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Garlic supplementation increases peripheral blood flow: a role for interleukin-6?

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Abstract

There is considerable epidemiological and clinical evidence that regular garlic supplementation reduces cardiovascular risk. In this study, we have investigated the hypothesis that dietary garlic supplementation increases tissue blood flow and this is mediated by the vasodilatory actions of interleukin-6 (IL-6). Venous occlusion plethysmography was used to measure resting calf blood flow before and after oral administration of 600 mg of garlic tablets once daily for 7 days in 13 young healthy female volunteers (treatment group) and 13 female controls matched for age and body mass index (BMI). Blood samples were obtained at the time of plethysmography to measure plasma levels of IL-6, nitrate, nitrite and c-GMP. In the treatment group, calf blood flow increased significantly from 3.01 (2.56 to 3.3) ml min^{-1} 100 mL^{-1} of tissue before garlic to 3.46 (3.0 to 4.03) ml min^{-1} 100 mL^{-1} of tissue after 7 days of garlic ($P = 0.001$). Plasma IL-6 concentrations increased significantly from 54.6 (32.3 to 151.6) mcg/mL before to 151 (135.75 to 422.3) mcg/mL after 7 days of garlic ($P = 0.02$). However, there was no significant change in the plasma levels of nitrate, nitrite and c-GMP after the garlic ($P = 0.4$, 0.9 and 0.65 respectively). In the control group, resting calf blood flow and plasma levels of IL-6, nitrite, nitrate and c-GMP remained unchanged after 7 days ($P = 0.62$, 0.92, 0.28 and 0.35 respectively). Calf blood flow showed a non-linear correlation with plasma IL-6 levels after garlic supplementation ($r = 0.86$, $p = <0.001$) but not before. There was no significant relationship between blood flow and plasma nitrate, nitrite and c-GMP in either groups and between blood flow and IL-6 in the control group. These data suggest that garlic supplementation increases resting tissue blood flow and this may be mediated by IL-6.

Keywords: Blood flow; Garlic; Interleukin-6; Vasodilatation

1. Introduction

There is considerable epidemiological and clinical evidence that regular dietary garlic (allum sativum) supplementation reduces cardiovascular risk [1]. Both animal and clinical studies have shown that garlic is a potent inhibitor of platelet aggregation [2, 3]. It normalizes serum lipids in both acquired and familial hyperlipidemia [4] and has both antioxidant [5] and vasodilatory [6] effects and also normalizes blood pressure in some hypertensives [7]. A recent meta-analysis has however shown that any effect of garlic on cholesterol is modest [8]. Although these properties of garlic may explain its cardioprotective effects, the physiological and pharmacological mechanisms mediating these effects are unknown. It has been suggested that the effects of garlic may be vascular endothelium-dependent and nitric oxide mediated. This is because its pharmacological actions appear to parallel the effects of nitric oxide. Water and alcohol extracts of garlic activate nitric oxide synthase in placenta villous tissue in a dose dependent manner. It was observed that levels of stable metabolites of nitric oxide (such as nitrate and nitrite) were significantly increased in supernatants after incubating garlic with placental villous tissue [9]. Clinical studies have also demonstrated that chronic garlic intake improves aortic elastic properties of the elderly, effects attributed to vascular endothelial dependent activation of the L-arginine-nitric oxide pathway [10]. Although amino acid analysis has shown that garlic is a rich source of L-arginine (precursor of nitric oxide), further studies suggest release of NO precursors are unlikely to explain its actions [11].

There is evidence that cytokines can induce the synthesis of NO and arachidonate derivates such as prostacyclin and thromboxane, which play an important role in the regulation

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of vessel tone [12]. Interleukin-6 (IL-6) has been shown to be a potent inhibitor of vascular smooth muscle contraction in the thoracic aorta from Sprague Dawley rats [13]. Furthermore, the vascular endothelial cell is an important source of IL-6 with hypoxia being a potent stimulant for increased production [14]. We hypothesized that the reported vascular effects of garlic might be mediated by cytokines and chose to examine IL-6, as it has been shown to have cardiovascular effects. In this study we have tested the specific hypotheses that [1] garlic increases resting tissue blood flow and [2] that this effect may be mediated by the vasodilatory effect of IL-6.

