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Hemorheology and walking of peripheral arterial occlusive diseases patients during treatment with *Ginkgo biloba* extract[1]

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KEY WORDS arterial occlusive diseases; *Ginkgo biloba*; hemorheology; erythrocyte deformability; fibrinogen; diabetic angiopathies

AIM: To study the effects of *Ginkgo biloba* extract 761 (*Gb*E)[5] from the points of view of hemorheology for patients of peripheral arterial occlusive diseases (PAOD). **METHODS:** The treatment with *Gb*E (240 mg·d^{-1}, *po*) and the pain-free walking distance (PFWD) were carried out for 24 PAOD patients (12 nondiabetic, ND and 12 diabetic, D) over 48 wk. The parameters erythrocyte stiffness (ES) and relaxation time (RT), the blood plasma viscosity (η), the plasma fibrinogen concentration (C_f) and the blood sedimentation rate (BSR), the PFWD, and maximal walking distance (MWD) were determined at 6 wk before treatment (−6), at the beginning of the treatment (0), and after 6, 11, 16, and 48 wk of treatment. **RESULTS:** At wk −6, ES and RT of both the ND- and D-group were not significantly different from a healthy control group. At wk 0, stiffness and RT were significantly higher than healthy control, and the mean PFWD was only 111 m. The η value was significantly elevated and C_f and BSR were enhanced. Throughout 11 wk of treatment ES, RT, η, and C_f decreased gradually and PFWD improved. Between 16 and 48 wk, ES, and RT were no longer significantly different from the controls, whereas η and C_f decreased gradually but remained higher than normal, BSR decreased, and the PFWD improved by a factor of 3.8 times (D) and 3.3 times (ND). **CONCLUSION:** *Gb*E gives therapeutic effects in PAOD patients.

In peripheral arterial occlusive diseases (PAOD), elevated blood viscosity, increased plasma fibrinogen levels, altered erythrocyte aggregation, and erythrocyte fluidity are related to the severity of the circulatory insufficiency[1,2]. For the treatment of PAOD, the use of vaso- and hemorheologically active drugs are considered to be a useful therapy[3], in addition to physical therapy and the elimination of risk factors. The *Ginkgo biloba* extract 761 (*Gb*E) from the leaves is one of these drugs. In experimental models of ischemia, edema, and hypoxia, *Gb*E reduced vascular, tissular and metabolic abnormalities as well as impaired blood rheology[4]. As to possible mechanisms of these effects of *Gb*E, antioxidative protection of the membrane ultrastructure[5], improved tissue oxygen delivery[6], and the modulation of some enzymatic systems and ionic pumps[4] have been discussed.

In the present study, the effect of *Gb*E on the pain-free walking distance, the erythrocyte stiffness, erythrocyte relaxation time and other parameters related to hemorheology was investigated. This study focused on the time course of the parameters observed during long term treatment of PAOD patients (Fontaine Stage II) over a period of one year.

MATERIALS AND METHODS

***Gb*E** The standardized *Gb*E is a mixture of more than ten substances. The two main classes of the components are flavonoids (24 %) and terpenes (6 %). 2.9 % of the terpenes is bilobalide and 3.1 % is ginkgolide A, B, and C. Besides, *Gb*E contains oligomer proanthocyanidine (5 % − 10 %), carbonate (*c* 9 %), ginkgo acid, biflavonoide, *etc*. The LD_{50} of

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*Gb*E (orally) was > 10 $g \cdot kg^{-1}$ in rat[8].

Patients, control groups, and timing of the study A group of 24 patients (18 males and 6 females) with PAOD of the lower extremities with a maximal walking distance of 100 – 300 m were randomized enrolled and treated with *Gb*E 120 mg bid (orally)[6,9]. All patients included in the study were classified as Fontaine stage-II. Among them, a subgroup of $n = 12$ was diabetics (average age 64 ± 6 a) and another subgroup of $n = 12$ (64 ± 5 a) was not diabetics. Among the former, 5 were continuously treated with insulin and 7 with euglucon during the investigation. 10 healthy volunteers (2 males, 8 females, 41 ± 11 a) as control group were investigated with same treatment in this study. Besides, another placebo group for the patients was carried out during our study. The medication and the placebo, were given under double-blind conditions.

The hemorheology data were collected at 6 wk before treatment (wk – 6), at the beginning of treatment (wk 0), and after 6, 11, 16, and 48 wk of treatment. Additional data were collected for η, C_f, BSR, PFWD, and MWD at wk 22 and 36. Since 21 patients had been treated with various vasoactive substances for more than one year before the beginning of the study, the time-span – 6 to 0 wk was included in the study as a wash-out period during which all patients were given a placebo. Since wk 0 they were treated for another 48 wk with *Gb*E.

