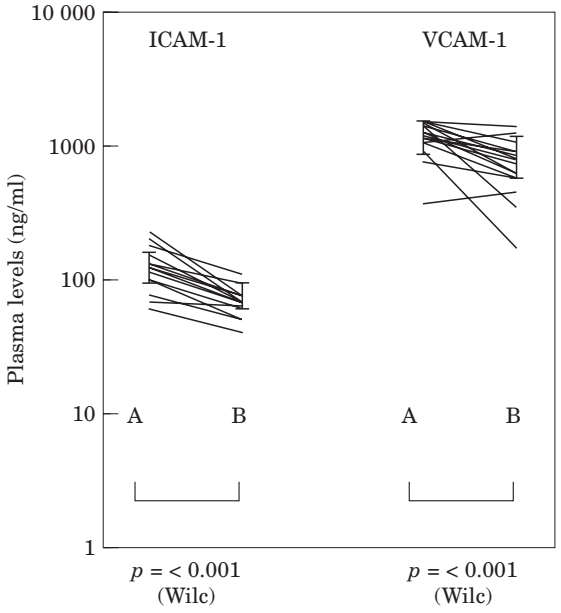

Fig. 2. Changes in plasma levels of ICAM-1 and VCAM-1 in patients with CVD before (A) and after (B) 60-d treatment with micronised purified flavonoid fraction. A logarithmic scale has been used because of the differences in the absolute levels of the two molecules. *p* levels are Wilcoxon ranked-sum test. The thick lines join the respective median levels. Vertical lines represent interquartile range.

the Middlesex Hospital. Patients who consented were asked to attend the department on three separate occasions at 2 weeks before treatment, the day of starting treatment and after finishing treatment. A medical history was taken and clinical examination performed. This included a systemic as well as venous examination. Patients had a full blood count, serum urea and electrolyte/liver function tests. All patients were assessed clinically according to the clinical classification of the Hawaii system. People with (duplex proven) varicose veins only (C2), with associated oedema (C3), skin changes (C4) and/or healed ulceration (C5) of >4 weeks were included. Active ulceration (C6) would have interfered with activation of leukocytes and these were thus not included.

All patients had a venous duplex examination and photoplethysmography within the previous 8 weeks to establish venous disease and to localise it anatomically. The minimum extent of duplex examination in our patients included saphenofemoral junction, long saphenous vein, saphenopopliteal junction, short saphenous vein, anatomical perforators. Femoropopliteal veins and other reflux sites as picked up by colour Doppler. Patients were assessed for other possible diagnoses, including arterial disease. Ankle–brachial pressure ratios were performed whenever there was clinical suspicion. All patients wore class II support stockings and continued to do so during the study.

Patient selection

The inclusion criteria for the study were: (1) age above 18 years; (2) patients affected with Hawaii stage C2 to C4; (3) C6 with a healed ulcer for at least 4 weeks,[8] (4) psychological stability and motivation.

The exclusion criteria were directed at: (i) patients who

Table 3. Comparison of mean visual analogue symptom scores in all patients before and after 60-d treatment with "purified micronised flavonoid fraction". Although decrease was seen in all the parameters, only the decrease in "heaviness" reached statistical significance. (Values in parentheses are standard deviation).

	Pain	Heaviness	Cramps	Paraesthesiae	Oedema
Before treatment	8.2 (3.3)	7.7 (2.6)	8.1 (2.8)	5 (3.3)	(6.8 (3.2)
After treatment	8.2 (3.3)	6.4 (2)	6.8 (3.4)	4.8 (2.8)	5.8 (2.4)
Probability (Wilcoxon)	0.094	0.004	0.07	0.19	0.057

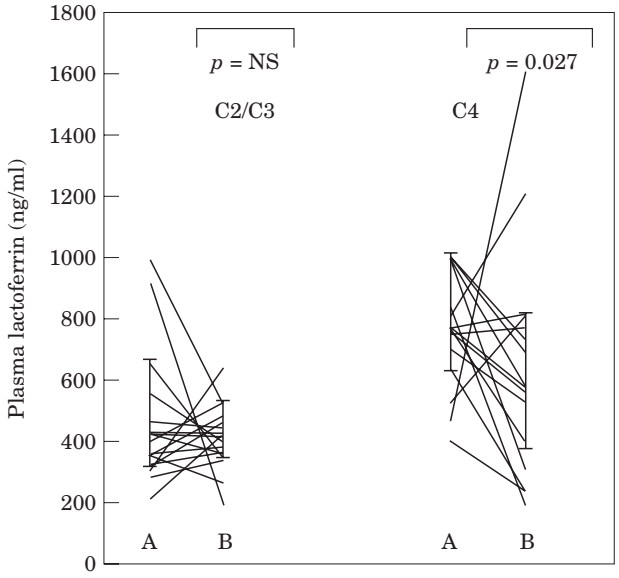

Fig. 3. Shows the differing changes in plasma lactoferrin in patients with (C4) and without (C2–3) skin changes before (A) and after (B) 60-d treatment with micronised purified flavonoid fraction. Probability levels are Wilcoxon ranked-sum test. The much increased plasma lactoferrin activity is lowered significantly in patients with skin changes.

