Ptaquiloside-induced early-stage urothelial lesions show increased cell proliferation and intact β-catenin and E-cadherin expression

Running title: Immunohistochemistry of ptaquiloside-induced lesions

Rui M. Gil da Costa\textsuperscript{a,b}, Paula A. Oliveira\textsuperscript{c}, Margarida M. S. M. Bastos\textsuperscript{b}, Célia C. Lopes\textsuperscript{a}, Carlos Lopes\textsuperscript{a}

a- Abel Salazar Institute for Biomedical Sciences (ICBAS), University of Porto, Largo Prof. Abel Salazar 2, 4099-003 - Porto, Portugal.
b- LEPAE, Chemical Engineering Department, Engineering Faculty, University of Porto, Rua Dr. Roberto Frias s/n, 4200-465 - Porto, Portugal.
c- Veterinary Sciences Department, CECAV, University of Trás-os-Montes and Alto Douro, Quinta de Prados 5001-801 - Vila Real, Portugal.

Corresponding author:
Rui M. Gil da Costa
Pathology and Molecular Immunology Dept. (DPIM)
Abel Salazar Institute for Biomedical Sciences (ICBAS), University of Porto,
Largo Prof. Abel Salazar 2, 4099-003 Porto, Portugal.
gildacosta@portugalmail.pt
Tel: +351 222 062 200, Fax: +351 222 062 232
Abstract

Bracken (*Pteridium aquilinum*) is a carcinogenic plant whose main toxin, ptaquiloside, causes cancer in farm and laboratory animals. Ptaquiloside contaminates underground waters as well as meat and milk from bracken-grazing animals and is a suspected human carcinogen. A better understanding of the underlying mechanisms of carcinogenesis can be achieved by studying the early stages of this process. Unfortunately, most research on ptaquiloside has focused on the late, malignant, lesions, so the early changes of ptaquiloside-induced carcinogenesis remain largely unknown. This study aims to characterize early-stage ptaquiloside-induced urinary bladder lesions both morphologically and immunohistochemically. 12 male CD-1 mice were administered 0.5 mg ptaquiloside intraperitoneally, weekly, for 15 weeks, followed by 15 weeks without treatment. 12 control animals were administered saline. Bladders were tested immunohistochemically for antibodies against a cell proliferation marker (Ki-67), and two cell adhesion markers (E-cadherin and β-catenin). Two exposed animals died during the work. Six ptaquiloside-exposed mice developed low-grade and two developed high grade urothelial dysplasia. No lesions were detected on control animals. Significantly increased (p<0.05) Ki-67 labelling indices were found on dysplastic urothelium from ptaquiloside-exposed mice, compared with controls. No differences were found concerning E-cadherin and β-catenin expression. Early-stage ptaquiloside-induced urothelial lesions show increased cell proliferation but there is no evidence for reduced intercellular adhesiveness, though this may be a later event in tumour progression.

Keywords: Natural toxin, ptaquiloside, animal model, cancer, dysplasia, biomarkers, cell proliferation, cell adhesion.
Introduction

Bracken (*Pteridium aquilinum*, a member of the Dennstaedtiaceae family) is one of the most widely distributed plants worldwide and the only one whose ingestion is known to naturally cause cancer in animals (Evans and Mason, 1965; Carvalho *et al*., 2006). The discovery of ptaquiloside, a norsesquiterpene glycoside of the illudane family (Niwa *et al*., 1983; Van der Hoeven *et al*., 1983), and its identification as a natural alkylating agent and a major determinant of bracken carcinogenicity (Hirono *et al*., 1987; Shahin *et al*., 1998) have driven recent research on bracken-associated carcinogenesis. Ptaquiloside is excreted in milk and residues are present in meat from bracken-fed cows (Alonso-Amelot *et al*., 1996; Fletcher *et al*., 2011), while ptaquiloside leaching from bracken due to rain contaminates underground waters (Rasmussen *et al*., 2005). Besides these important human exposure routes to ptaquiloside, concerns have also been raised regarding the inhalation of air-borne bracken spores (Simán *et al*., 2000). Several epidemiological studies have suggested that bracken ingestion by some human populations or exposure to bracken toxins such as ptaquiloside may be related to a higher cancer incidence (Hirayama, 1979; Villalobos-Salazar, 1985; Galpin *et al*., 1990; Alonso-Amelot and Avendaño, 2001). Two reports by British governmental agencies have recently addressed the risks posed by ptaquiloside exposure. Human exposure to ptaquiloside via drinking water in the United Kingdom was assessed by the British Food and Environmental Research Agency, (Ramwell *et al*., 2010), while a report by the British Committee on the Toxicity of Chemicals in Food, Consumer Products and the Environment (COT) addressed the risks placed by ptaquiloside-contaminated foodstuffs, in particular by milk (COT, 2008) and considered it prudent to regard bracken as potentially carcinogenic at all levels of ingestion.