2. Methods

2.1. Subjects

We used computer assisted strain gauge plethysmography to compare resting calf blood flow in 13 healthy young women volunteers before and after 7 days of oral dietary supplementation with garlic (treatment group) and 13 age and body mass index (BMI) matched female controls. All the subjects were normotensive, non-users of oral contraceptives and non-smokers. Specific questions were asked about dietary intake and previous medical history. Exclusion criteria were any history of cardiovascular disease, evidence of current infectious or inflammatory diseases and hematological or neoplastic disorders. Also, current or previous administration of medications that may affect vascular endothelial cell function or endogenous nitric oxide donors as well as regular intake of garlic, either as supplements or as part of the normal diet, were also grounds for exclusion. Allocation of subjects to the 2 arms of the study was non-randomized and not blinded. However, the women were matched for both age and BMI. Compliance, in the treatment group, was assessed by tablet count at the post-administration visit. All the women were selected on the basis of a regular 28-day menstrual cycle, and garlic supplementation commenced between days 17 and 26 (mean 16 $\pm$ 1.2 SD). Calf blood flow in the control group was measured 7 days apart. We chose the calf because it had previously been found to be less susceptible to involuntary movement artifact, and also because it offered a larger volume of skeletal muscle for blood flow assessment than the forearm [15]. In addition we now have a very large database on blood flows in this tissue. To avoid the possible influence of the menstrual cycle on the observations, all the studies were carried out during the luteal phase of the cycle in the two groups Serum progesterone levels were also monitored to confirm ovulatory status.

In the treatment group, calf blood flow was measured before and after oral ingestion of 600 mg of enteric-coated garlic tablets daily for 7 days. Each tablet contained 0.6g of dried garlic per 100g i.e., 1800 μg allicin. (Kwai, Lichtwer Pharma, GmgH, Berlin). The post treatment measurement was made on the last day of garlic administration (day7). Venous blood samples were also taken at the times of the calf blood flow measurement to assay plasma levels of nitrate and nitrite (stable metabolites of NO), cyclic GMP (a second messenger of NO), and IL-6. Calf blood flow assessment and venous blood sampling were also performed on the control group on days 1 and 7 of the study. The freshly drawn samples were spun at 3000 revolutions per minute at 4°C, for 15 min. The plasma was stored at -70°C until assayed. Informed consent was obtained from all the volunteers, and the local Research Ethics Committee approved the study.

2.2. Study protocol

Calf blood flow was measured in a quiet room with the temperature kept constant at 23 to 24°C. The women rested supine for at least 30 min before the study. Observations were made in the supine position with the right mid-calf supported at the level of the heart. Arterial blood pressure was measured non-invasively in the ipsi-lateral calf and arm, using a Dinamap Vital Sign Monitor (Type 1800, Critikon, Tampa, FL, USA). The average values of systolic, diastolic and mean arterial blood pressures were calculated from triplicate measurements. Blood flow was measured using the Filtrass strain gauge plethysmograph system (Filtrass, DOMED, Munich, Germany) [16]. This system is based on the standard strain gauge plethysmography system described by Gamble et al. [16]. The device is mercury free with an integrated automatic calibration device. This allows a touch free calibration, thus reducing artifacts due to investigator manipulation. The sensor is calibrated automatically in triplicate, by a computer driven program at the start of each study. The relative merits of the Filtrass over the standard strain gauge in terms of the quality of calibration and reproducibility have been documented [16]. The Filtrass program is computer-assisted and allows the selection of pre-recorded protocols for measuring blood flow and other microvascular parameters, such as filtration capacity in the calf or forearm. Briefly, the congestion pressure cuff, which is attached to a compressor pump built into the apparatus, was placed around the right thigh and enclosed in a rigid corset to reduce filling volume and thus filling time [15]. Changes in calf circumference, in response to a rapid increase in cuff pressure, were measured using a passive inductive transducer with an accuracy of ± 5 μm. The files were recorded and saved for subsequent "off-line" analysis.

