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STUDIES ON THE BIMODAL EFFECT OF HUMIC SUBSTANCES IN THE BLOOD CLOTTING SYSTEM

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SUMMARY

This study was performed in order to evaluate the dose-dependent effect of different humic acid (HA) preparations on blood coagulation. Using the thrombelastographic method we examined HA from Altteich Peat (Eastern Saxony, Germany), sodium humate from an ombrotrophic mire in Mecklenburg-Western Pomerania, Germany, and the IHSS Reference HA from Waskish Peat (Minnesota, USA). The results revealed a strikingly bimodal behaviour of the tested HA: at high concentrations (250-1000 µg/ml) they prolong the reaction time, i.e. the period of time necessary until the beginning of clot formation, by two- to twelvefold (anticoagulant effect) whereas they at low concentrations (0.9-31.3 µg/ml) tend to reduce the reaction time by an average of 25 % (procoagulant effect).

KEYWORDS: humic substances, blood coagulation, thrombelastography, bimodal effect

INTRODUCTION

Previous studies have shown that negatively charged polymers such as naturally occurring humic acids (HA) and synthetic HA-like polymers prolong the blood clotting time in vitro and in vivo (Klöcking, 1991; Klöcking et al., 1994). We were able to demonstrate that the anticoagulant effect of HA is based on the inhibition of coagulation factors IIa, VIIa and Xa (Klöcking et al., 2004). The focus of this study is to examine HA of different origin and different kinds of preparation for dose-dependent effects on blood coagulation.

MATERIAL AND METHODS

Test substances

The test substances comprise six HA preparations extracted and in different manner purified from the Altteich peatland in Eastern Saxony (Germany). The HA were extracted with sodium hydroxide at pH 9. Further preparation steps were done in different ways:

HA AM-A-2xW — was precipitated with 0.1 mol/l hydrochloric acid and washed two times with demineralized water.

HA AM-A-10xW — was prepared in the same way, but washed ten times with water.

HA AM-B-2xW	was precipitated with oxalic acid and washed two times with water.
HA AM-B-10xW	was prepared in the same way, but washed ten times with water.
HA AM-C-2xW	was precipitated with citric acid and washed two times with water.
HA AM-C-10xW	was prepared in the same way, but washed ten times with water.

Reference humic substances

IHSS Waskish Peat HA (IR107H) and sodium humate 76 (Na-HA 76) were used as reference humic substances. Waskish Peat HA comes from Sphagnum peat of Pine Island Bog in Koochiching County, Minnesota (USA) and was purchased from the International Humic Substances Society (IHSS). Na-HA 76 was isolated according to Klöcking et al. (1977) from the water of Dierhäger Moor, an ombrotrophic mire of the coastal region of Mecklenburg-Western Pomerania (Germany).

Thrombelastography

Thrombelastography (TEG) first developed by Hartert (1948) is a method for testing the coagulation in the blood or in the blood plasma. The principle of the thrombelastographic measurement has been previously described (Hartert and Schaeder, 1962; Chandler, 1995). In this study we measured the reaction time R – one of the TEG basic parameters – using the Hellige Thrombelastograph D. The reaction time R represents the time until the first evidence of clot formation is detected. It is shortened under *hyper*coagulable conditions and prolonged under *hypo*coagulable conditions, for example by anticoagulants.

Test conditions

A twofold dilution series of the test substances is prepared in Tris-HCl buffer pH 7.4. A volume of 0.1 ml of the test substance dilution and 0.2 ml of human citrate plasma are applied into the special thrombelastography cup, mixed and incubated at 37° C for two minutes. Controls receiving HA-free Tris-HCl buffer instead of the test substance dilution are prepared in the same way. The TEG is started by addition of 0.05 ml $CaCl_2$ (0.05 mol/l). After a running time of 60 minutes, the TEG is evaluated using the Hildgard TEG-Meter. The reaction times in Figure 1-4 represent mean values of at least three independent experiments.