Oral administration of ptaquiloside to rats led to the formation of ileal and urinary bladder (Hirono *et al*., 1987) tumours. However, intravenous administration induced mammary gland
tumours (Shahin et al., 1988), showing the administration route to be an important factor in ptaquiloside-induced carcinogenesis. In guinea pigs fed bracken, the urinary bladder was also shown to be the main target organ (Bringuier et al., 1995) and in cattle the upper digestive tract was an additional site for cancer development (Masuda et al., 2011). In mice, there was a surprisingly different pattern of organ involvement, with the development of a lymphoproliferative malignancy and pulmonary tumours (Pamucku et al., 1972). Bracken-associated bladder tumours have been intensively studied morphologically, especially in cattle (Carvalho et al., 2006). Some studies also attempted a molecular approach in rat (Freitas et al., 2002) and bovine (Corteggio et al., 2010; Roperto et al., 2010; Corteggio et al., 2011) tumours, so as to understand the oncogenesis of these lesions. However, despite experimental data showing that ptaquiloside causes H-ras activation via mutations at codons 58, 59 and 61 (Prakash et al., 1996; Shahin et al., 1998), too little is known about the molecular changes leading to the appearance of the malignant phenotype. Malignancy is generally thought to derive from the stepwise accumulation of mutations through a sequence of consequent phenotypic changes, from pre-neoplastic lesions to benign and, finally, malignant neoplasms (Stricker and Kumar, 2010). Deregulated cell proliferation and reduced expression of intercellular adhesion molecules are some of the early changes found in pre-neoplastic lesions and epithelial neoplasms. Increased cell proliferation and activation of the WNT pathway with β-catenin nuclear translocation are important characteristics of human bladder cancer (Mallofré et al., 2003; Ahmad et al., 2011). Recently, β-catenin and ras mutations were shown to cooperate in driving bladder carcinogenesis in transgenic mice (Ahmad et al., 2011). Considering ptaquiloside causes H-ras mutations, it is now tempting to check for putative β-catenin changes in ptaquiloside-induced lesions. Our recent experiments with ptaquiloside in mice have resulted in the induction of urothelial dysplasia, a lesion which represents the early-stages of urothelial carcinogenesis (Gil da Costa et al., 2011). Hence, this report
describes the expression of a cell proliferation marker (Ki-67), and two important molecules involved in epithelial cell adhesion (E-cadherin and β-catenin) in ptaquiloside-induced early-stage urothelial lesions, in mice.

Materials and methods
Ptaquiloside isolation
An improved method designed for ptaquiloside isolation from bracken (Ojika et al., 1985) was adopted with minor modifications. Briefly, 1000 g (dried weight) of bracken crosiers (collected in the mountainsides of Arcos de Valdevez, Portugal, 41º 49´ 12´´ N, 8º 24´ 11´´ W, a location where bovine enzootic haematuria, a syndrome associated with ptaquiloside toxicity, is known to occur), were blended in water (10 L) and stirred at room temperature for 1 hour. A bracken sample was deposited at the University of Trás-os-Montes and Alto Douro herbarium (reference nº 18248). The aqueous extract was filtered and adsorbed on a resin (Amberlite XAD-2, Supelco) (3 L). The resin was eluted with methanol (10 L). The concentrated methanol extract was dissolved in water (400 ml) and extracted with n-butanol (5 x 500 ml). The concentrated n-butanol extract was separated by column chromatography on silica gel (Merck), starting with chlorophorm followed by ethyl acetate, ethyl acetate-methanol (92:8 %, v/v) and methanol. All fractions were controlled by ¹H and ¹³C nuclear magnetic resonance (NMR), with an Avance III 400 (Brucker) spectrometer. Fractions found to contain ptaquiloside were chromatographed twice on octadecyl-sylane (ODS) silica gel (Fujy-Silysia) with methanol-water (20:80 and 40:60 %, v/v) and methanol to obtain pure ptaquiloside. The compound’s identity was confirmed by means of its previously reported ¹H and ¹³C NMR and mass spectrometry data (Niwa et al., 1983; Oelrichs et al., 1995), obtained with a Q-TOF 2 (Micromass) mass spectrometer. Six milligrams aliquots were prepared for each experimental week, freeze-dried and kept at -20ºC until use.
Animals

Twenty-four four-weeks-old male CD-1 mice were provided by Charles River (Barcelona, Spain). Animals spent one week being acclimated under routine laboratory conditions before starting the experiments. Mice were kept at 4 litter-mates to a cage and were fed a standard balanced diet and water ad libitum. The animals were maintained at the University of Trás-os-Montes and Alto Douro (Vila Real, Portugal) in a 12 h light/12 h dark cycle (8 am–8 pm). All experiments were performed in accordance with national legislation (DL 129/92; DL 197/96; NP 1131/97) and the European Convention for the Protection of Animals used for Experimental and Other Scientific Purposes and related European Legislation (OJ L 222, 24.8.1999).

