Carcinogenicity of the fern *Pteridium aquilinum* collected from enzootic bovine haematuria-free hilly area in India

R. K. Dawra, N. P. Kurade* and O. P. Sharma
Indian Veterinary Research Institute, Regional Station, Palampur 176 061, India

Chronic exposure in low doses to bracken fern (*Pteridium aquilinum*, family Pteridaceae) has been associated with urinary bladder tumours and intermittent haematuria in bovines of hilly areas. In spite of the widespread distribution of the fern in the Himalayas and southern hills of the country, the disease is restricted to well-defined pockets. The possible reason for absence of the disease in other hilly areas even in presence of *P. aquilinum* could be due to lack of carcinogenic potential in the plant growing in such areas. In the present study, the fern was collected from an area where cases of bovine urinary bladder cancer and associated haematuria (enzootic bovine haematuria) have not been recorded. In these areas animals are left for grazing during rainy season. This fern was fed to guinea pigs for 30 months at the rate of 30% (w/w). The fern caused toxic and neoplastic changes in urinary bladders of the exposed animals. The incidence of tumours was 18.6%. The fern was found positive for the carcinogen ptaquiloside (3.74 ± 0.6 mg/kg). The low level of ptaquiloside as compared to the values reported from elsewhere (0 to 9,776 mg/kg) in the fern was found positive for ptaquiloside and caused toxic and neoplastic changes in the urinary bladders of the exposed animals. *Pteridium aquilinum* was collected from the forest areas near Palampur (32°06′N; 76°32′E) during July–August and dried in shade. At the collection stage, the fern fronds were fully opened but the spores formation had not started. The dried fern was ground and stored in polyethylene bags. From the pooled, thoroughly mixed ground bracken fern powder, three samples were collected for ptaquiloside analysis. Thirty-two healthy male guinea pigs obtained from the institute laboratory animal colony were divided into two groups of 16 each. One group (control) was given normal guinea pig feed (containing recommended nutritional requirements for guinea pig, compounded using ingredients like maize, oilseed cake, gram, wheat bran, vitamin and mineral supplements), while the other group (experimental) was fed ration in which bracken fern powder was mixed at the rate of 30% (w/w). The proportion of various ingredients in the experimental feed was adjusted so that the final composition after mixing fern was comparable to the normal feed. The animals of both the groups were fed in this way for 30 months. Thereafter, the animals in both the groups were kept on normal guinea pig feed for 24 months. Overnight urine was collected at monthly intervals using metabolic cages and was screened for presence of erythrocytes by microscopy of urine sediments. Any animal which died during the experiment and those killed (using overdose of diethyl ether) at the end of the experiment were subjected to detailed necropsy. Urinary bladders were fixed by injecting 4% buffered formalin and other affected tissues were collected in 10% formalin. The tissues were then processed following routine histopathology procedures. For animal experiments, CPCSEA and Institute Animal Ethics Committee guidelines were followed.

Ptaquiloside content in the fern powder was estimated by HPLC using Waters HPLC system, with 510 and 515 pumps, rheodyne injector, 490E multichannel detector, C18 reverse phase column (4.6 × 250 mm) and acetonitrile-H2O gradient (20 : 80 for first 20 min followed by increase to 100% acteonitrile in the next 20 min). The biological activity have been isolated from bracken fern. Ptaquiloside content was found to vary (from 0 to 9,776 mg/kg) in the *Pteridium* samples collected from different regions of the world. Variation in the disease incidence in the hilly areas has also been linked to variation in the ptaquiloside content of the fern from different areas. However, the ptaquiloside content, role and carcinogenic capacity of *Pteridium aquilinum* from ‘no disease’ or nonenzootic’ areas in India to cause disease has not been investigated so far. In the present study, we have investigated the carcinogenic potential of *Pteridium aquilinum* collected from an area, where there is no incidence of the disease (nonenzootic areas). The fern was found positive for ptaquiloside and caused toxic and neoplastic changes in the urinary bladders of the exposed animals.

