Studies on Hemorrhagic Diathesis of Experimental Bovine Bracken Poisoning

II. Heparin-Like Substance Level in Blood

Otohiko YAMANE, Takatoshi HAYASHI, Satori SAKO, Seijiro TATEMATSU, Kenzo TAKEDA and Hiroshi FUKUSHIMA

Department of Veterinary Internal Medicine, Faculty of Agriculture, Tottori University, Tottori-shi, Tottori 680

(Received for publication December 11, 1974)

Abstract. Quantitative determination of the blood level of a heparin-like substance was made using a group of three calves with experimentally-induced bracken poisoning. The average blood level of the heparin-like substance in eight normal calves was 3.2 ± 0.95 µg/ml (mean ± s.d.). The blood level of the heparin-like substance exceeded 6.0 µg/ml in all the affected animals in the hemorrhagic stage. It did not increase in the initial stage, when the clotting time prolonged mildly. This was due to the effect of a decrease in the platelet count observed in this stage. It was very likely that the prolonged clotting time in the hemorrhagic stage might have been caused by the synergistic effect of an increase in the blood level of the heparin-like substance and the stimulated sensitivity of the affected blood to the heparin-like substance as a result of the decrease in the platelet count.

Prolongation of blood-clotting time is well known to occur in bovine bracken poisoning. The authors [18] pointed out that a decrease in number and function of platelets, an increase in amount of a circulating anticoagulant agent, and the inactivity of the coagulant factor produced in the liver played an important role in the hemostatic disorders of this disease. In the previous paper, it was demonstrated that the circulating anticoagulant agent which increased in amount in the late stage of this disease was a heparin-like substance. The present investigation was performed to confirm directly an increase in the blood level of the heparin-like substance in the case of experimental bovine bracken poisoning.

Materials and Methods

Three calves shown in Table 1 were fed approximately 2.0 kg of fresh fronds of bracken per head per day. The experimental methods used are shown in Table 2. Hematological routine tests were performed at intervals of three days to a week. Quantitative determination of the heparin-like substance was made by Murakami and co-worker’s method (improved Bassioni-Warren technique).

In addition, a group of normal eight calves was used for obtaining the average value of the blood level of the heparin-like substance.

Results

1. Clinical and hematological findings

These findings are shown in Figs. 1, 2 and 3. In calf No. 21, edema and hyperemia began to be seen in the visible mucous membrane on the 12th day and became remarkable on the 22nd day. Anorexia began to
be noticed on the 51st day. On the 53rd day three petechiae of rice grain size were recognized in the conjunctiva, as well as nasal discharge was. Stagerring appeared on the 54th day. Three miliary petechiae were seen following a decline of general symptoms on the 55th day, when the animal was sacrificed. Hematologically, platelets began to show a tendency to decrease in count on the 28th day. The animal suffered from thrombocytopenia exhibiting a gradually decreasing curve. The decreasing curve of the leukocyte count was approximately linear like that of the platelet count. Red cells decreased a little in count only in the serious stage.

In calf No. 22, edema was seen in the conjunctivae on the 9th day and hyperemia on the 12th day. Petechiae were found scattered in the vaginal mucosa on the 53rd day. Anorexia and nasal discharge appeared on the 55th day following a loss of vigor. The animal died on the 57th day. Hematologically, platelets began to decrease in count on the 18th day and leukocytes on the 23rd day. Thereafter thrombocytopenia

<table>
<thead>
<tr>
<th>Calf No.</th>
<th>Breed</th>
<th>Sex</th>
<th>Age (months)</th>
<th>Body weight (kg)</th>
<th>Experimental period</th>
<th>Date of death</th>
</tr>
</thead>
<tbody>
<tr>
<td>21</td>
<td>Holstein</td>
<td>Female</td>
<td>6.5</td>
<td>133</td>
<td>15 June- 8 Aug., 1973</td>
<td>8 Aug. R</td>
</tr>
<tr>
<td>22</td>
<td>Holstein</td>
<td>Female</td>
<td>6.5</td>
<td>152</td>
<td>15 June- 9 Aug., 1973</td>
<td>9 Aug. T</td>
</tr>
</tbody>
</table>

