The Hemolytic Activity of Bracken Extracts in Guinea Pigs

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ABSTRACT. This study was conducted to elucidate the hemolytic activity of a new toxic substance in bracken fern. A crude extract (CE) was prepared from the methanol extracts of bracken by the column chromatography. When the CE was injected subcutaneously in guinea pigs, the hemoglobinuria and hemolysis were observed within 6 hr, and 3 days later edema and hemorrhages in the urinary bladder were observed. The CE was then fractionated by high performance liquid chromatography (HPLC), and three (HF, BF and CF) of the fractions showed the toxic activities in guinea pigs. The HF caused the hemolysis, whereas both the BF and the CF caused the hemorrhagic cystitis without any hemolytic activities. The HF was further fractionated by the HPLC, resulting of the 3 fractions (HF-I, II and III). The hemolysis was caused only with the HF-II, and HF-II as well as HF did not cause the hemorrhagic cystitis. HPLC analysis revealed that both BF and CF contains braxin B and braxin C, respectively, and both HF and HF-II do not contain braxin A, B or C. These facts suggest that bracken fern contains a new toxic substance (hemolysin) which induces the acute hemolysis in guinea pigs.—KEY WORDS: bracken fern, guinea pig, hemolysin, hemolytic effect.

Bracken fern, *Pteridium aquilinum* (L) Kuhn, is known to induce serious poisoning in livestock and experimental animals. Among the experimental animals, guinea pigs have been reported to be the most susceptible to bracken poisoning with hematuria, edema and hemorrhage in the urinary bladder similar to that observed in cattle [6, 10–12, 15, 21].

In the previous studies we have reported that 3 glycosides (braxin A, B and C) isolated from methanol extracts of bracken fern caused hemorrhagic cystitis in the guinea pig urinary bladder [10–13, 21]. Recently we found that when the crude extract (CE) of bracken fern was injected subcutaneously (s.c.) in guinea pigs, it often caused hemoglobinuria during 1–5 hr after the injection. Blood examination shows that the hemolysis occurred during the period, but the hemolysis was not induced by s.c. injection of braxin A, B or C. These findings suggest that bracken fern may contain additional toxic substance(s) which induce vascular hemolysis and hemoglobinuria.

In order to confirm this possibility, we tried to isolate the hemolytic substance in bracken fern and the hemolytic effect was compared with that of saponin in guinea pigs.

MATERIALS AND METHODS

**Animals**: Male Hartley guinea pigs weighing 200–250 g were used. The animals were given chow pellets (CG-3 Nihon Clea, Japan) and tap water *ad libitum*, but fasted for 24 hr before the experiment, except for drinking water.

**Preparation of bracken extracts**: Fronds of bracken fern were collected at Hyoen pasture in a suburban area of Tottori prefecture in Japan. The fronds were immediately freeze-dried at -20°C, followed by a freeze-drying. The dried materials were stored at -20°C. The freeze-dried bracken fern was hashed in ice-cold methanol containing 0.5% acetic acid and was applied to column chromatography with RP-18 (Lobar, Merck, Darmstadt, Germany) eluted with methanol and 0.01% acetic acid to obtain CE. The CE was separated into fractions using the high performance liquid chromatography (HPLC) with a preparative column of ASAHIPAK HIKARISIL-C18 (20 × 250 mm, Asahi Chemical Industries Ltd., Tokyo, Japan) and TSK-GEL ODS-80TM (21.5 × 300 mm, Tosoh, Tokyo, Japan), and was filtered with suction. The methanol extract was washed repeatedly with hexane and ether, and was applied to column chromatography with RP-18 (Lobar, Merck, Darmstadt, Germany) eluted with methanol and 0.1% acetic acid to obtain CE. The CE was separated into fractions using the high performance liquid chromatography (HPLC) with a preparative column of ASAHIPAK HIKARISIL-C18 (20 × 250 mm, Asahi Chemical Industries Ltd., Tokyo, Japan) connected to INERTSIL PREP-ODS (20 × 50 mm, Gasukuro Kogyo, Tokyo, Japan), eluted with methanol and 0.02% acetic acid (3.5:6.5). The eluates were collected using a fraction collector and then divided into 9 fractions (Fig. 1). Each of the fractions was evaporated to dry and was preserved in methanol containing 0.2% acetic acid at -20°C for the examination of their toxic activities.