*Pteridium aquilinum* was collected from the forest areas near Palampur (32°06′N; 76°32′E) during July–August and dried in shade. At the collection stage, the fern fronds were fully opened but the spores formation had not started. The dried fern was ground and stored in polyethylene bags. From the pooled, thoroughly mixed ground bracken fern powder, three samples were collected for ptaquiloside analysis. Thirty-two healthy male guinea pigs obtained from the institute laboratory animal colony were divided into two groups of 16 each. One group (control) was given normal guinea pig feed (containing recommended nutritional requirements for guinea pig, compounded using ingredients like maize, oilseed cake, gram, wheat bran, vitamin and mineral supplements), while the other group (experimental) was fed ration in which bracken fern powder was mixed at the rate of 30% (w/w). The proportion of various ingredients in the experimental feed was adjusted so that the final composition after mixing fern was comparable to the normal feed. The animals of both the groups were fed in this way for 30 months. Thereafter, the animals in both the groups were kept on normal guinea pig feed for 24 months. Overnight urine was collected at monthly intervals using metabolic cages and was screened for presence of erythrocytes by microscopy of urine sediments. Any animal which died during the experiment and those killed (using overdose of diethyl ether) at the end of the experiment were subjected to detailed necropsy. Urinary bladders were fixed by injecting 4% buffered formalin and other affected tissues were collected in 10% formalin. The tissues were then processed following routine histopathology procedures. For animal experiments, CPCSEA and Institute Animal Ethics Committee guidelines were followed.

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fern samples were extracted and the extract was partially purified for analysis by the procedure of Olerichs et al. 14. Partial purification of the extract from fern is required to remove the interfering compounds. The ptaquiloside in the extract was converted to pterosin B before analysis.

Grossly, in urinary bladder congestion, oedema and haemorrhages to varying extent were observed in exposed animals. In three animals the wall of the bladder was thickened at some places and the mucosa revealed ulceration and severe congestion and haemorrhages. In other organs mainly congestive lesions were observed.

The histopathological changes observed in the urinary bladder and intestine of the exposed animals are summarized in Table 1. The animals (n = 3) dying between 0 and 16 months of exposure did not show any change in the intestinal mucosa. But in these animals, mild to severe congestion along with oedema and haemorrhages were observed in the lamina propria of urinary bladder (Figure 1 a). The urothelium revealed areas of desquamation and mild proliferation (Figure 1 a). The animals (n = 5) dying between 17 and 30 months also did not show any changes in the intestines except one animal where proliferative changes were observed in the caecum. Changes in urinary bladder were similar to those described for animals which died between 0 and 16 months. In one animal urinary bladder revealed irreversible type of hyperplastic urothelium (Figure 1 b).

Animals (n = 8) dying or sacrificed between 31 and 54 months showed changes both in intestine as well as in urinary bladder. In three animals, proliferative changes were observed in mucosal lining epithelium of intestine. One animal revealed proliferative changes in ileal and the other in caecal mucosal lining epithelium. Squamous metaplasia of ileal lining cells was observed in the third animal. Proliferation of goblet cells and congestion in lamina propria along with inflammatory cell infiltration were observed in most of the animals. Almost all the animals exposed to the fern revealed mild to severe changes of congestion, oedema and extravasation of erythrocytes in the lamina propria of urinary bladder.

Six animals (including animal No. 8 killed on 30 months) revealed irreversible type of proliferative changes in the transitional epithelium. The hyperplastic urothelial cells and their nuclei were uniform in shape and contained variable number of nucleoli (Figure 1 c). Blood capillaries were seen growing in the urothelium at some places (Figure 1 d). The urothelial proliferative changes were also associated with desquamation at some places. The lamina propria beneath the proliferating epithelium was infiltrated with mononuclear cells mainly lymphocytes, plasma cells and occasionally macrophages (Figure 1 c, d).