Results

HA were examined in the range of 7.8 to 1000 µg/ml for dose-dependent effects on the reaction time. Figure 1 demonstrates for the Altteich peat HA AM-A-2xW that concentrations of 7.8 to 31.3 µg/ml shorten the reaction time by maximum 18 % of the control, whereas concentrations of 500 and 1000 µg/ml enhance significantly the R-values by six- to twelvefold.

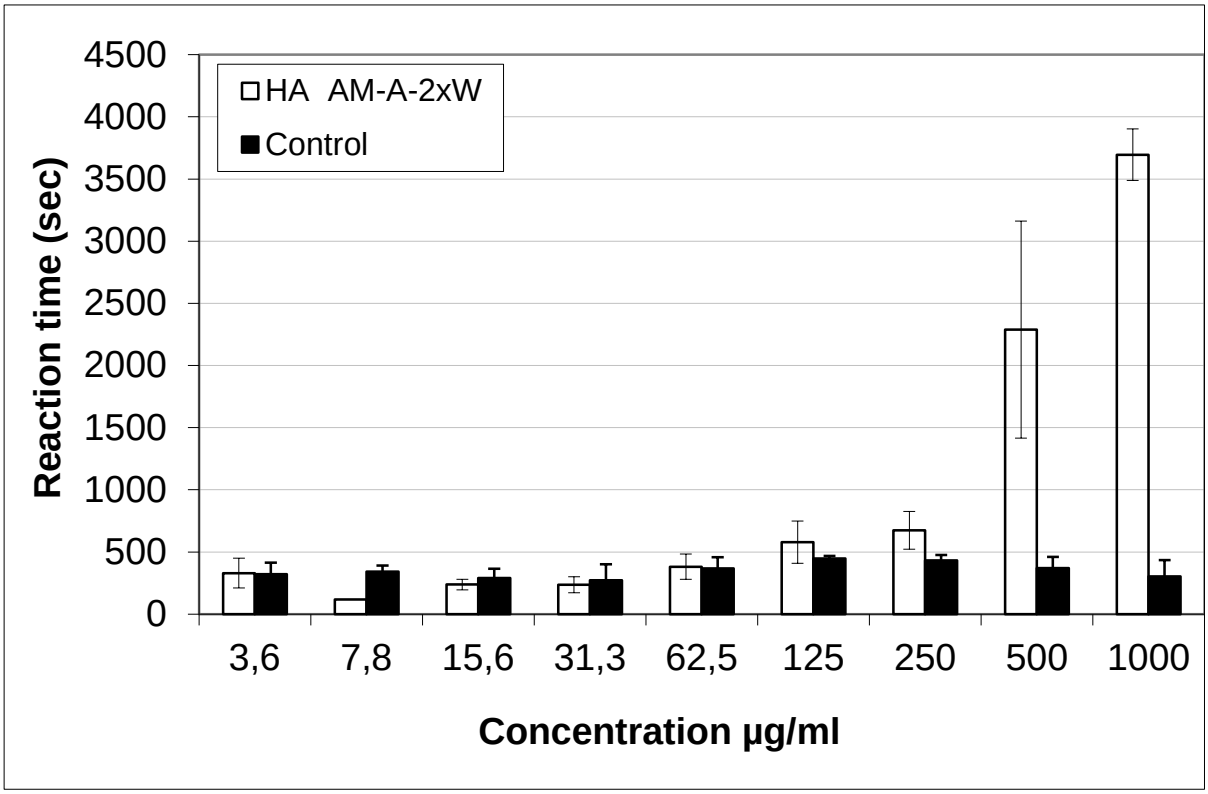

Fig. 1. Influence of Altteich Peat HA *(AM-A-2xW)* on the TEG reaction time in human blood plasma, compared with HA-free controls.

Similar results were received after increasing the number of washing steps from two to 10 (HA-A-10xW) (Fig. 2).

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The Altteich peat HA AM-B-2xW at concentrations of 15.6 and 31.3 µg/ml diminished the reaction time by maximum 25 % of the control, whereas concentrations of 62.5 to 1000 µg/ml enhanced it by two- to 13-fold. Similar results were received after increasing the washing steps from two to 10 (HA AM-B-10xW).

