

An investigation of changes in ocular blood flow in rabbits with long-term hyperhomocysteinemia using color doppler ultrasonography

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Aim: Hyperhomocysteinemia was induced in rabbits using methionine. Ocular blood flow changes were examined by color Doppler ultrasonography (CDU).

Materials and methods: Fifty New Zealand rabbits were divided into 5 groups. Groups 1-4 received one of the following chemicals: methionine only, methionine plus vitamin B6, methionine plus vitamin B12, or methionine plus folic acid. Controls received no chemicals. Serum homocysteine levels were measured on treatment days 1 and 60. Orbital vessels were examined by CDU on day 60.

Results: In the 4 study groups, homocysteine levels were significantly elevated on day 60 compared to the levels on day 1 ($P < 0.05$). Elevations of homocysteine levels in group 1 were greater than those of groups 2 and 4 on day 60 ($P < 0.05$). When results of ocular blood flow changes were compared, no statistically significant difference was found based on flow velocities in the ophthalmic artery. Measurements from the ciliary artery revealed a statistically significant decrease in flow velocity in the study groups ($P < 0.05$).

Conclusion: Using CDU, we found that flow velocities in the ciliary artery were decreased. This condition is attributed to atherosclerotic and thromboembolic alterations of the ocular vessels due to hyperhomocysteinemia.

Key words: Homocysteine, atherosclerosis, vitamins, rabbit, color doppler ultrasonography

Uzun dönem hiperhomosisteinimli tavşanlarda oküler kan akımı değişikliklerinin renkli doppler ultrasonografi ile araştırılması

Amaç: Bu çalışmada, tavşanlara metionin verilerek oluşturulan hiperhomosisteineminin oküler kan akımı üzerindeki etkilerini renkli Doppler ultrasonografi kullanarak araştırdık.

Yöntem ve gereç: Toplam 50 Yeni Zelanda tavşanı beş gruba ayrıldı. Grup 1-4'e sırasıyla; sadece metionin, metionin ve B6 vitamini, metionin ve B12 vitamini, metionin ve folik asit uygulandı. Kontrol grubunun diyetine kimyasal madde eklenmedi. Serum homosistein seviyeleri tedavinin 1. ve 60. günlerinde çalışıldı. Orbital damarlar 60. günde renkli Doppler ultrasonografi ile değerlendirildi.

Bulgular: Çalışma gruplarının dördünde homosistein seviyeleri 60. günde 1. günden anlamlı şekilde yüksekti ($P < 0,05$). 60. günde homosistein seviyesindeki yükselme Grup 1'de Grup 2 ve 4'den fazla idi. Oküler arter akım hızlarındaki değişiklikler karşılaştırıldığında gruplar arasında istatistiksel olarak anlamlı fark gözlenmedi. Çalışma gruplarında silier arterin akım hızlarında istatistiksel olarak anlamlı düzeyde azalma izlendi ($P < 0,05$).

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Sonuç: Renkli Doppler Ultrasonografi kullanarak silier arterde kan akım hızlarının azaldığını gösterdik. Bu durum, hiperhomosisteinemiden dolayı oküler damarlarda gelişen aterosklerotik ve tromboembolik değişikliklere dayandırılabilir.

Anahtar sözcükler: Homosistein, ateroskleroz, vitaminler, tavşan, renkli doppler ultrasonografi

Introduction

Homocysteine is a sulfur amino acid produced by the metabolism of methionine, which is one of the essential amino acids obtained from dietary proteins. Homocysteine was found to be one of the risk factors causing vascular disease when McCully (1) reported in the 1970s that patients with severe homocysteinemia died secondary to atherosclerosis and thromboembolic disease. A mild to moderate elevation of the serum homocysteine level was documented as an independent risk factor for atherosclerosis, peripheral vascular disease, and cardiovascular disease (2-4).

In evaluating the plasma homocysteine level and its interaction with conventional vascular risk factors, the European Concerted Action Project (2) showed that hyperhomocysteinemia is an independent risk factor for vascular disease, similar to smoking or hyperlipidemia. Based on the evidence of the relationship of elevated plasma homocysteine levels with atherosclerosis and thromboembolism, hyperhomocysteinemia was considered as an additional risk factor for occlusive disease of the retinal artery and vein and was demonstrated in several studies (5-7). However, most of these results are related only to laboratory studies of symptomatic patients.