Three animals revealed malignant transformation in the lining epithelium of bladder mucosa in the form of adenocarcinoma in two animals and transitional cell carcinoma in one animal. The neoplastic growth in case of adenocarcinoma had invaded the lamina propria in one case (Figure 2 a, b) and muscular layer in another (Figure 2 c). The cellular reaction was more prominent in the former than in the latter. The animal with transitional cell

<table>
<thead>
<tr>
<th>Animal no.</th>
<th>Died/ sacrificed#</th>
<th>Vascular changes</th>
<th>Neoplastic changes</th>
<th>Lesions in intestine</th>
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<tr>
<td></td>
<td></td>
<td>Congestion</td>
<td>Oedema</td>
<td>Haemorrhage</td>
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<td>P</td>
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<tr>
<td>5</td>
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<td>54*</td>
<td>Mild</td>
<td>A</td>
<td>P*</td>
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A, Absent; P, Present; AC, Adenocarcinoma; TCC, Transitional cell carcinoma; EPC, Epithelial proliferative changes; IC, Inflammatory change; #, time in months after start of feeding; *, Killed, other animals died during course of experiment; †, Developed haematuria. AC of sebaceous gland observed in animal No. 11.
carcinoma revealed severe proliferation of transitional epithelium in the form of sheet (Figure 2d) and the growth was infiltrated in the lamina propria. The urothelium of control animals did not reveal any histological changes.

The hepatic changes were highly variable in different animals. Half of the animals exposed to the fern revealed severe congestion of sinusoids and periportal vessels. Hepatocytes revealed mild to severe degenerative
changes in most of the animals and also fatty change in a few of them. In some animals necrosis of the hepatocytes was evident in the periportal as well as in midzonal area. The animals which died or were killed in the later part of the experiment also revealed proliferation of the lining epithelium of bile ducts.

Myocardium of a few animals revealed mild to moderate degenerative changes and congestion in most of the animals. Lungs in most of the animals revealed severe alveolar congestion along with haemorrhages and oedema in a few. In most of the animals there was perivascular and peribronchial infiltration with mononuclear cells. Areas of severe emphysema and collapse were also observed in most of the animals.

Essentially, all the animals revealed severe congestion in the cortex of the kidneys. A few of them also revealed congestion in medulla and pelvis. In one animal the epithelial lining cells of pelvis revealed proliferative changes and in the other there was severe congestion and oedema along with extravasation of erythrocytes in the subepithelial tissue of pelvis. One animal (No. 11) revealed adenocarcinoma of sebaceous gland. In the control group no significant histopathological changes were observed.

During the exposure period, screening of urine samples showed microhaematuria in one animal (Animal No. 9; Table 1). Three more animals developed haematuria between 31 and 54 months (Table 1).

The fern used for feeding was analysed for ptaquiloside/kg fern powder. The fern sample used was analysed for ptaquiloside and was found to contain 3.74 ± 0.6 mg of ptaquiloside/kg fern powder.

EBH is a major disease of cattle and buffaloes in hilly areas of the country. The incidence of the disease varies from 0.8 to 11.5% in the different areas. No treatment is available and the disease continues to be a major animal health problem in the affected areas. It adversely affects the productivity and economy of the region. Understanding all aspects related to the aetiology of the disease can help in evolving strategies for control of the disease. The occurrence of the disease has been associated with prolonged exposure to P. aquilinum in low doses. In the hilly areas there are pockets where P. aquilinum is present in the pastures but disease is absent. One of the reasons could be lack of carcinogenic potential of P. aquilinum present in these areas. The present study was undertaken to answer this question. In this study exposure of experimental animals to P. aquilinum caused changes in urinary bladder and ileocaecal region of the intestine. In the intestine these changes did not progress to neoplasia. In the earlier studies using rat as experimental model, neoplastic changes in intestine have been reported in animals exposed to bracken fern (P. aquilinum). In the urinary bladder the early changes in the present study were indicative of toxicity and appeared in animals dying before 16 months. The animals (3/16) dying after 30 months showed neoplastic changes in the urinary bladder. The tumours were mainly restricted to urinary bladder and overall incidence was low (18.6%). In the earlier study by us15, P. aquilinum was collected from an area where the disease was present. Exposure of guinea pig resulted in chronic intermittent haematuria, transitional cell carcinoma and metastases. In the earlier study15, haematuria appeared after 11 months of exposure and in some animals it developed into macrohaematuria. In the present study microhaematuria did not develop into macrohaematuria and the number of animals affected was less (4/16) as compared to the earlier study (7/21)15. Bracken fern induced bladder tumours in guinea pigs have also been reported by Bringuier et al.16. In their study, bracken fern was collected at the stage when upper portion of the fronds were still curled and these were dried and incorporated in the feed at the rate of 30% (w/w). In the present study, the fern fronds used were fully opened and could be categorized as mature. The exposure period used was 30 months as compared to 5 months by Bringuier et al.16. The body weights of the animals used in the two studies were comparable. The fern sample used was analysed for ptaquiloside and was found to contain 3.74 ± 0.6 mg/kg fern powder. The young curled fronds have been found to have higher amounts of ptaquiloside than the opened fronds17. Ptaquiloside values for the material used in the earlier studies15,16 are not available but the incubation periods in these studies were shorter and the incidence of the tumours was higher. In the another study12 using rats as an experimental model, the fern was collected at the curled stage and was incorporated at the rate of 25% for 162 days. After a wait period of 105 days, 85% of the animals had tumours while in the same study another group fed the same fern collected from a different location had lower incidence of tumours. In the earlier studies, the ptaquiloside contents were not known but it appears from tumour incidence that the concentration of the ptaquiloside in the material used is critical in determination of the incidence rate and duration of the exposure.