have conditions that would change the status of leukocyte/endothelial activation; (ii) subjects who were taking compounds similar to flavonoids to avoid bias and (iii) patients who had other major systemic illnesses. These criteria were: (1) history of alcohol or drug abuse; (2) known history of allergy or intolerance to diosmin or any other venotonic agent; (3) active venous ulceration; (4) diabetes mellitus; (5) impaired hepatic function (ALT or AST 3-fold above the normal limit) or impaired renal function (serum creatinine >120 μmol/l); (6) any concomitant active disease or abnormality in laboratory test (judged as clinically significant by the investigator); (7) patients treated with other vasoactive drugs within the 15 days prior to inclusion; (8) patients with an acute/chronic inflammatory or infectious disease; (9) deep venous thrombosis within the past 12 months; (10) superficial venous thrombosis within 3 weeks; (11) patients using steroids, non-specific anti-inflammatory drugs (NSAIDs), other "vaso-active" drugs, Vitamins A, C and E or anticoagulants; (12) previous poor compliance to treatment; (13) participants of a trial within past 3 months were excluded; (14) pregnancy, breastfeeding or lack of active contraception also excluded the patient.

Daflon® 500 mg twice daily (Servier, France) was administered for 60 days. Evaluation of venous symptoms employing a visual analogue scale was performed before and after treatment. Specimens (30 ml each) were taken from foot veins before and after standing the patient supported for 30 min.

Collection of specimens

A 18G cannula (Vasculon 2, Viggo-Spectramed, Helsinborg, Sweden) was placed in the distal long saphenous vein or dorsal foot vein of one leg. The cannula was flushed with heparinised saline solution. The patient then stood supported against the side of the couch for 30 min without moving the calf muscles (it was previously shown by the authors by direct pressure measurement that this raises the venous pressure in the superficial veins of the leg to between 70 and 80 mmHg), after which 2 ml of blood was taken and discarded from the cannula, and a further 10 ml was collected into two tubes containing ethylenediamine <TK; 4 tetra-acetic acid and one tube containing citrate <TK; 1 (Vacutainer Becton Dickinson Vacutainer Systems Europe, BP No 37-38341 Meylan Cedex, France). Blood samples were carefully placed directly into the sample tubes after removing the stoppers to prevent excessive cell agitation.

ELISA tests for soluble markers

Blood for ELISA tests for soluble plasma markers was collected in EDTA bottles. The specimens were spun at 20,000 r.p.m. for 10 min to separate the plasma and promptly frozen at −20 °C before analysis. The

Markers measured were E-selectin, P-selectin, VCAM, ICAM-1, VW factor and lactoferrin. Commercial kits were used for these tests (ICAM-1/VCAM/E-selectin by R&D Systems, 4–10 The Quadrant, Barton Lane, Abingdon, Oxon OX14 3YS, U.K.; VW factor kit by Diagnostica Stago, 9 rue des Freres Chausson, 92600 Asnierès-sur-Seine, France; lactoferrin assay by OXIS International, Portland, OR, U.S.A.) were used for these analyses.

Demographics

There were equal numbers of patients with and without skin changes ($n=10$). Seven patients in each group had superficial venous insufficiency (SVI). Of the remaining six patients the majority (5) had combined SVI and deep venous insufficiency (DVI). Most patients either had long saphenous (LSV) involvement ($n=9$) or combined LSV and short saphenous (SSV) involvement ($n=7$). Two patients had CVD involving neither of the two major superficial anatomic systems. These patients had other superficial veins (e.g. lateral superficial vein of the thigh) involvement without reflux in the mentioned major superficial systems.

Results

Significant reduction was seen in plasma levels of VCAM and ICAM-1 activity following therapy. Reduction in the level of ICAM-1 32% (141 ng/ml: 73 ng/ml) and VCAM, 29% (1292 ng/ml: 717 ng/ml) were seen. Reduction in plasma lactoferrin (36% decrease, 760 ng/ml: 560 ng/ml) occurred in the skin-changes group. The levels of VW factor also decreased in patients with skin changes but not in simple venous disease.