Apparatus and measurement parameters The erythrocyte stiffness (Pa) and relaxation time (ms) were measured photometrically with the automated Microscopic Photometric Monolayer Technique (MPMT)[5,7]. The erythrocyte-stiffness gives a measure of the erythrocyte resistance to elongation due to the static suspension flow, whereas the erythrocyte-relaxation time describes the dynamic shape recovery process of elongated erythrocytes after a sudden stoppage of the flow. Thus, they reflect the static and dynamic response of the erythrocytes to deforming shear forces[5,7].

The plasma viscosity was assessed with a falling ball viscometer. The plasma sample volume used was 500 μL. The average of three measurements was taken for each sample. The data were obtained at (20 ± 2.5) ℃ and were temperature-corrected according to the instructions of the manufacturer (Haake Messtechnik, Karlsruhe, Germany).

The plasma fibrinogen concentration was measured with a photometric Chromotimer (Behringwerke AG, Marburg, Germany). The blood sedimentation rate, BSR ($mm \cdot h^{-1}$), was determined according to the method of Westergren[10].

To quantitate the clinical success of the therapy, the pain-free walking distance (PFWD) and the maximal walking distance (MWD) were measured with a belt ergometer (Wooday GmbH, Weil am Rhein, Germany) at a speed of 3 $km \cdot h^{-1}$ and with a 10 % incline.

HEPES buffer A buffer was used as standard buffer: NaCl 137, KCl 4, $CaCl_2$ 1.8, Na_2HPO_4 0.8, NaH_2PO_4 0.2, $MgSO_4$ 0.7, HEPES 8.4 ($mmol \cdot L^{-1}$), water-free glucose 1 g $\cdot L^{-1}$, and NaOH 1 $mol \cdot L^{-1}$, pH 7.4. The osmolality was 290 ± 10 $mOsmol \cdot kg^{-1}$. Bovine serum albumin (Serva Feinbiochemica GmbH & Co, Heidelberg, Germany) 1 $g \cdot L^{-1}$ was added to the HEPES-buffer prior to use except for the preparation of the erythrocyte monolayer.

Erythrocyte-suspension and monolayer preparation Heparinized venous blood 0.5 mL (10 $kIU \cdot L^{-1}$) was dispersed in HEPES buffer 10 mL. After three times centrifugation at 1700 × *g* for 10 min, 20 μL of erythrocytes were obtained from the sediment and re-suspended in HEPES buffer 50 μL without albumin. The final suspension (20 μL) was pipetted onto the bottom of the flow chamber of the MPMT. During a 10-min sedimentation the erythrocytes formed a monolayer of cells which attached to the glass. All other erythrocytes above the monolayer, which were not in contact with the glass, were flushed away with a short HEPES buffer flow (maximal wall shear stress 3 Pa). The stiffness and the relaxation time data were obtained from the erythrocytes in the monolayer. They represent the average measurements of 4000 – 5000 erythrocytes. The measurements were undertaken at (20 ± 0.5) ℃ with HEPES buffer as perfusion fluid. The data were obtained within 1 h after blood withdrawal.

Statistics The two-tailed Wilcoxon test was used.

RESULTS

Erythrocyte stiffness and relaxation time At wk −6, the beginning of the wash-out period, the erythrocyte-stiffness and relaxation-times were not significantly different in both patient groups compared to the control group. After the placebo treatment, both parameters had clearly increased and differed significantly from the controls (Fig 1).