Experimental design

A total of 24 male CD-1 mice were divided into two groups of twelve mice each. Group 1 animals were injected intraperitoneally, every week in the same day, with 0.5 mg ptaquiloside freshly dissolved in phosphate buffered saline (PBS) pH=7.4 for 15 consecutive weeks. Group 2 animals were control mice injected with PBS only. The health status of the mice was monitored daily for the duration of the study. The drinking water was changed once a week, and the volume drunk was recorded. Weekly food intake was also noted. Body weights were measured once a week. Animals presenting with bite-wounds were placed on separate cages. Dead animals were removed immediately and necropsied. All surviving mice were sacrificed 15 weeks after ending the treatment, by isofluorane inhalation, and complete necropsies were conducted.
Histological analysis

The urinary bladders were inflated in situ by injecting 10% phosphate-buffered formalin (100 μl), ligated around the neck to maintain proper distension and immersed in formalin for 12 hours. After fixation, the urinary bladder was cut into two strips, and routinely processed with other organs. Tissue sections (2 μm) were stained with haematoxylin and eosin (HE) and examined under a light microscope by two researchers. Histological changes of the bladder epithelium were divided into the following types: normal urothelium, low-grade urothelial dysplasia, high-grade urothelial dysplasia and fibrovascular polyps.

Immunohistochemistry

Urinary bladder sections from group 1 animals were tested immunohistochemically for reaction with anti-Ki-67 (TEC-3, Dako), anti-E-cadherin (4A2C7, Zymed) and anti-β-catenin (CAT-5H10, Zymed) antibodies, using a standard peroxidase protocol. Anti-Ki-67, anti-E- cadherin and anti- β-catenin antibodies were diluted at 1:50, 1:150 and 1:600, respectively, and used on tissues after heat-induced epitope retrieval (HIER). For HIER, slides were incubated for 20 minutes in a steamer at 100°C with a citrate buffer pH=6.0. One thousand urothelial cells were counted and the Ki-67 labelling index was expressed as the percentage of Ki-67-positive cells. Normal duodenum sections from group 2 animals provided positive controls while negative controls were made by omitting the primary antibodies.

Statistical analysis

Statistical analysis was carried out using the SPSS 11.0 statistical software (SPSS Inc. USA). The distribution of Ki-67 labeling indices was studied using the Kolmogorov-Smirnov test. Since this variable didn’t depart significantly from normality, one-way analysis of variance
ANOVA) was used to detect significant differences between experimental groups. The level of significance considered was p<0.05.

Results
Minor wounds of the dorsal and prepuce areas due to aggressive behaviour between male animals were observed in both groups. Two wounded mice from group 1 died on weeks 14 and 15, respectively. Table 1 summarizes the results concerning body weight, water and food intake. No macroscopic lesions were detected on the urinary bladders from either group, but histological examination demonstrated the presence of low-grade zonal urothelial dysplasia in 6 out of 10 (60%) and high-grade zonal urothelial dysplasia in 2 out of 10 (20%) ptaquiloside-exposed mice. Findings from other organs (mainly consisting of a B-cell lymphoproliferative malignancy) have already been reported (Gil da Costa et al., 2011). Dysplastic lesions were characterized by increased urothelial cellularity and loss of normal urothelial polarity and umbrella-cells differentiation. High-grade dysplastic lesions also showed prominent anisokaryosis with cells presenting indented or reniform nuclei. Urothelial denudation was multifocally present. Two group 1 mice with dysplastic changes also developed luminal, sessile lesions, covered with urothelium and supported by an abundant, dense and oedematous fibrovascular stroma, classified as fibroepithelial polyps (Figure 1-A). No lesions were found in the control group (Figure 2-A). Ki-67 nuclear immunostaining was found in ptaquiloside-exposed animals and positive cells were mostly in (but not restricted to) the basal layer (Figure 1-B), while controls showed nearly no immunostaining (Figure 2-B). Although the KI-67 labelling index was low in both groups, group 1 showed a statistically significant increase (p<0.05) in mean Ki-67 labelling index values (0.18±0.30%), compared with group 2 (0.01±0.03%) animals, (Figure 3). Immunohistochemical examination for E-cadherin and β-catenin expression showed that dysplastic urothelium from ptaquiloside-exposed animals
(Figures 1-C and 1-D, respectively) showed the same membrane-associated, strong, diffuse immunostaining observed in normal urothelium from group 2 (Figures 2-C and 2-D, respectively).