Table 2. Experimental methods

<table>
<thead>
<tr>
<th>Test performed</th>
<th>Methods used</th>
</tr>
</thead>
<tbody>
<tr>
<td>Symptoms</td>
<td>Every day</td>
</tr>
<tr>
<td>RBC</td>
<td>Electronic cell counting</td>
</tr>
<tr>
<td>WBC</td>
<td>Thoma-Zeiss</td>
</tr>
<tr>
<td>Platelets</td>
<td>Ress-Ecker</td>
</tr>
<tr>
<td>Whole blood clotting time</td>
<td>Lee-White</td>
</tr>
<tr>
<td>Heparin level</td>
<td>Bassiouni-Warren (Murakami et al.)</td>
</tr>
<tr>
<td>Plasma recalcification time</td>
<td>Citrated plasma</td>
</tr>
</tbody>
</table>

Fig. 1. Symptoms and changes in blood in calf No. 21
and leukopenia occurred with a gradual decrease in both cells. Red cells decreased a little in count only in the serious stage.

In calf No. 23, edema hyperemia were seen in the conjunctiva on the 39th day. A few miliary petechiae were scattered in the conjunctiva on the 53rd day. Petechiae appeared also in the gingiva and vagina on the 56th day, by which time nasal discharge and anorexia had been noticed. The animal was sacrificed on the 58th day. Hematologically, platelets began to decrease on the 8th day and leukocytes on the 33rd day. Thereafter, thrombocytopenia and leukopenia occurred with a gradual decrease in both cells. The red cell count showed no definite tendency.

2. Determination of blood clotting time and heparin-like substance level

The results obtained are shown in Fig. 4, 5 and 6. Blood clotting time before receiving bracken was 19'30", 16'00" and 22'00" for Calves 21, 22 and 23 respectively. A mild prolonged clotting time was seen in Calf 22 after the 34th day but never in Calves 21 and 23 until the appearance of
petechiae in the mucous membrane. Thereafter, the prolongation of clotting time occurred in all the animals at the same time with the appearance of petechiae.

Recalcification time before receiving bracken was 108", 105" and 108" for Calves 21, 22 and 23, respectively. The clotting time was prolonged mildly in all the animals in the initial stage (edema and hemorrhage) and markedly in the hemorrhagic stage.

The heparin-like substance level was 2.6 to 3.5μg/ml before receiving bracken and showed no tendency to increase in any animal until the appearance of petechiae. On the 41st day a mild increase in level was seen only in Calf 21. In Calves 21, 22 and 23, the heparin-like substance level increased to 6.1, 6.6 and 3.7μg, respectively, in the hemorrhagic stage and to 5.6, 6.8 and 4.7μg in the serious stage. The average heparin-like substance level in a group of 8 normal calves was 3.2±0.95μg/ml (mean ± standard deviation).

3. Effects of heparin level and platelet count upon blood clotting time

Heparin level in blood and platelet count were examined in vitro for effect upon blood clotting time by means of a procedure. In this procedure, plasma heparin levels along with varying platelet counts were determined by estimating recalcification time. The results are shown in Fig. 7.

In blood plasma with no heparin added, the clotting time did not change until the platelet count decreased to 15×10^4/mm³.
After the platelet count reached $10 \times 10^4$/mm$^3$, it was prolonged in accordance with the decrease in platelet count. When heparin was added to the blood plasma, the clotting time was prolonged in good correlation with the dose of heparin added. Its prolongation was especially remarkable in the plasma containing less than $10 \times 10^4$ platelets per cubic millimeter.