Calf blood flow was measured using the principles described in a previous protocol [17]. In the current protocol, venous congestion pressure was raised rapidly to 40 mmHg and the pressure held for 20 s. Since this pressure occludes venous return but not arterial blood inflow, the initial swelling rate will equal arterial blood flow [18]. In order to avoid subject discomfort and prolongation of the protocol and also to enable us to relate the data to our existing large database,

no attempt was made to exclude blood flow from the foot by the application of a supra-systolic congestion pressure via an ankle cuff. Moreover, since the circulation to skin, ankle and foot tissue was included in the estimation of blood flow, in both groups, our belief is that the exclusion would make little difference to the outcome. The change in circumference of the calf was estimated from the slope of the first 3 s of the volume response to the pressure step. This procedure was repeated 3 times, with the congestion pressure kept at zero for 5 min between each of the measurements. The Filtrass analysis program calculates the change in circumference and uses it to estimate volume change, assuming that the segment of calf being studied is a cylinder of uniform diameter and constant length. Units of blood flow (QaU) were milliliters per minute per 100millitres of tissue volume ($ml.min^{-1}.100\ mL^{-1}$).

2.3. Biochemical assays

Nitrite concentrations were assayed spectrophotometrically by the Griess reaction [19]. This involved the addition of a mixture of naphthalethylenediamine diahydrochloride (BDH Laboratories Ltd, Poole, UK) and sulfanilamide, and reading the absorbance at 540 nm. For the assay of nitrates, the samples were shaken with cadmium powder (325 mesh, from Aldrich Chemicals Ltd; Gillingham, UK), which reduced nitrate to nitrite. Total nitrite was measured as before [18]. Measurement of c-GMP was by radioimmunoassay (Amersham International plc, Buckinghamshire, UK) using the method previously described by Brooker et al. [20].

Plasma IL-6 was determined using a commercial radioimmoassay kit (Milenia IL-6 EIA) obtained from DPC Biermann, GmbH, Germany. Milenia IL-6 is an immunometric assay, designed for the quantitative measurement of IL-6 in plasma or serum. The assay uses a horseradish peroxidase-labeled polyclonal antibody, directed against the IL-6 molecule. A chromogenic substrate (3, 3,′5, 5′ -tetramethylbenzidine, TMB) reactive with the enzyme label was added to the sample. The resulting color, read spectrophotometrically at 450 nm, was directly related to the endogenous IL-6 concentration. The antibodies in the Minelia IL-6 procedure are highly specific for human IL-6, with no detectable cross reactivities to recombinant mouse IL-6 or to other cytokines that may be present in patient samples. The detection limit of the assay, defined as the concentration three standard deviations above the response at zero doses was approximately 4.0 μg/mL.

2.4. Statistical analysis

Values for blood flow, plasma nitrate, nitrite, cGMP and IL-6, which were not normally distributed, are expressed as median (interquartile range). The differences between the paired measurements were assessed using analysis of variance for repeated measures (ANOVA). Relationship between blood flow and the biochemical parameters were compared. Differences were considered to be statistically significant when *P*-values were less than 0.05. The SPSS statistical package, Version 10 was used for statistical analysis.

3. Results

The 2 groups of women were of similar age (27.5 ± 2.9 vs. 28.1 ± 4.8 years, $P = 0.49$) and BMI (22.9 ± 1.9 vs. 21.2 ± 6.1, $P = 0.33$) for the treatment and control groups respectively. In the treatment group, 8 of the women were Caucasians (White European origin) and the remaining 5 of black African origin. There were 7 Caucasians and 6 black African women in the control group. All the women complied with and completed the treatment regime and no adverse outcomes were reported. Calf blood flow increased significantly from 3.01 (2.56 to 3.3) QaU (median [interquartile range]) before garlic to 3.46 (3.0 to 4.03) QaU after 7 days of garlic supplementation ($P = 0.004$) (*Fig. 1a*). The increase in blood flow was similar in black and white women ($P = 0.18$). There was no significant difference in systolic (121.3 ± 2.7 vs. 109.8 ± 2.2 mm Hg, $P = 0.31$) and diastolic (60.0 ± 2.6 vs. 57.4 ± 3.4 mm Hg, $P = 0.3$) blood pressures before and after garlic. Similarly, garlic did not appear to have any significant effects on heart rate (65.0 ± 1.9 vs. 63.0 ± 1.3 beats per minute $P = 0.18$, before and after garlic respectively). In the control group, calf blood flow (2.59 (2.17 to 3.14) vs. 2.58 (2.16 to 3.2) QaU, systolic blood pressure (112 ± 7.0 vs. 115 ± 17.0 mmHg), diastolic pressure (61 ± 7.0 vs. 63 ± 5.0 mmHg), and heart rate (64 ± 3.0 vs. 66 ± 5.0 beats per minute), remained unchanged after 7 days ($P = 0.6$, 0.56, 0.41, and 0.22 respectively). Baseline blood flow values were similar in both groups ($P = 0.19$). However, blood flow was significantly greater in the treatment group than in the control group after 7 days ($P = 0.001$). (Table 1).