Several methods, such as carotid angiography, fundus fluorescein angiography, laser Doppler velocimetry, ophthalmoplethysmography, and color Doppler ultrasonography (CDU) are used to assess changes in ocular blood flow (8-11). CDU is also used in the diagnosis of vascular diseases of orbits, such as arteriovenous malformations, orbital varix, retinal detachment, diabetic retinopathy, choroid detachment, ophthalmic arterial stenosis, central retinal artery and vein occlusion, ocular ischemic syndrome, and glaucoma (10-13).

In the present study, we induced hyperhomocysteinemia in rabbits by administering

methionine and examined changes in the ocular blood flow using CDU. We also studied the effects of folic acid, vitamin B₆, and vitamin B₁₂ on homocysteine levels and ocular blood flow parameters.

Methods

Animals and diets

Fifty (25 female, 25 male) New Zealand rabbits were divided into 5 groups with 10 rabbits in each. Only methionine (100 mg/kg per day) was given to group 1, methionine (100 mg/kg per day) plus vitamin B₆ (30 mg/kg per day) were given to group 2, methionine (100 mg/kg per day) plus vitamin B₁₂ (80 mg/kg per day) were given to group 3, and methionine (100 mg/kg per day) plus folic acid (20 mg/kg per day) were given to group 4 orally as additions to the diets. Control animals received no chemicals. These rabbits were followed for 2 months. Weights and food consumption were recorded weekly. On days 1 and 60 of treatment, rabbits were deprived of food overnight and blood samples were taken from the marginal ear vein to measure homocysteine levels. All experimental protocols were approved by the Institutional Animal Care and Use Committee of Erciyes University.

Homocysteine levels were measured by high-performance liquid chromatography (HPLC). HPLC was performed on a solvent delivery system and a fluorescent detector (385 nm excitation, 515 nm emission); 0.1 mol/L acetic acid-acetate buffer, pH 5.5, containing 30 mL/L methanol was used to elute the constituents as the mobile phase. Flow rate was 0.7 mL/min. L-homocysteine calibrators (5-100 µL mol/L) were prepared in phosphate-buffered saline, pH 7.4, and in pooled EDTA plasma. Homocysteine peaks, which were separated with the analytic HPLC column, were provided in 3.3 min. Homocysteine concentrations were calculated according to peak levels provided from the fluorescent detector. The variation coefficient was 3.5% (14).

Evaluation by CDU

All examinations were performed using a CDU unit (Powervision, Toshiba, Tochigi, Japan) with a 10-MHz linear probe. The orbital vessels examinations were performed on day 60 of treatment. The rabbits were examined in supine position after an intramuscular sedative agent was administered. Ultrasound conductive gel was applied to the external surface of the eyelids, and care was taken to exert the least transducer pressure as possible on the closed eyelids. We first scanned the whole orbit using gray scale imaging and then applied the CDU with color scale to identify the orbital vessels.

We determined peak systolic velocity (PSV), end-diastolic velocity (EDV), and resistive index (RI) for the ophthalmic artery (OA) and the ciliary artery (CA). PSV was defined as the maximum systolic peak velocity and EDV was defined as the minimum flow velocity at the end of the diastolic phase prior to the next cardiac cycle.

The OA is typically identified as a large caliber pulsatile vessel adjacent to the optic nerve, showing an immediate increase in systole followed by a dicrotic notch and slow diastolic flow. The CA was both on the nasal (medial) and temporal (lateral) sides of the optic nerve posterior to the choroid in their retrobulbar areas.

Statistical analysis

Data are presented as means (\pm standard deviations), and the 5 groups were compared using one-way analysis of variance with t tests for pairwise comparisons. Shapiro-Wilk test was used for testing

data normal distribution, and found to comply. The post-hoc Scheffe test was used to compare differences in proportions among the groups. Data were analyzed using the SPSS 9.0 (SPSS, Chicago, IL, USA) software package. $P \leq 0.05$ was considered statistically significant.

Results

Plasma homocysteine levels

Table 1 lists the plasma homocysteine levels. In all 4 study groups, homocysteine levels were significantly elevated on day 60 compared to those on day 1 ($P < 0.05$). In group 1, elevations of the homocysteine levels were higher than those of groups 2 and 4 on day 60 ($P < 0.05$). In group 4, the decrease of homocysteine levels was significantly lower than that of groups 1, 2, and 3 on day 60 ($P < 0.05$).