The Altteich peat HA AM-C-2xW at concentrations of 31.3 to 125 µg/ml reduced the reaction time by maximum 46 % of the control. Concentrations of 500 and 1000 µg/ml prolonged it by only 7 %. After increasing the number of washing steps from 2 to 10, both the anticoagulant and the procoagulant effect of HA AM-C-10xW were reduced significantly. The reaction time was shortened only at concentrations of 125 and 250 µg/ml HA by 34 % and 53 %, respectively, and was increased only at a concentration of 1000 µg/ml by 14-fold.
Figure 3 illustrates the effect of Na-HA 76 on the reaction time. At concentrations of 7.8-31.3 µg/ml it reduced the reaction time by maximum 20 % - 40 % and prolonged it at concentrations of 500 and 1000 µg/ml by six- to nine-fold.

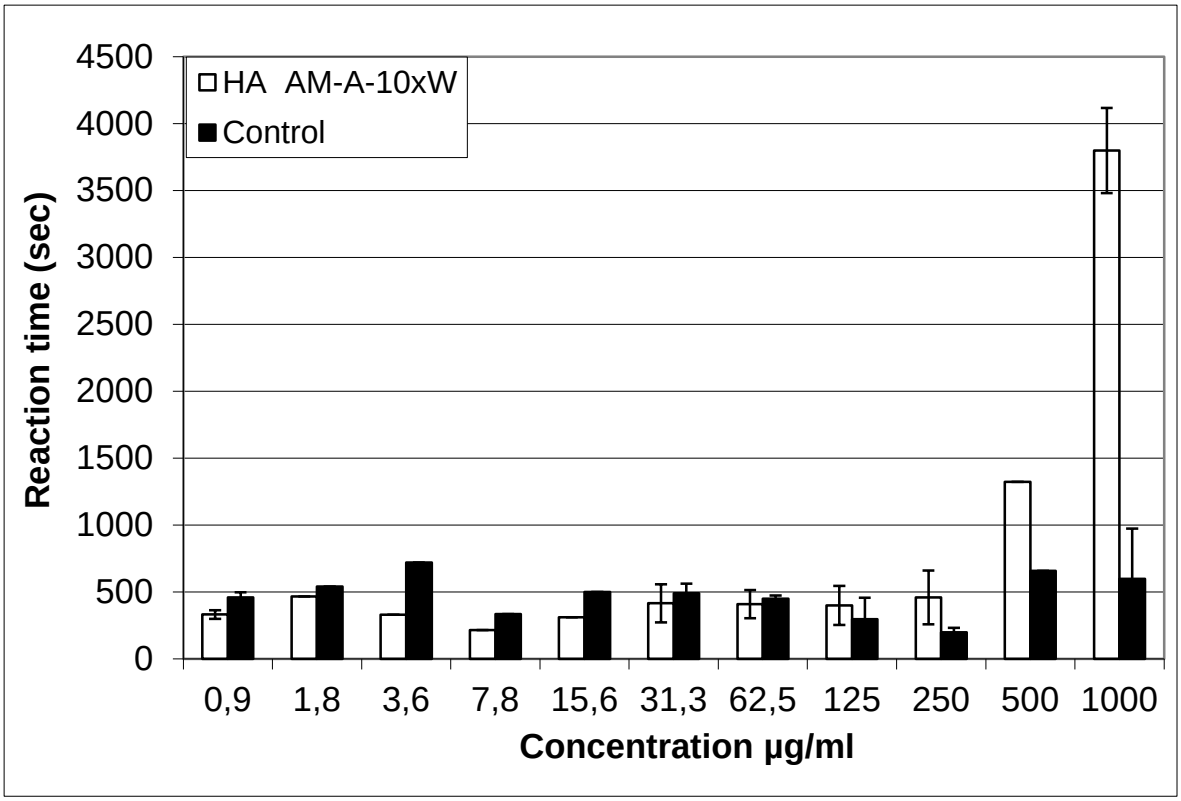

Fig. 2. Influence of Altteich Peat HA *(AM-A-10xW)* on the TEG reaction time in human blood plasma, compared with HA-free controls.