In the treatment group, plasma IL-6 concentrations increased significantly from 54.6 (32.3 to 151.6) μg/mL before, to 151 (135.75 to 422.3) μg/mL after 7 days of garlic supplementation ($P = 0.02$) (*Fig. 2a).* There was no significant change in the plasma levels of nitrite 15.5 (11.0 to 26.50) μg/mL, vs. 13.6 (4.7 to 33.0) μg/mL, $P = 0.4$, nitrate 12.6 (9.85 to 19.65) μg/mL, vs. 10.9 (7.45 to 31.2) μg/mL, $P = 0.96$ and c-GMP 240 (157.0 to 200) μg/mL (143.0 to 310.0) μg/mL, $P = 0.65$ for before and after garlic supplementation, respectively (Table 1). IL-6 concentrations showed a significant nonlinear correlation with calf blood flow after garlic supplementation ($r = 0.86$, $p = <0.001$, first order quadratic equation $y = ax/b+x$) (*Fig. 3a)* but not before garlic administration. There was also a significant correlation between changes in blood flow and plasma IL-6 concentrations ($r = 0.8$, $P = 0.001$). There was no significant relationship between blood flow and IL-6 levels on days one and seven in the control group ($r = 0.16$, $P = 0.59$,

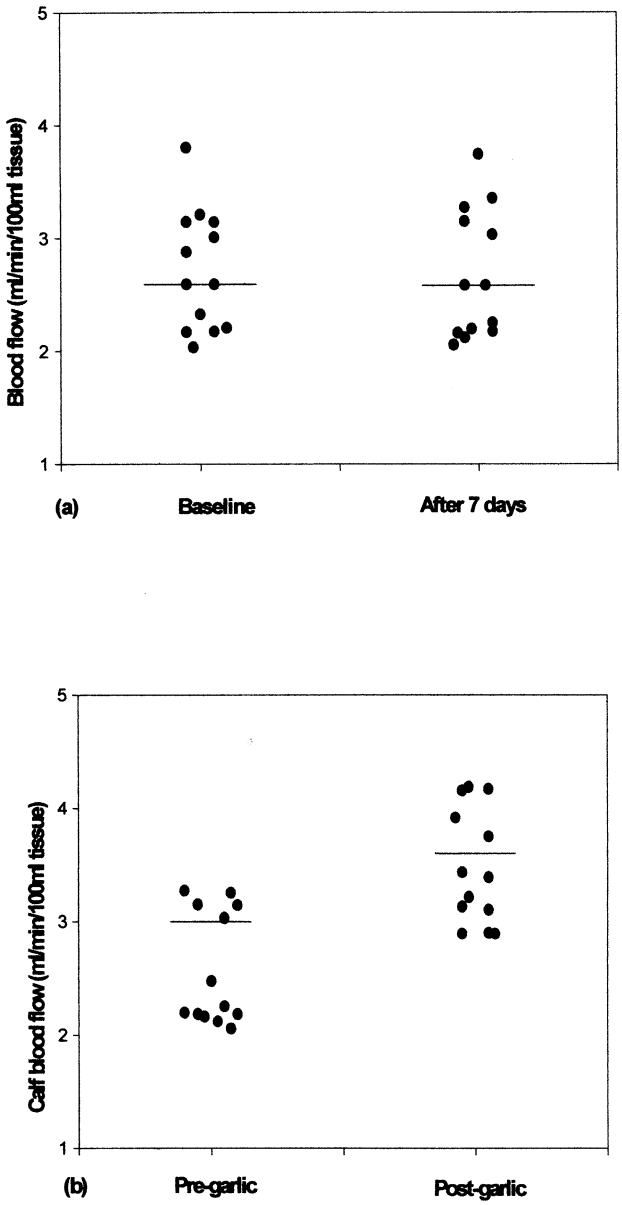


Fig. 1. Resting calf blood flow (a) before and after 7 days in the control group (n = 13) and (b) after 7 days of garlic supplementation (n = 13).

and r = 0.19, $P = 0.54$ respectively). Circulating levels of IL-6, nitrite, nitrate and c-GMP were not significantly different in the control group after 7 days ($P = 0.62$, 0.92, 0.28 and 0.35 respectively). Plasma levels of IL-6 were significantly greater in the treatment group than in the control group at 7 days ($P = 0.002$). The values for nitrate, nitrite and c-GMP were not significantly different from the controls ($P = 0.6$, 0.6 and 0.5 respectively). (Table 1).

