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Effect of Synthetic Humic Acid-Multimetal Complex on Human Plasma Prothrombin Time

F.-J. Lu,[1] T.-S. Huang,[2] J.-H. Lee[1]

[1]College of Medicine, National Taiwan University, 1 sec 1 Jen-Ai Road, Taipei, Taiwan, Republic of China
[2]Department of Biochemistry and Medicine, College of Medicine, National Taiwan University, Taipei, Taiwan, Republic of China

Residents in the southwest coast of Taiwan are liable to suffer from obstructive vascular disease, locally known as blackfoot disease (Tseng et al. 1961). Epidemiologic researchers use to attribute the occurrence of blackfoot disease to the high arsenic content in the local well water (Chen et al. 1962). Recent studies, however, have concluded that the fluorescent humic substances (HS) found in the well water are also associated with this disease(Lu 1990a). In previous papers, we have reported that both natural well water HS and synthetic humic acid (HA) could affect the prothrombin time of normal human plasma(Lu et al.1990). In the process of synthesizing HA, if As_2O_3 is added, the effect of HA on shortening prothrombin time would become more apparent. As_2O_3 alone, however, has no effect(Lu 1990b). It appears that HA acting as the core of the complex, would accelerate the coagulation of blood, while arsenic is itself an auxiliary agent only, it would function when combined with HA. HS found in the well water of the endemic region in Taiwan could induce peripheral vasculopathy of mice (Lu 1990a, 1990c). They contain more than thirty kinds of elements, and arsenic is only one of them(Lu et al.1988). Therefore, we conjecture that some containing elements in HS may also have the same potentiating ability as that of arsenic.

It will be reported in this paper that the synthetic HA-MultiMetal complex has the ability to shorten human plasma prothrombin time. If it contains metal elements such as As,

Correspondence to: F.-J. Lu

Fe, Zn, Cu, Cr, and Al, this ability would be more obvious. The potentiating ability of the mixtures of As, Al, Cu, Cr, Fe and Zn is stronger than that of As alone.

MATERIALS AND METHODS

The procedure of synthesizing HA follows Wang's method (Wang et al. 1976). Take out three conical flasks. Put 1.0g of protocatechuic acid [3,4- $(OH)_2C_6H_5COOH$ or 3,4-dihydroxy benzoic acid, Sigma Co.] and 100ml of H_2O in one of the three flasks, and adjust its pH value to 7.0 with $Ca(OH)_2$ solution. Fill the second flask with 1.0g of protocatechuic acid and 100ml of H_2O and 3.3mg of As_2O_3 (Wako Pure Chemical, grade of atomic absorption), and adjust the pH value to 7.0 with $Ca(OH)_2$ solution. Fill the last flask with 1.0g of protocatechuic acid, 100ml of H_2O and a mixture of metals i.e. 40μg of Fe as $FeCl_3$, 60μg of Cu as $Cu(NO_3)_2$, 80μg of Zn as $Zn(NO_3)_2$, 90μg of Cr as $K_2Cr_2O_7$, 140μg of Al as $AlCl_3$ and 3.3mg of As_2O_3. All metals are Wako Pure Chemical atomic absorption grade. Adjust the pH value to 7.0 with $Ca(OH)_2$ solution. Cover the mouths of these flasks with cotton stoppers and put them in a water bath at 25℃ for oxidative polymerization. During the reaction period, slowly shake the flasks and adjust the pH values of their contents to 7.0 with $Ca(OH)_2$ solution everyday. Stop the reaction process on the 17th day and acidify the solution to pH 2.0 with conc. HCl solution. Leave the contents of the flasks to settle down for a while, then centrifuge them and discard supernatant portions. The precipitates obtained are crude synthetic HA- and crude synthetic HA-Single/MultiMetal complexes. Use diethyl ether to extract the untreated protocatechuic acid and other impurities from the precipitates. Dissolve the precipitates with 0.5 N NaOH solution or 0.1 M $Na_4P_2O_7$ (pH=7.0) solution. Centrifuge again. The supernatants are acidified to pH 2.0. Wash the precipitates with 0.1 N HCl-HF solution, then dissolve them with distilled water and dry in a rotary evaporator. Synthetic HA or synthetic HA-Single / MultiMetal complexes were further purified by Sephadex G-25 column chromatography before experiment. In short, synthetic HA or its metal complexes were dissolved in alkaline solution and then were adjusted to pH 7.2-7.4. Three to five ml of each solution were then passed through a G-25 column (65×5.5 cm) and eluted with distiled water

at a speed of 0.5ml/min. Every 5ml was collected as a fraction. There were two absorption peaks at 280nm. The first peak appeared at tube 36 and the second one at tube 48. The first peak was dried and stored for experiments. The estimated synthetic HA molecular weight was about 6,000 Dalton similar to that of previous report (Hanninen et al. 1987). The HA-As complex contains As 0.228 μg per mg of HA. The HA-Multimetal complex contains As 0.122 μg, Zn 1.772 μg, Fe 3.519 μg, Cr 0.443 μg, Cu 12.570 μg, Al 27.85 μg per mg of HA. The contents of various metals and As were measured by ICP-AES (JOBIN YVON 38 plus, French).