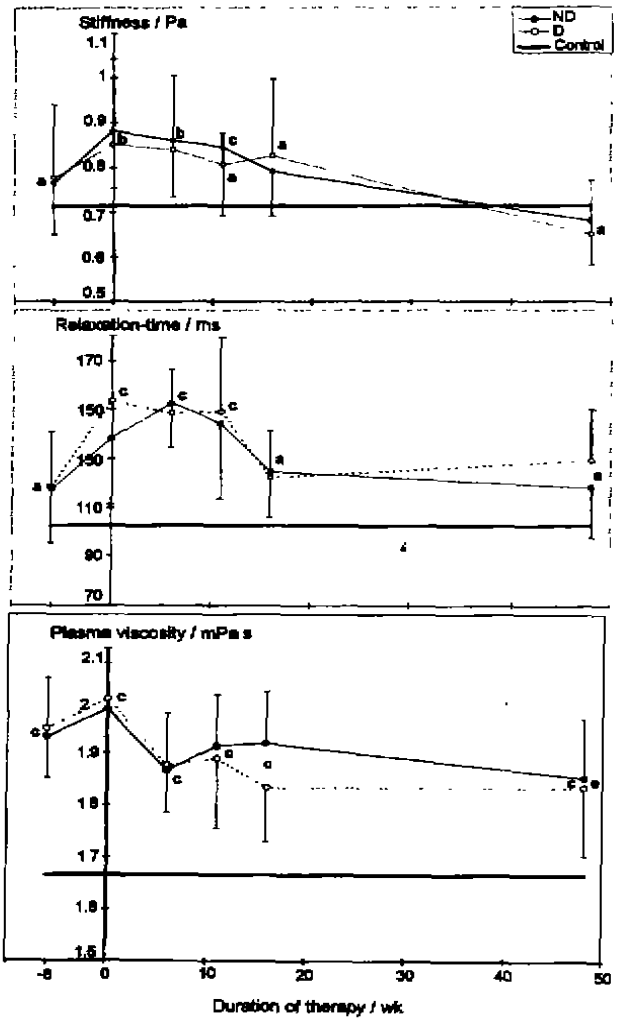

Fig 1. Hemorheology of diabetic (○) and nondiabetic (●) PAOD patients before and during treatment with *Gb*E 240 mg·d^{-1}. $\bar{x} \pm s$. $^{a}P > 0.05$, $^{b}P < 0.05$, $^{c}P < 0.01$ *vs* healthy control (—). $^{e}P < 0.05$ *vs* wk 0.

During the first wk 11 of treatment erythrocyte stiffness decreased gradually in both patient groups. At 11 wk it remained higher than that in the control for the ND-group and was not significantly different for the D-group. After wk 16 there was no significant difference in both groups compared to the control group. It decreased further and was close to the control level after 48 wk (Fig 1). A similar trend in the time course was seen in the erythrocyte relaxation time data (Fig 1). However, during the first 11 wk of treatment the differences compared to the controls were larger for both the ND and the D-group. At wk 16 it was no longer significantly different from the controls and only minor changes were observed during the following period of the treatment.

Plasma viscosity, plasma fibrinogen, and BSR At the beginning of the *Gb*E treatment the plasma viscosity was the highest. After 6 wk of treatment the level decreased and remained lower throughout the following treatment period. At the end of the treatment period, the plasma viscosity was lower than that at wk 0. However, it was still higher than the controls in both patient groups at any time throughout the study (Fig 1).

Both patient groups showed an elevated plasma fibrinogen with concentrations at the upper limit of healthy control group. No effect was found during the wash out period. The plasma fibrinogen level decreased gradually throughout the treatment period (Fig 2).

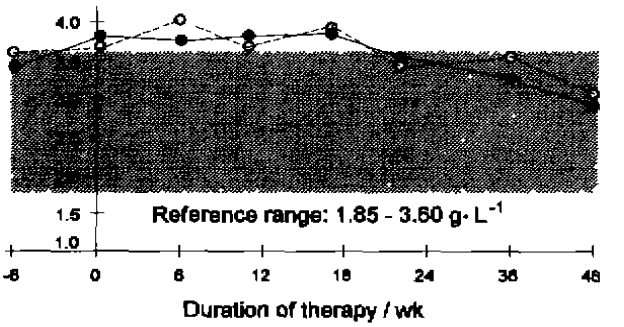

Fig 2. Plasma fibrinogen level (g·L^{-1}) of diabetic (○) and non-diabetic (●) patients before and during *Gb*E therapy. $n = 12$ patients in each group.

During the treatment with *Gb*E, the BSR values decreased in both the nondiabetic and the diabetic patients. The coefficient of variation decreased markedly. This decrease was statistically significant in the nondiabetic patients after wk 48 (Tab 1).

Pain-free and maximal walking distances

Tab 1. BSR of diabetic (D) and nondiabetic (ND) PAOD patients before and during treatment with *Gb*E.
[f] $P < 0.01$ *vs* wk 0.

	Duration of therapy (wk)	−6	0	6	11	16	22	36	48
ND	BSR ($mm \cdot h^{-1}$)	12.8	10.2	12.5	11.5	13.6	10.3	8.4	4.1[f]
	coefficient of variation	0.88	0.66	0.96	0.78	0.81	0.56	0.35	0.40
D	BSR ($mm \cdot h^{-1}$)	8.9	9.3	11.6	14.9	11.2	9.3	9.5	5.8
	coefficient of variation	0.92	0.59	0.75	0.79	0.69	0.69	0.44	0.33

In both patient groups, MWD and PFWD increased with the duration of the *Gb*E therapy (Fig 3). After 16 and 48 wk of treatment the difference was statistically significant. There was no significant difference between the two groups. The MWD was correlated linearly ($r = 0.993$, $P < 0.001$) with the PFWD. It was 82 % of the MWD (the correlation analysis included all 24 patients and the data at wk 0, 6, 11, 16, and 48).