The fraction showed the hemolytic activity was further fractionated by using HPLC with a preparative column of INERTSIL PREP-ODS (20 × 250 mm, Gasukuro Kogyo, Tokyo, Japan) connected to INERTSIL PREP-ODS (20 × 50 mm, Gasukuro Kogyo, Tokyo, Japan) and TSK-GEL ODS-80TM (21.5 × 300 mm, Tosoh, Tokyo, Japan), eluted with methanol and 0.02% acetic acid (4:6).

**Assessment of the toxic activities in guinea pigs**: The samples were vacuum dried and dissolved in 1 ml of ice cold physiological saline. Then they were filtered through a sterile membrane filter (0.22 μm, Sartorius, Goettingen, Germany), and were injected subcutaneously into guinea pigs. Control animals were treated with saline. The hemolysis was examined by measuring the plasma
hemoglobin concentrations in treated animals. Blood samples were collected every 1 hr until 6 hr after the injection. Since the repeated blood collection is limited in guinea pigs and only a small amount of samples is available, the plasma hemoglobin concentrations were determined according to the method recently developed by us [published in this issue of J. Vet. Med. Sci.] which enables us to collect blood repeatedly and allowed us to measure the plasma hemoglobin concentration in small amount of sample (10 µl). The animals were observed for the occurrence of hemoglobinuria and other symptoms. The macroscopic changes in the urinary bladder were carefully observed immediately after the animals were sacrificed by bleeding under ether anesthesia on the 3rd day. In addition, the erythrocytes in treated animals were examined microscopically for the occurrence of Heinz body with the new methylene blue stain.

In vitro assessment of the hemolytic activity: The in vitro hemolytic activities of the bracken extracts were examined and the effect was compared with that of saponin (Wako Pure Chemicals Industries, Ltd., Osaka, Japan). Blood samples (10 ml) were collected from guinea pigs using a heparinized disposable syringe, and centrifuged at 1,000 x g for 15 min (Hitachi 05PR-22, Tokyo, Japan). After the plasma and the buffy coat were gently removed, the red blood cells (RBC) were washed several times with physiological saline. The final RBC suspension was adjusted to the original hematocrit value with the saline. After preincubation for 5 min at 37°C, 100 µl of the bracken extracts (HF, HF-II) or saponin solution was added to the RBC suspension (900 µl). In control group, 100 µl of physiological saline or methanol containing 0.1% acetic acid solution was added to the RBC suspension. The RBC suspensions were incubated at 37°C in a shaking incubator (Iuchi SB-20, Osaka, Japan), and the hemoglobin concentrations were measured during incubation for 4 hr. In addition the in vitro hemolytic activity of HF-II were examined in the presence of 10% serum obtained from guinea pigs.

Statistical analysis: Data are expressed as the mean ± S.E. The significance of the differences in two groups was determined by Student’s t-test for unpaired values.

RESULTS

About 0.9–1.2 g of CE was obtained from 100 g of the dried bracken. The toxic effects of the CE were investigated by the s.c. injection in guinea pigs during 3 days. With the CE prepared from 200 g of dried bracken, the plasma hemoglobin concentration (PHC) increased rapidly and reached the maximum at 1 hr, and then gradually decreased until 6 hr (Fig. 2). At 1 hr, the PHC increased significantly to 394 ± 21 mg/dl (n=4), whereas in saline control it was 28 ± 7 mg/dl (n=4) as shown in Fig. 3. The hemoglobinuria was observed within 5 hr after the injection, and the hematuria and the hemorrhagic cystitis were observed after 2 or 3 days.

The CE was separated into the 9 fractions by the HPLC with the preparative columns (Fig. 1), and the toxic activities of the each fraction were examined in guinea pigs. It was found that three (HF, BF and CF) of the fractions showed either the increase of PHC or hematuria and hemorrhagic cystitis. With the HF prepared from 200 g of dried bracken, the PHC was changed similarly to that with CE with time (Fig. 2), and at 1 hr after the injection it increased significantly to 349 ± 39 mg/dl (n=4) as shown in Fig. 3. The hematuria and hemorrhagic cystitis were not observed with the HF. The yield of the HF was about 0.045-0.047% of the dried bracken. Both the BF and the CF did not cause the hemolysis (Figs. 2 and 3), but caused the hematuria and
hemorrhagic cystitis. On the HPLC analysis, the braxin A, B and C was not in the HF, whereas the braxin B and braxin C was in the BF and CF, respectively (data not shown).