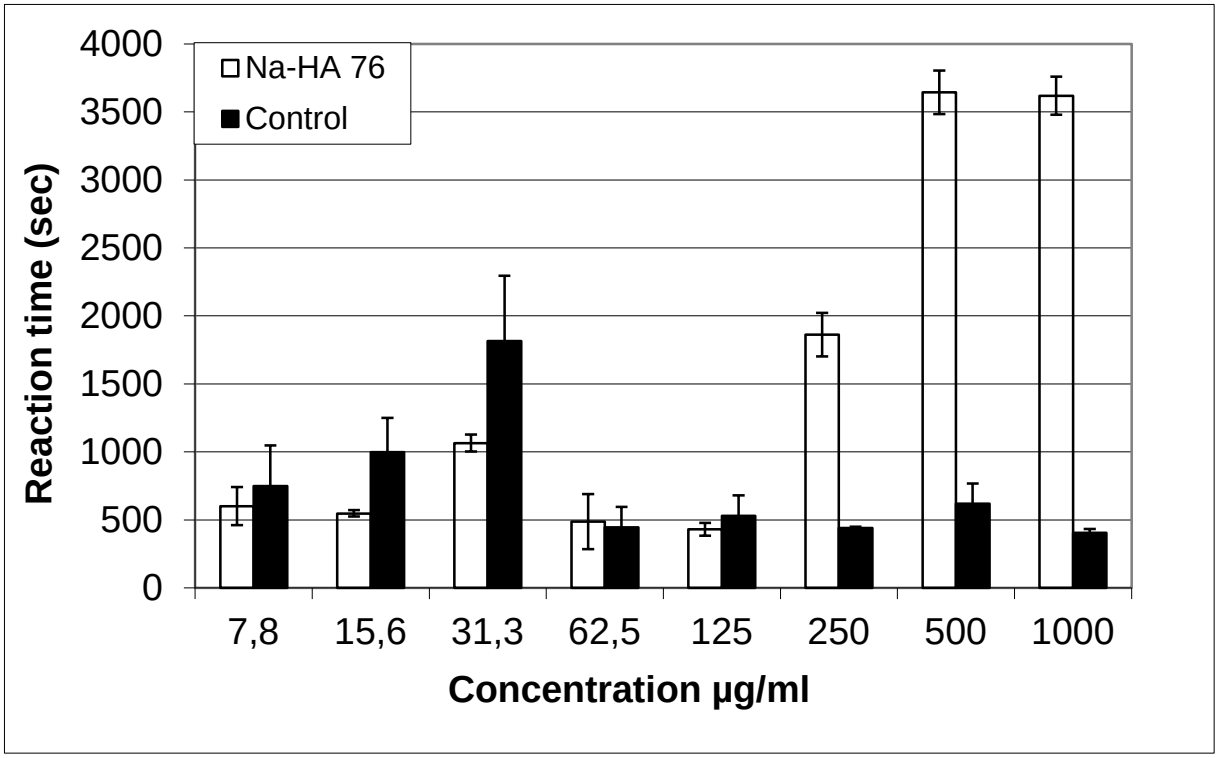

Fig. 3. Influence of sodium humate *(Na-HA 76)* on the TEG reaction time in human blood plasma, compared with HA-free controls.

An analysis of the IHSS reference HA from Waskish peat reveals a different coagulation profile compared to Altteich peat HA and Na-HA. Waskish peat HA at concentrations of 62.5-1000 µg/ml prolongs the reaction time by two to twelve times, i.e. it develops a strong anticoagulant effect over a wide concentration range. In contrast, a significant hypercoagulable state could not be observed (Fig. 4).

