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Comparison of the effect of different homocysteine concentrations on clot formation using human plasma and purified fibrinogen

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Dear Sir,

Up to the present we have scarce knowledge about the effects of homocysteine (hcys) on the haemostatic process. In the literature there are few papers describing studies on the effects of homocysteine on proteins that participate in blood coagulation and fibrinolysis in humans (1–3). However, the harmful effect of high homocysteine concentrations on the intima of the vessel wall is well established (4,5).

In healthy adults the plasma concentration of total hcys is less than 15 μM. However, marked differences exist in the range of reference values found in the literature, due to the diversity of methodology used, differences in sample handling and, mainly the diversity of nutritional and ethnic factors present in the different populations studied.

A plasma hcys level ≥5 μM defines hyperhomocysteinemia. Homocysteine concentrations between 15–30 μM are considered mild hyperhomocysteinemia, and may be found in subjects with coronary, cerebral and peripheral vascular disease or renal insufficiency (6); values above 100 μM are identifiable as homocysteinuria.

We explored the effect of hcys on fibrin formation by adding D,L homocysteine (Sigma Chemical Company, St. Louis, MO, USA) to pooled plasma from 20 healthy donors with an initial plasma hcys concentration of 10.5 μM and fibrinogen concentration of 3 g/l to yield final concentration levels of 13 μM, 19 μM and 52 μM, classified as low (LH), medium (MH) and high (HH) levels. The hcys concentration was measured using a Microplate Enzyme Immunoassay (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Plasma samples were incubated for 30 minutes (min) with hcys at 37 °C, with the clotting conditions described elsewhere (7). The data were analyzed first comparing the variances applying the Fisher-test, then if the variances were similar, the t-test for similar variance was applied or vice versa; a $p < 0.05$ was considered statistically significant.

We have found that at 52 μM hcys, the slope and final turbidity characteristics of fibrin polymerization were slightly decreased compared to control values. The lysis process also was

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mildly affected at medium and high hcys concentration, being slower compared to that of the control. Clot structure analyzed by scanning electron microscopy revealed no differences in either the general architecture or the fibrin fiber diameter at these hcys concentrations (results not shown).

Since the differences found were statistically significant but relatively small, we added hcys to plasma at very high concentration, i.e. as in homocysteinuria, and we examined the effects of different hcys concentrations on clotting of purified fibrinogen to detect an effect on fibrin polymerization. In these experiments, the hcys concentration was measured using the fluorescence polarization immunoanalysis method (FPIA) (Axsym system – Abbott). To a single healthy plasma donor, with an initial hcys concentration of 7.3 μM and 4 g/l of fibrinogen concentration, hcys was added to a level of 251 μM, obtained by FPIA. The plasma sample without adding hcys gave a value of 6 μM (the blanks were diluted by the same factor of 1.13, that corresponds to the solution of hcys added). Using the same protocol as before, but different incubation times: 30 min, 1 and 4 hours (h), no effects were observed on fibrin polymerization at this concentration and at the different time incubations used (Table 1; 4 h). After polymerization, the clots were extensively washed and processed for fibrin cross-linking analysis by SDS-PAGE in an 8% gel, and no differences were seen in the pattern of γ-dimer and α-polymer formation.

For the studies carried out with purified fibrinogen, we incubated fibrinogen with increasing hcys concentrations to observe changes in the polymerization process, using the clotting conditions described elsewhere (7), with minor modifications: 5 units/ml of aprotinin was added to the purified protein, since it had plasminogen as a contaminant, and 5 mM $CaCl_2$ final concentration, incubated with the purified fibrinogen for 1 min before adding thrombin. The minimum concentration that affected fibrin formation was around 408 μM hcys, at which all polymerization parameters were altered (Table 1). As done in plasma, after purified fibrinogen polymerization was completed, the clots were reduced with 2% DTT-2% SDS and analyzed by SDS/PAGE, revealing no impairment either in γ-dimer or in α-polymer formation. It seems that hcys has no effect on the activity of FXIII with respect to fibrin cross-linking. Studies are in progress in our laboratory to analyze the possible causes of the alterations of fibrin polymerization in the presence of very high hcys concentration in the purified system, and evaluate the fibrinolysis process in both plasma and purified fibrinogen, as a continuation of these studies.

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Table 1

Effects of high levels of homocysteine on fibrin polymerization.

	Control	Control + hcys
Plasma[1]		
Lag phase (sec)	30 ± 0	30 ± 0
Slope (OD units/sec)× 10^{-3}	4.5 ± 0.4	4.7 ± 0.0
Maximum ι (OD units)	0.537 ± 0.038	0.557 ± 0.012
Purified fibrinogen[2]		
Lag phase (sec)	150 ± 0	250 ± 17
Slope (OD units/sec)× 10^{-3}	1.1 ± 0.0	0.69 ± 0
Maximum ι (OD units)	0.212 ± 0.011	0.131 ± 0.019

[1] 251 μM Hcys,

[2] 408 μM Hcys.