CDU findings

Table 2 contains the CDU findings. When the results were compared, no statistically significant difference was observed between flow velocities in the OA ($P > 0.05$). However, the measurements of flow in the CA in the study groups showed a statistically significant decrease in flow velocities ($P < 0.05$). PSV in group 1 was lower than that in group 4 and controls ($P < 0.05$). Although the PSV in groups 2 and 3 was lower than that in group 4 and controls, the difference was not statistically significant ($P > 0.05$). EDV in groups 1 and 3 was significantly lower than EDV measured in group 4 ($P < 0.01$). Similarly, EDV of group 2 was lower than that in group 4 and controls, but this difference was

Table 1. Homocysteine levels in the treatment groups on days 1 and 60 of treatment.

	Day 1, $\mu\text{mol/L}$	Day 60, $\mu\text{mol/L}$	P
Group 1	9.906 \pm 4.226	104.250 \pm 53.32	< 0.05
Group 2	9.513 \pm 4.075	65.873 \pm 14.89 ^a	< 0.05
Group 3	8.355 \pm 4.091	84.922 \pm 17.35	< 0.05
Group 4	8.615 \pm 5.227	19.033 \pm 8.825 ^{a,b,c}	< 0.05

^aStatistically significant compared to group 1 ($P < 0.05$).

^bStatistically significant compared to group 2 ($P < 0.05$).

^cStatistically significant compared to group 3 ($P < 0.05$).

Table 2. Velocity measurements (cm/s) in orbital vessels.

Vessels	Group 1	Group 2	Group 3	Group 4	Control	F	P
OA							
PSV	14.6 ± 3.6	12.1 ± 0.9	14.1 ± 3.0	15.0 ± 3.0	12.7 ± 3.9	0.9	0.43
EDV	8.3 ± 2.5	6.9 ± 0.6	7.8 ± 2.5	8.9 ± 2.2	7.1 ± 2.8	0.9	0.42
RI	0.42 ± 0.09	0.43 ± 0.03	0.45 ± 0.10	0.40 ± 0.09	0.42 ± 0.09	0.3	0.82
CA							
PSV	18.7 ± 3.6 ^{*d}	23.7 ± 3.9	19.6 ± 5.2	27.7 ± 6.3 ^{*e}	26.8 ± 6.3 ^{*da}	5.0	0.03
EDV	10.5 ± 2.7 ^{*d}	11.8 ± 2.6	11.3 ± 4.5 ^{*d}	20.1 ± 4.9 ^{*ac}	17.6 ± 7.6	5.8	0.01
RI	0.44 ± 0.07	0.50 ± 0.05 ^{*d}	0.40 ± 0.10	0.28 ± 0.02 ^{*b}	0.36 ± 0.17	4.1	0.08

Each group had 10 eyes examined. Results expressed as velocity (cm/s) ± SD.

Note: "a" refers to the group that is statistically different from group 1; "b" is the group that is statistically different from group 2; "c" is the group that is statistically different from group 3; "d" is the group that is statistically different from group 4; and "e" is the group that is statistically different from controls.

*P < 0.05 indicates statistical significance.

OA, ophthalmic artery; CA, ciliary artery; PSV, peak systolic velocity; EDV, end-diastolic velocity; RI, resistive index.

not statistically significant. RI values of the CA in the first 3 groups were higher than those in group 4 and controls; however, statistical significance was present only between groups 2 and 4 (P < 0.05).

Discussion

High levels of plasma homocysteine are toxic to the vessels. The exact mechanism by which homocysteine affects the vascular system is poorly understood. Numerous studies have documented that hyperhomocysteinemia is a risk factor for both arteriosclerosis and venous thrombosis (2,3,15-18). Because the central retinal vein shares a common, fibrous adventitia with the central retinal artery, it is likely that both arterial and venous disease could contribute to the development of a central retinal vein occlusion. Any sclerotic thickening of the central retinal artery could easily compress the adjacent central retinal vein and begin the sequence of events that leads to thrombus formation (19). As a risk factor for both arterial and venous disease, hyperhomocysteinemia may represent a "double hit" in the multifactorial pathogenesis of central retinal vein occlusion (20).