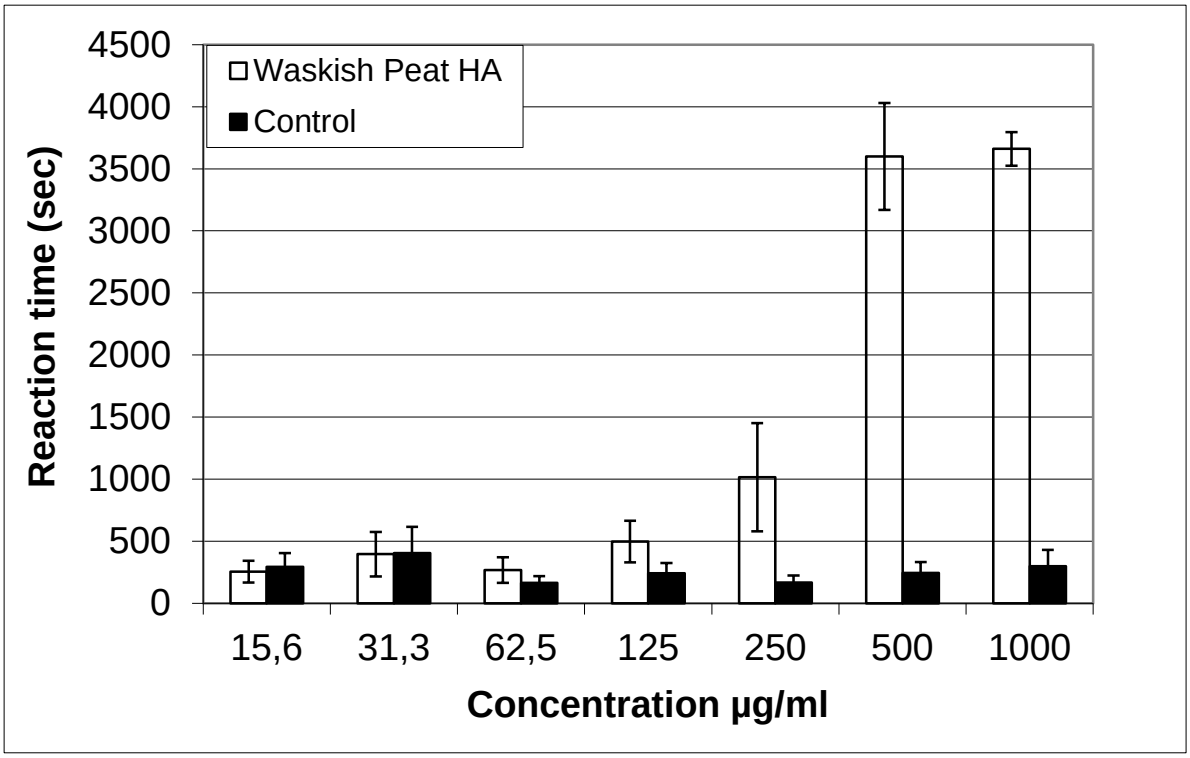

Fig. 4. Influence of the IHSS reference HA from Waskish Peat on theTEG reaction time in human blood plasma, compared with HA-free controls.

DISCUSSION/CONCLUSION

Humic substances represent a class of multifunctional polyanionic compounds having significant impact on living organisms. Based on their heat-storing, anti-infectious, anti-inflammatory and some other health-promoting properties they are widely used in veterinary medicine including aquaculture. It has also been known for a long time that humic substances affect blood coagulation, however, the predominating activity – procoagulant vs. anticoagulant – is still a matter of debate.

Despite differing origin and preparation methods used, the majority of humic substances tested in our experiments present a uniform picture characterized by a striking bimodal behaviour of the tested HA. At high concentrations (250-1000 µg/ml) they prolong the reaction time by two- to twelvefold indicating an anticoagulant effect whereas they at low concentrations (0.9-31.3 µg/ml) tend to reduce the reaction time by an average of 25 % which indicate a procoagulant effect.

From the pharmacological point of view, Altteich peat HA and Na-HA from Dierhäger Moor may work as both styptic (procoagulant) and anticoagulant agents. The observed concentration-dependent opposite behaviour of bioactive substances is known from literature as bimodal effect of humic substances (Junek et al., 2009). It is the first that such a bimodal effect of humic substances was demonstrated in blood coagulation. In this way, our discovery could provide the answer to a long standing discussion about the predominating activity of humic substances in blood coagulation. It will be an issue for further studies to elucidate the mechanism of the observed effect.

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