Eleven out of 16 animals died during the course of experiment. Most of the animals died during the early part of experiment. The mortality may be due to specific or combined effect of mild nutritional stress, various unknown antinutritional factors/toxins in the plant and/or spontaneous secondary infection due to immunosupression/lowered immunity in the animals, as evidenced by the lesions in other organs.

Excessive loss of cells from the damaged urothelium is followed by proliferation for repair around the damaged area by the remaining healthy cells. This repair process may over-compensate for the damage and temporarily produce a urothelium that is many layers thick. This type of pathology has been described for the cytotoxic bladder carcinogens/chemicals in rats18,19. However, the mechanism of progression of this change to an irreversible...
stage is not known. In our study, irreversible type of urothelial proliferative change was observed in all the nine animals which survived more than 30 months. Three animals revealed malignant transformation and the other six revealed hyperplastic changes which were classified as irreversible as described by Hicks and Chowaniec\(^{19}\). These lesions were termed irreversible because in five animals such changes were observed 6 to 14 months after withdrawal of fern exposure. The early changes in the urinary bladder of exposed animals were mainly vascular such as congestion, oedema and haemorrhages. These changes in some animals were associated with proliferation of urothelium as well as degeneration and in others with necrosis and sloughing of urothelium. In other organs, viz. lungs, liver, spleen and kidneys major changes were also vascular. In the light of these observations, there is need for further studies to determine if fern toxins primarily act on vascular tissue and the other changes observed are secondary manifestation.

The cellular reaction observed around the preneoplastic hyperplastic urothelium in the present study suggests the role of cellular immunity in the progression of tumour development. Hicks and Chowaniec\(^{19}\) also observed focal aggregates of small lymphocytes, plasma cells and macrophages in the lamina propria beneath the preneoplastic urothelium. The cellular infiltration was comparatively more in initial stages of tumour growth as compared to the advanced stages as is evident in tumour infiltrating muscular layer. This observation points to a possible role of blocking factors in fern-induced carcinogenicity responsible for immune lymphocyte toxicity and progression of tumour growth as described by Currie and Basham\(^{20}\) and Bansal et al.\(^{21}\) in human bladder tumours.

Though the main seat of lesions in EBH is urinary bladder, the pathological changes have also been observed in other organs such as liver, kidneys, lungs and spleen. The cause of these lesions in field cases and their relation with the disease is not clear. In the present study the pathological changes in organs such as liver, kidneys, lungs and spleen were comparable to those described by Nandi\(^{22}\), Tripathi et al.\(^{23}\) and Singh et al.\(^{24}\) in field cases of EBH.

Microhaematuria was observed in all the animals which developed tumours in the urinary bladder (Table 1). One more animal without histological evidence of haemorrhages but with severe congestion and urothelial proliferation revealed microhaematuria.

The present study demonstrates the carcinogenic potential of \(P.\ aquilinum\) from a disease-free or nonenzootic area in India. The lower incidence of tumours in the present study could be because of lower ptaquiloside content. Low ptaquiloside content in \(P.\ aquilinum\) and grazing during the period when there are plenty of other greens available could be the reasons for absence of the disease from these areas.

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