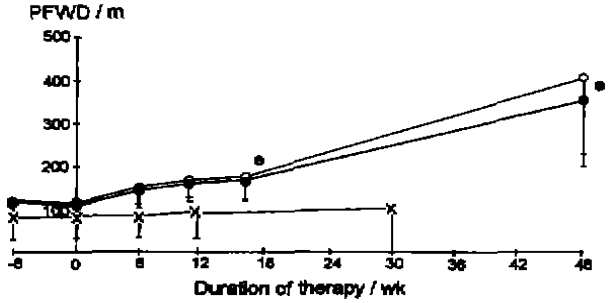

Fig 3. Pain-free walking distances ($\bar{x} \pm s$) of diabetic (○) and nondiabetic (●) PAOD patients treated with *Gb*E. (×): placebo group (*$P < 0.05$ *vs* wk 0).

DISCUSSION

Erythrocyte stiffness and relaxation time Throughout the period of investigation, the elevated erythrocyte stiffness and relaxation time of the PAOD patients were normalized after 16 wk of treatment with *Gb*E. It was assumed that the production of free radicals increased in PAOD[12]. And some investigators reported that free radicals cause an irreversible impairment of erythrocyte deformability[8]. Based on the measurement technique MPMT, it was shown in an earlier study of a group of healthy donors orally treated with *Gb*E, that the drug exerted a protective effect on erythrocytes against hydrogen peroxide damage[5]. So we supposed that the changes of ES and RT investigated in this study might be attributed to the antioxidative effect of the drug on the erythrocytes. The stiffening of erythrocytes and the increase of relaxation time observed after the wash-out phase might result probably from free radical damaging. After the beginning of the *Gb*E treatment, it took a period up to 16 wk until the erythrocyte stiffness and relaxation time had been normalized. This period represents about 93 % of the erythrocyte's lifetime in the circulation. It might be concluded that the normalization of ES and RT be due to the replacement of irreversibly damaged erythrocytes by undamaged new born ones. These newborn erythrocytes might be antioxidativly protected by *Gb*E. This could explain the slow time course of the normalization of the erythrocyte parameters ES and RT during *Gb*E treatment.

The deterioration of the erythrocyte stiffness and relaxation time during the wash-out period and their improvement during the treatment might thus be used as an indicator for pathologic rheological alterations in diabetic and nondiabetic PAOD patients as well as for the therapeutic benefits of medication.

Plasma viscosity, plasma fibrinogen, and BSR In addition to the cellular components of blood, plasma viscosity also affects the blood fluidity. The elevated plasma viscosity level was attributed to the high plasma fibrinogen level[14]. In this study, the elevated plasma viscosity as well as the plasma fibrinogen concentrations and BSR of the PAOD patients were improved after the treatment with *Gb*E. This might be considered as another evidence for the therapeutic effects of *Gb*E. The decrease of BSR of the D patient group, however, was not statistically significant. This could be mentioned that the BSR of the diabetic group was less affected during the treatment. These points should be studied in more detail in future.

PFWD The measurement of the MWD and the PFWD provided a clinical parameter to judge the success of the therapy. An effect of this physical exercise on blood rheology parameters could be excluded. In an earlier study, a group of 35 PAOD (Fontaine II) patients was given a

placebo over a period of 30 wk but exercised on the ergo belt (same settings as in the study presented here). Only minor, not significant, effects were been observed[9]. The new data presented here, during the *Gb*E treatment the PFWD and the MWD increased and alleviated the pain sensation following the MWD test in both the ND- and the D-group of PAOD patients.

Several *rheological and biochemical* parameters of the blood and clinical symptoms observed at the beginning of the *Gb*E therapy for both diabetic and nondiabetic PAOD patients were abnormal: 1) high erythrocyte stiffness and relaxation times, presumably resulting from a free radical oxidative damage of the erythrocyte membrane, 2) higher plasma viscosity accompanied by a high plasma fibrinogen and higher BSR, and 3) short pain-free and maximal walking distances. Following the *Gb*E therapy, the erythrocyte stiffness and relaxation times of the patients were mostly normalized, the blood plasma revealed a tendency towards normalization, and walking distances improved markedly. Considering that *Gb*E enhanced capillary erythrocyte velocity in intravital microscopy studies[15] and improved oxygen delivery in ischemia, as determined by transcutaneous oxygen partial pressure measurements in PAOD patients[6], there is now one further evidence for an effective long-term *Gb*E treatment in diabetic and nondiabetic PAOD patients.

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银杏提取物对外周动脉阻塞性病人的血液流变学和行走的研究[1]

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关键词 动脉阻塞性疾病; 银杏; 血液流变学; 红细胞变形; 纤维蛋白原; 糖尿病性血管病