Changes in E-selectin levels (decrease) and P-selectin levels (some increase following therapy) were not significant statistically. The levels of reduction in endothelial markers following flavonoid therapy was seen to be similar in each of the different supine–standing–supine positions.

Discussion

We observed a decrease in some soluble endothelial activation markers in patients with CVD following 60 d therapy with oral purified flavonoid fraction. In particular VCAM-1 and ICAM-1, adhesion molecules important in endothelial interaction with neutrophil, monocytes and lymphocytes were decreased significantly. This effect was seen in all patients, irrespective of their clinical stage. Lactoferrin levels were appreciably reduced following therapy in patients with skin changes only. This probably represents damping of the much-increased granulocyte activity in these patients compared to those with simple CVD. The effect on E-selectin levels was also seen more in the C4 group. Unknown effects on platelet activity might explain the slight rise seen in P-selectin following therapy. Similar effects may explain the differing phenomena seen with VW factor following therapy (rise in VW factor in C3 patients and fall in C4 group).

We did not make any distinction between patients with superficial venous insufficiency and those with deep venous insufficiency in interpreting the results. Previous studies from this department did not report on any difference between these two groups[9] for a similar clinical stage. We compared equivalent clinical stages (C) which are representative of the severity of CVD. Some of these markers, especially VW factors, are not very sensitive indicators of endothelial activation. Some, like P-selectin and VW factor, are almost certainly affected by other local phenomena like platelet activation. The physiological significance of these soluble markers remains an issue of debate. They may represent protease cleavage from cell surface subsequent to endothelial stimulation. They may also represent molecules released to block receptors and to prevent their attachment to the endothelium.

It is only relatively recently that the effectiveness of therapy for CVD is being tested with objective parameters. It has been shown that colour-duplex ultrasonographic and air-plethysmographic criteria improve in patients 1 month following surgical therapy. This improvement was shown to be maintained at 2 years.[10] It would be interesting to measure the mid to long-term effect of therapy on endothelial markers. Another study of the effect of compression therapy/scleropathy on the microangiopathy (Leu *et al.*)[11] showed non-significant changes post-therapy. The parameters studied were capillary microscopy, Doppler fluxmetry and $tcPo_2$ measurement. Elevated levels of VCAM-1, VW factor, E-selectin, IAM-1 and lactoferrin have been reported in patients with CVD compared to controls.[12] However, our study is the first to demonstrate a measurable change in these parameters in CVD following therapy. This shows the feasibility of using the soluble markers of endothelial activation as a parameter to measure the response to various forms of treatment.

Experimental blockade of adhesion molecules either with soluble ligands or with specific monoclonal antibodies has a dramatic effect on inflammation and

immune mediated tissue damage.[13,14] The observation that some markers are specifically lowered following therapy in patients with skin changes demonstrates the possibility of flavonoids to ameliorate and/or arrest some of the skin damage that occurs in CVD. Inflammatory mechanisms have been implicated in the valvular damage seen in CVD,[15] and these compounds may have a role in ameliorating this damage as well. These aspects merit further study.

Further studies are needed to assess the role of soluble endothelial markers in prognosticating the clinical course of CVD. The role of purified micronised flavonoid fraction and similar compounds also needs to be re-examined in the context of non-surgical treatment for CVD.

Experimentally, flavonoids have been shown to have several anti-inflammatory actions. Our study is the first to demonstrate a mode of action for these compounds in the clinical setting. In the hamster cheek pouch, Daflon® significantly inhibited the macromolecular permeability-increasing effect of histamine, bradykinin and LTB4. Flavonoid-treated animals also tended to have a lower number of leukocytes adhering to the venular endothelium.[16] In addition flavonoids could decrease the production of free radicals[17] and alter cytokine release.[18] They can alter the composition of the venous wall[19] and have some haemorrheological effects.[20] Our study demonstrates the effect of micronised purified flavonoid fraction on decreasing leucocyte degranulation and endothelial activation. The effects of these compounds on duplex ultrasonographic parameters and on resolution of lipodermatosclerosis need to be quantified in prospective studies. The lack of side-effects of these compounds makes them especially attractive as medications for CVD.

In conclusion, the increase in some endothelial activation markers studied was dampened by the administration of purified micronised flavonoid fraction for 60 d in patients with CVD. This suggests that these compounds may downregulate the activated endothelium in these patients. Double-blind randomised trials are needed to assess the role of these compounds in CVD. Our study demonstrates the feasibility of using changes in levels of soluble endothelial markers as parameters for assessing the response to therapy in CVD.

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