No significant correlations were observed between blood flow and the plasma nitrate, nitrite and c-GMP concentrations in both groups and between blood flow and IL-6 in the controls (days one and seven) (*Fig.3b)*. Multiple regression analysis showed that only IL-6 levels after garlic administration, were independently related to calf blood flow in the treatment group but not the controls.

4. Discussion

We have demonstrated, in an observational study using strain gauge plethysmography, that there is a significant correlation between dietary garlic supplementation and increases in calf blood flow in healthy individuals. Moreover, the increase in blood flow was associated with a significant elevation of plasma levels of IL-6. The data failed to demonstrate any relationship between the increase in blood flow and activation of the L-arginine- nitric oxide pathway.

Increases in red cell flux, in response to garlic administration and assessed using intravital microscopy and Doppler fluximetry, in both animal and human models, have been reported previously [21, 22]. In the latter, a randomized placebo-controlled double blind crossover study, the increase in microvascular flow was associated with vasodilatation of the pre-capillary resistance vessels. This was attributed to reduced viscosity and favorable rheological effects of garlic, such as inhibition of platelet adhesion and increased fibrinolytic activity at the microvascular level [23, 24]. Support for this contention has recently been gained from the observations that garlic and aspirin were equally effective in inhibiting aggregation of activated platelets in mouse pial microvessels [25, 26]. Microvascular blood flow is vital for nutritive exchange at the tissue level. Moreover, there is increasing evidence that tissue or organ dysfunction is preceded by a gradual deterioration of microvascular function. Clearly, therapeutic interventions that either prevent reduction of or restore microvascular blood flow to normal could help prevent organ or tissue failure.

As stated above, the effects of garlic on the improvement of skin microcirculation, studied using laser Doppler fluximetry, have been reported [21, 23]. However, while the skin provides an accessible organ for investigating some aspects of peripheral hemodynamics, its usefulness as a measure of nutritive flow is limited by its dual function of nutrition and thermoregulation, and also by the presence of arterio-venous anastomoses. We chose to study the calf because the high muscle to skin blood flow ratio means more of the blood flowing through it will traverse microvascular beds thereby largely representing nutritive (microvascular) blood flow. In the light of this, we feel that our data reflects the effects of garlic on tissue nutritive perfusion. Moreover, the improvement in blood flow was significantly related to, and therefore possibly mediated by, increases in the circulating levels of IL-6.

Interleukin-6 is a multifactorial cytokine that plays an important role in host defenses, immune responses and hematopoeisis. It is expressed by a variety of normal and activated cells including vascular endothelial cells [27] and resembles IL-1β in its biological activities, which include