Several studies demonstrated that an elevated plasma homocysteine level is a risk factor for thromboembolism in ocular vascular structures (5-7). In 1993, Wenzler et al. (21) described data on 4 patients with central retinal artery occlusion and 2 with central retinal vein occlusion who had elevated homocysteine levels after methionine-loading testing. Biousse et al. (22) presented data on a 24-year-old man with bilateral central retinal vein occlusion and increased plasma homocysteine levels suggested that elevated homocysteine is an independent risk factor for retinal vascular occlusive disease (23,24). Vine et al. (7) demonstrated that a statistically significant association exists between the presence of hyperhomocysteinemia and central retinal vein occlusion. Their study adds further support for a cardiovascular risk profile for persons with central retinal vein occlusion. They suggested that all patients with central retinal vein occlusion should be screened for possible hyperhomocysteinemia. Unlike other risk factors, hyperhomocysteinemia is readily reversible in most individuals by treatment with inexpensive vitamin preparations containing folic acid (25,26).

Although the rabbit retinal vasculature is quite different from the human retinal vessels, in this study, we induced hyperhomocysteinemia in rabbits and assessed the effects of oral vitamin supplementation (vitamins B₆ and B₁₂, and folate) on hyperhomocysteinemia. Folic acid supplementation clearly reduced plasma homocysteine levels, whereas vitamin B₁₂ was less effective and vitamin B₆ had no effect. The results of this study indicate that supplementation with physiologic doses of folic acid could reduce homocysteine levels. Our findings were similar to those reported in other studies (27,28).

In this study, the effects of hyperhomocysteinemia on ocular blood flow in rabbits were determined using CDU. We also assessed whether folic acid is effective on hyperhomocysteinemia as examined in several studies and whether vitamins B₆ and B₁₂ have any impact on the blood flow rates in vascular structures. The results showed, significantly, that hyperhomocysteinemia leads to an obvious reduction in CA flow rates. Oral supplementation with vitamins B₆ and B₁₂ cannot eliminate this effect. However, oral supplementation with folic acid plus methionine can prevent the decrease in flow rates. In this study, supplementation of folic acid through the diet led to a significant reduction in homocysteine levels compared to the groups given vitamins B₆ and B₁₂, which is in agreement with the literature. No significant difference was found in blood homocysteine levels between groups given only vitamin B₆ or B₁₂ and methionine. Similar to the laboratory findings, a significant difference was not observed in CDU and CA flow rates in rabbits given vitamins B₆ and B₁₂ compared to rabbits in group 1. Our study indicated that, when all the groups were compared with the control group and each other, no statistically significant difference in OA flow rate was found between the group in which hyperhomocysteinemia was induced by the administration of only methionine and each of 3 groups given vitamin supplementation.

However, the decrease of some flow parameters in CA in the study groups was statistically significant, which could be explained by the possible effect of small vessels rather than the large ones in hyperhomocysteinemia.

This was no surprise to us because one of the essential points in planning this study was the indication whether hyperhomocysteinemia, which was shown to be a risk factor for thromboembolic events in central retinal artery and veins in human studies as reported in the literature, causes alterations in flow rates. With such a result, not only will the findings in clinical studies be supported, but also the involvement of the central retinal artery and vein will be determined in cases with hyperhomocysteinemia during the pre-symptomatic period; this was thought to be a guide for the treatment of this case, which is reversible with vitamin supplementation. As expected, this difference was observed.

In the light of all these findings, the fact that the involvement of CA, particularly the ocular involvement in pre-symptomatic cases, might be a criterion for the evaluation of risk factors and require further investigation. The CDU used in different clinical studies of hyperhomocysteinemia revealed deterioration in the elasticity of vascular structures. This might suggest that the CA is involved with other vascular structures. However, this issue needs to be clarified by further studies.

Using CDU, we demonstrated the hemodynamic changes associated with hyperhomocysteinemia, that is, decreased flow velocities in the CA. As indicated, detailed longitudinal studies must be initiated to confirm the close association between changes in blood flow and elevation of plasma homocysteine levels. Further studies may clearly show the beneficial effects of vitamin supplementation on the retrobulbar blood supply in patients with hyperhomocysteinemia.

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