In order to measure prothrombin times, various concentrations of synthetic HA (with or without metal mixtures) were prepared by mixing appropriate amounts of HA with normal human pooled plasma and reagents (Behring). In the test, 0.03ml of synthetic compounds at various concentrations are first mixed with normal human plasma respectively. Heat the mixtures for one minute in a water bath at 37℃, then add thromborel S solution which has been preheated for 15 minutes at 37℃. Finally, use Fibrintimer (Behring) to measure prothrombin time.

The statistical analysis was preformed by ANOVA and then multiple comparison.

RESULTS AND DISCUSSION

Experimental data show that when synthetic HA-MultiMetal complex contains six elements (As, Fe, Cu, Cr, Al, Zn), its effect on the prothrombin time of human plasma is most obvious. When the complex is at a concentration of 30 ug/ml, it can reduce normal pool human plasma prothrombin time from the 13.1 seconds of the control experiments to 10.3 seconds (the mean of 3-14 experiments), and synthetic HA-As complex at the same concentration can only reduce the 13.4 seconds of the control experiments to 11.6 seconds; while synthetic HA without any metal content needs to have a 100 ug/ml concentration to reduce the 13.5 seconds of control experiments to 11.2 seconds (Figure 1). Protocatechuic acid, the monomeric component of HA , does not have any effect on the prothrombin time (Figure 1). As_2O_3 or As_2O_5 (1 ug/ml - 500 ug/ml) or metal mixtures of As, Fe, Zn, Cu, Cr, Al (3.71 ug/ml - 37.1 ug/ml) used in

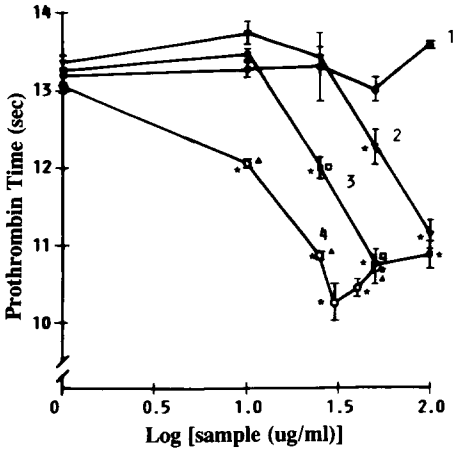

Figure 1. Effect of synthetic humic acid and synthetic HumicAcid-MultiMetal complex on normal human pooled plasma prothrombin time. Data are expressed as mean±SD (n=3-14). 1. protocatechuic acid. 2. synthetic humic acid. 3. synthetic HumicAcid-As complex. 4. synthetic HumicAcid-MultiMetal (As, Fe, Zn, Cu, Cr, Al) complex * $P<0.001$ vs control ▲ $P<0.001$ vs 3 or 2 □ $P<0.001$ vs 2.

the control experiments also did not influence the length of prothrombin time (data not shown). The experimental results prove that synthetic HA itself has the ability to shorten prothrombin time; if it contains metal, such ability will be increased, especially when it contains more than one metal element. Arsenic is only one among the many auxiliary agents of HA.

Blackfoot disease is an arterial occlusive disease with unknown etiology (Tseng, 1961). Our previous studies had shown that local HS could induce peripheral vasculopathy of mice (Lu 1990a) in vivo, and shorten human plasma prothrombin time in vitro (Lu et al. 1990). HA was demonstrated in the bone of rats fed with HA. (Yu 1982). It is interesting that Kashin-Beck disease, an endemic arthritis found in Mainland China, is proposed to be caused by HA (Zhai et al. 1990). In rats fed with ^{125}I-HA solution, we demonstrated that ^{125}I-HA was absorbed into the body and distributed to various organs and tissues. (unpublished data). Furthermore, HA was shown to be a

plasmin inhibitor (Lu et al. 1992). It was also shown that HA can cause endothelial damage and stimulate endothelin production (Chin et al. 1993). So, HA may not only cause endothelial damage but also enhance blood coagulation and hamper fibrinolysis. Therefore, it is highly possible that HA-metal complexes in the local well water play an important role in the pathogenesis of Blackfoot disease.

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