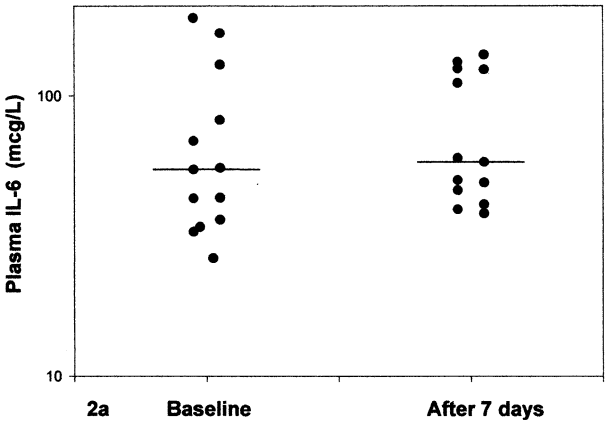
Table 1
Summary of measured variables

	Baseline (n = 13)	After 7 days (n = 13)	p-value
(a) Control			
Blood flow (ml/min/100ml)	2.59 (2.17–3.14)	2.58 (2.16–3.2)	0.6
Plasma IL-6 (mcg/mL)	54.6 (35.1–105.5)	58.0 (43.5–124.5)	0.62
Plasma-Nitrite (mcg/mL)	17.0 (11.25–20.25)	15.0 (12.9–21.45)	0.92
Plasma Nitrate (mcg/mL)	14.0 (5.45–33.0)	20.9 (10.9–31.4)	0.28
Plasma cGMP (mcg/mL)	183.0 (138.0–242.0)	228.0 (131.5–304.0)	0.35
	Pre-garlic (n = 13)	**Post-garlic (n = 13)**	**p-value**
(b) Treatment group			
Blood flow (ml/min/100ml)	3.01 (2.56–3.3)	3.46 (3.0–4.03)	0.001
Plasma IL-6 (mcg/mL)	54.6 (32.3–151.6)	151 (135.75–422.3)	0.01
Plasma Nitrite (mcg/mL)	15.5 (11.0–26.5)	13.6 (4.75–33.0)	0.96
Plasma Nitrate (mcg/mL)	12.6 (9.85–19.65)	10.9 (7.45–31.2)	0.4
Plasma cGMP (mcg/mL)	240 (157.0–327.0)	200 (143.0–310.0)	0.65

Comparison of measured peripheral blood flow and plasma concentration of IL-6, c-GMP, nitrite and nitrate after 7 days in (a) controls and (b) of garlic supplementation group. Values are expressed as median (interquartile range). P-values less that 0.05 are considered statistically significant (Wilcoxon paired-ranked test).

inhibition of vascular smooth muscle contraction [28, 29]. Ikeda et al. [30] previously investigated the circulatory effects of IL-6 and demonstrated that serum levels were elevated in myocardial infarction. Increased IL-6 levels are thought to contribute to the cardiovascular manifestations of severe sepsis, by inhibiting myocardial and peripheral vascular smooth muscle cell contractility, which these workers hypothesized, was mediated by NO [31]. However, in the present study, the changes in IL-6 following garlic supplementation were not associated with corresponding changes in plasma levels of stable metabolites of NO. Thus, the effect of garlic on blood flow was likely to be independent of the activation of the NO pathway. Furthermore, there is considerable evidence that IL-6 contributes to the vascular smooth muscle relaxing effects of calcium channel blockers. For example, Roth et al. [32] reported that manidipine enhanced growth factor -dependent transcription of the IL-6 gene in human mesangial cells, while Walz et al. [33] demonstrated that Verapamil stimulates secretion of IL-6 in cultured rat smooth muscle cells. Similarly, Rödler et al., in a more comprehensive study, have shown that four calcium channel blockers, Amlodipine, Felodipine, Isradipine and Manidipine activate the transcription of the genes encoding IL-6 and IL-8 in primary human vascular smooth muscle cells and fibroblasts at nanomolar concentrations. By contrast, the calcium-channel blockers, propranolol and frusemide (both antihypertensives) failed to affect the IL-6 genes. In the light of these observations, the significant rise in IL-6 following garlic supplementation and the direct relation between blood flow and IL-6 levels following garlic, that were seen in the present study, suggest that it may well be involved in the vascular effects of garlic.

The likely sources of the garlic-induced increases in IL-6 are vascular smooth muscle cells and the endothelium. Studies to determine the expression of IL-6 mRNA by vascular endothelial cells and smooth muscle cells after incubation with garlic, could enable the cell types involved in garlic induced-IL-6 production to be identified. There is supportive evidence that release of IL-6 by vascular endothelium provides a protective mechanism for vascular homeostasis particularly under hypoxic conditions. For example, Yan et al. [34] have demonstrated that hypoxia-mediated expression of IL-6 may have protective vascular effects under ischemic conditions. It prevents hypoxia-induced suppression of endothelial cell barrier function, by inhibiting hypoxia-induced decline of intracellular cAMP [34]. Furthermore, it maintains the expression of the anticoagulant cofactor thrombomodulin and suppression of procoagulant tissue factors [33]. It also inhibits the expression of leukocyte adhesion molecules, such as E-selectin and acts as vasodilator through its effects on smooth muscle cells [34]. The present data support the observations of Ohkawa