The HF was further fractionated by the HPLC, resulting in 3 fractions (HF-I, HF-II and HF-III) as shown in Fig. 4. With the HF-II prepared from 200 g of the dried bracken, the PHC was changed similarly to those with CE and HF with time (Fig. 5), and at 1 hr after the injection it increased significantly to $277 \pm 84$ mg/dl (n=3), whereas the HF-I and III were in the control level of $29 \pm 07$ mg/dl (n=3) as shown in Fig. 6. The yield of the HF-II was about 0.017–0.018% of the dried bracken.

In contrast with HF and HF-II, the s.c. injection of saponin (up to 100 mg/kg) did not caused the hemolysis. In the in vitro studies with the guinea pig RBC showed that HF (4 mg/ml) and HF-II (2 mg/ml) did not cause hemolysis during 4 hr incubation, whereas saponin at concentration of 0.1 mg/ml caused obvious hemolysis within several minutes. The in vitro hemolytic activity also were not observed after the HF-II were incubated in the presence of serum obtained from guinea pigs (Fig. 7).
In the microscopic examinations, Heinz body was not observed in the erythrocytes of guinea pigs treated with the bracken extracts (CE, HF and HF-II) during the experimental period.

**DISCUSSION**

In previous papers we have reported that braxin A, B and C isolated from bracken fern induced hematuria and hemorrhagic cystitis in guinea pigs [10–13, 21]. But there are few reports on hemolytic toxicity of bracken fern. In the present study, it was clearly demonstrated that the bracken extracts caused hemolysis in guinea pigs after the s.c. injection and that bracken fern contains a new toxic substance (hemolysin) which is different from braxin A, B and C.

The crude extracts (CE) prepared from methanol extract of dried bracken was fractionated by HPLC (Fig. 1), and three fractions (HF, BF and CF) were found to have toxic activities in guinea pigs. Among the fractions only the HF showed the hemolytic effect, whereas both BF and CF caused hematuria and hemorrhagic cystitis. In addition, HPLC analysis confirmed that the HF does not contain braxin A, B or C, and BF and CF contains braxin B and braxin C, respectively. These findings show that the CE was successfully separated into the 3 fractions containing the hemolysin (HF), braxin B (BF) and braxin C (CF).

The HF was further purified by HPLC, resulting in 3 fractions (HF-I, II and III). The hemolysis was observed in the animals given the HF-II, but not by HF-I or HF-III. None of the fractions (HF-I, II and III) caused the hemorrhagic cystitis 3 days later. Since the HF-II still contained several compounds in the detailed HPLC analysis (data not shown), further fractionations and toxicity studies were conducted. However, at present, we have not been able to isolate the hemolysin. On the HPLC with the ODS columns, the HF and HF-II, which contains the hemolysin, were eluted faster than BF and CF. This suggests that the hemolysin in bracken is more polar and hydrophilic than braxin B and braxin C, and that the isolation of the hemolysin seems to be difficult.

There are many kinds of plants which cause hemolysis in animals, and saponins have been identified as the hemolysin [1, 5, 9, 14]. In the present study, however, saponin up to 100 mg/kg did not cause the hemolysis by s.c. injection in guinea pigs, though it caused severe hemolysis by intravenous injection as reported by us [published in this issue of J. Vet. Med. Sci.]. Additionally, in the *in vitro* experiments, saponin (0.1 mg/ml) caused the hemolysis obviously, whereas the hemolytic activity was not observed with HF-II (2 mg/ml) even in the presence of serum (Fig. 7). These results suggest that the properties of bracken hemolysin is different from that of saponin used in the present study and that the bracken hemolysin induces the hemolysis by indirect actions or its metabolite(s).

It has been reported that onions induce hemolytic anemia in canine and other domestic animals [4, 7, 8, 18], and three hemolytic compounds isolated from onions [19] have been reported to cause the Heinz body formation. It was also reported that the forage brassicas caused hemoglobinemia with the Heinz body formation in the ruminants such as cattle and sheep [2, 3, 9, 16]. The Heinz body is lead by the production of the methemoglobin and superoxide. The methemoglobin produced is susceptible to further oxidation and denaturation, resulting in the formation of Heinz body [7, 8, 17, 20]. In the present study, with the CE, HF and HF-II the Heinz body was not observed in the erythrocytes during the experimental periods. Therefore, it is suggested that the mechanism for the hemolysis by the bracken is different from those by onions and forage brassicas. Further investigations concerning both the properties of the hemolytic substance(s) in bracken and the mechanism of the hemolysis will be necessary.

We thus conclude that bracken fern contains a new toxic substance (hemolysin), which is different from braxin A, B or C.

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**REFERENCES**