**Discussion**

An increase in blood heparin level was reported by Evans and Howell [9] in a calf fed the alkaline ether-soluble fraction of bracken fronds by drench and by Evans [10] in a calf fed dried bracken fronds. In our previous paper, the authors demonstrated an increasing curve in the circulating anticoagulant test and suggested an increase in blood heparin level. These findings, how-
ever, were not explained on the basis of results obtained by quantitative determination.

In the present experiment, the blood level of heparin-like substance was determined quantitatively in 3 calves affected with experimentally-induced bracken poisoning.

Two techniques are known for the quantitative determination of heparin. The one [12, 14] is based on the coagulation system and the other [3, 4, 8, 13, 16] is not. The former is extremely limited in use and has some drawbacks, because it cannot be applied to a very small amount of blood, or no change in any coagulant system except a system of heparin is required. On the contrary, the latter technique is never vulnerable to the change of coagulant factors, but gives a correct heparin level, although it has a drawback to detect chondroitin sulfate together. Such chondroitin sulfate, however, is negligible, since its absorption coefficient is much lower than that of heparin.

From these facts, it is known that the latter technique [16] is the more reliable one. In the present experiment, this technique was used. Blood heparin levels of animals were studied by Eiber and Danishefsky [8] and Kimura [13] (5μg/ml in the dog) and detected by Allen et al. [2], but the present paper is the first to report the bovine blood heparin level. It was found that the free heparin level exceeded 6.0μg/ml in the hemorrhagic stage in all the animals examined in the present experiment. Since these levels are apparently higher than those determined in a group of 8 normal calves and another group of 3 calves before feeding bracken, it is evident that there was an increase in the heparin-like substance level in calves fed this plant.

The inhibition of thromboplastin formation [17] (the first phase of blood coagulation) and antithrombin activity [1], which inhibits the fibrinogen-fibrin transformation (the second and third phase of blood coagulation) are known to be induced by heparin activity in the mechanism of blood coagulation. Concerning this point, the authors [18] previously demonstrated the presence of anti-tissue thromboplastin and anti-plasma thromboplastin in calves fed bracken fern.

Whole blood clotting time and recalcification time are generally used for a screening test of blood coagulation disorders. It is known that if there are disorders in some coagulant factors, prolongation will occur both clotting times, especially to recalcification time [15]. It is expected that recalcification time will be prolonged at least when the blood heparin level increases. In the present experiment, both times were prolonged in the initial stage (edema and hyperemia), inspite of the absence of an increase in heparin level. This result indicates that the prolonged clotting time in the initial stage was due to the effect of the decrease in platelet count.

It is anticipated that the marked prolonged clotting time in the hemorrhagic stage may depend not only upon the increase in heparin-like substance level but also upon thrombocytopenia associated. This can be explained by two facts that a marked thrombocytopenia is present in the hemorrhagic stage of this disease, and that when there is a decrease in antiheparin activity [7] of the fourth platelet factor associated with thrombocytopenia, the blood becomes more sensitive to heparin. Conley et al. [5, 6] demonstrated that the heparin level required for inhibition of blood plasma coagulation was determined by the platelet count of the blood plasma. From the results of the present experiment in vitro, it was confirmed in bovine blood plasma that the prolongation of clotting
time began when the platelet count decreased to less than $10 \times 10^4$/mm$^3$, and that a gradual addition of heparin caused a marked prolongation of clotting time rapidly.

The results of an experiment in vitro cannot always be applied directly to the living organism. It is very likely, however, that the prolongation of clotting time in the hemorrhagic stage of a calf fed bracken may have been caused by the synergistic effect of an increase in heparin-like substance level and an enhancement of the sensitivity of blood to heparin, which depended upon the decrease in platelet count. In the present experiment, all the animals in the hemorrhagic stage showed a platelet count of less than $10 \times 10^4$/mm$^3$ following a rapid prolongation of clotting time. Critical thrombocytopenia has frequently occurred also in the field case of bovine bracken poisoning in the late stage of disease.

References