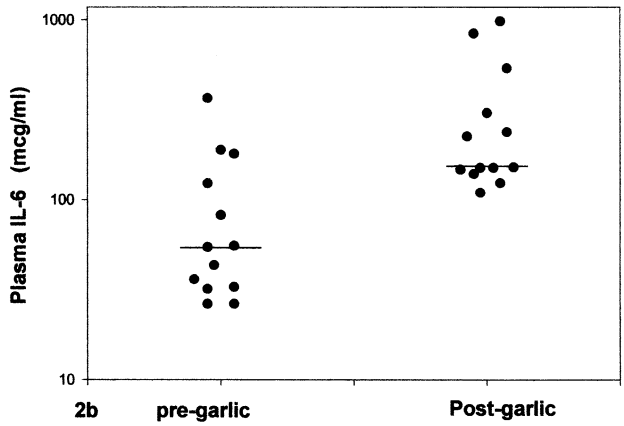
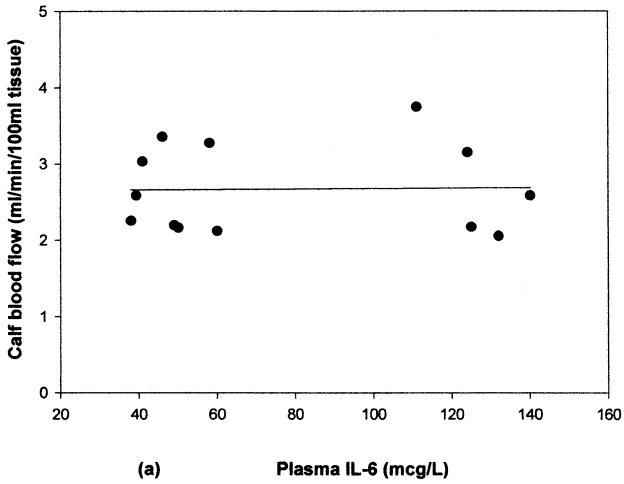


Fig. 2. Plasma concentrations of interleukin-6 (mcg/ml) (a) in the control group and (b) before and after 7 days of garlic supplementation ($p = 0.02$).



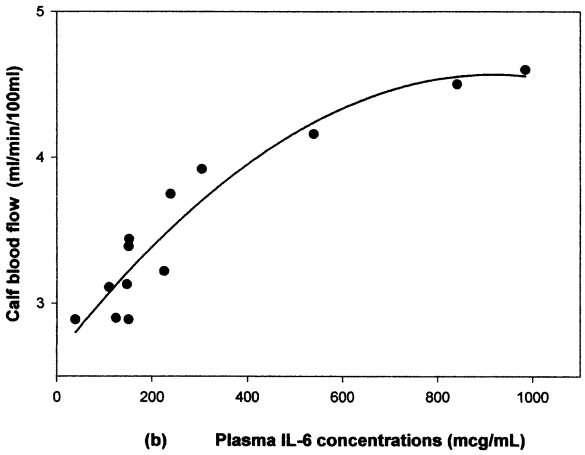


Fig. 3. Relationship between resting calf blood flow and plasma concentrations of interleukin-6 after 7 days (a) in the controls and (b) in the treatment group ($r = 0.86$, $p < 0.001$).

et al. that IL-6 is a potent inhibitor of vascular contraction mediated by cAMP synthesis [13]. If garlic were shown to induce IL-6 synthesis at the cellular level and to prevent hypoxia-induced suppression of cAMP, this would provide further evidence for the contemporary view of garlic as a cardioprotective dietary component.

In spite of the significant increase in calf blood flow and plasma IL-6 concentrations following garlic supplementation, we recognize that there are potential limitations of this study. Firstly, plasma levels of garlic were not measured to allow a correlation between circulating garlic and IL-6 levels. Secondly, the circulating levels of IL-6 may not reflect tissue activity of garlic. Thirdly, although the measured blood flow was presumed to represent nutritive blood flow to the calf muscle, we cannot exclude contributions from non-nutritive blood flow through the arterio-venous shunts in the skin of either the calf or the feet. Fourthly, although, we failed to demonstrate any changes in metabolites of NO metabolism, changes in blood flow before and after infusion of NO synthase inhibitors may be required to completely exclude any contributions by the NO pathway. Although robust conclusions cannot be drawn from these data, as the number of women in the study is small, the level of statistical difference in blood flow and IL-6 observed is so large that the findings are likely to represent real biological effects of garlic. While the data are interesting, their interpretation is limited by the fact that the study is non-randomized and non-blinded. Furthermore, whereas time related changes were controlled, the placebo effect was not. A randomized placebo controlled study, which includes male subjects, is therefore required to further investigate the effects of garlic on these parameters.

In summary, we have provided evidence from this observational study that garlic supplementation is associated with a significant increase in resting calf blood flow in female healthy controls which is associated with increased plasma levels of IL-6 but independent of the nitric oxide pathway.

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