Discussion and conclusions

Dysplasia represents an early pre-neoplastic lesion in the process of epithelial carcinogenesis. In the human urinary bladder, urothelial dysplasia precedes the development of in situ carcinoma (Hodges et al., 2010) and in the mouse a similar process occurs (Oliveira et al., 2006a). In what concerns bracken-induced bladder carcinogenesis, researchers have devoted most of their efforts to characterize spontaneous or experimentally induced malignant neoplasms rather than its benign or pre-neoplastic precursors. By studying the early molecular changes presented by such initial lesions, new insights may be gained into the oncogenesis of bladder tumours. Our experiments with CD-1 mice resulted in an 80% incidence of urothelial dysplasia in the ptaquiloside-exposed group (Gil da Costa et al., 2011). These results are in contrast to the absence of bladder lesions reported by (Pamucku et al., 1972) and show that mice, like other mammalian species so far tested, are susceptible to develop ptaquiloside-induced bladder lesions. This discrepancy may be due to several factors, especially the administration of whole bracken versus isolated ptaquiloside and the different administration routes (oral versus intraperitoneal). In this experiment, intraperitoneal administration was elected as an alternative to oral gavage. Considering that oral and parenteral administration to rats have resulted in neoplastic lesions of different target organs (Hirono et al., 1987; Shahin et al., 1998), this option allowed us to explore these differences. On the other hand, oral gavage is associated with high mortality, especially when long administration periods are required. Further experiments are under way, employing longer periods between exposure and euthanasia, so as to study the progression of these lesions into invasive carcinomas. It was
also interesting to notice the development of benign mesenchimal tumours (fibrovascular polyps), as such lesions are present, alone or together with epithelial tumours, in 48.8% of cattle affected by spontaneous bracken-related bladder neoplasms (Carvalho et al., 2006). Other authors (Hirono et al., 1987) also found urinary bladder mesenchimal tumours (sarcomas) in rats orally exposed to ptaquiloside, but Bringuier et al. (1995), who fed guinea pigs with bracken, did not. Despite these discrepancies, the present results indicate that ptaquiloside targets the urinary bladder’s stroma as well as the urothelium. In human and murine urinary bladder oncogenesis, early-stage lesions such as urothelial dysplasia and in situ carcinoma already show a characteristically increased cellular proliferation (Mallofré et al., 2003; Sun and Herrera, 2002). Also in the present study, a significantly higher Ki-67 labelling index was found in ptaquiloside-exposed animals (group 1), 80% of which had early-stage urothelial lesions, compared with control animals (group 2). These results show that increased cell proliferation is an early change in pre-neoplastic ptaquiloside-induced bladder lesions. Deregulated cell proliferation is thought to result from the inactivation of tumour-suppressor genes and the activation of growth-promoting proto-oncogenes (Stricker and Kumar, 2010). One important growth-promoting pathway in human urinary bladder cancer is WNT, deregulated in approximately 25% of human urothelial cell carcinomas (Ahmad et al., 2011). Activation of the WNT pathway leads to translocation of β-catenin from the cell membrane and the cytoplasm into the nucleus, where it acts as a transcription factor for proliferation-associated genes (Stricker and Kumar, 2010). Recently, a β-catenin mutation was shown to cooperate with ras mutations to drive bladder carcinogenesis in a mouse model (Ahmad et al., 2011). β-catenin is normally associated with the cell-surface protein E-cadherin, on the cell membrane, as part of the epithelial intercellular adherens junctions. In result, loss of normal β-catenin function may also lead to reduced intercellular adhesiveness, with increased cell motility and the appearance of an invasive phenotype.
Reduced E-cadherin expression seems to be a rather late event in human bladder tumorigenesis, associated with late-stage, invasive lesions (Sun and Herrera, 2002). This is also consistent with the observation that decreased E-cadherin expression accompanies the degree of tissue transformation during carcinogenesis of the bladder in the rat (Oliveira et al., 2006b). In the present study, anti-β-catenin and anti-E-cadherin antibodies were used to check whether their normal position on the cell membrane was maintained in ptaquiloside-induced urothelial dysplasia. Results show that the E-cadherin-β-catenin complex was maintained and that no nuclear β-catenin translocation occurred. This, on the one hand, indicates that activation of the WNT pathway is not an early event in ptaquiloside-induced bladder carcinogenesis in mice. On the other hand, as in human bladder carcinogenesis (Sun and Herrera, 2002), reduced E-cadherin expression is not a feature of early ptaquiloside-induced urothelial lesions. Increased Ki-67 labelling indexes observed in group 1 animals do not seem to depend on the WNT pathway and must have other underlying causes. Additional research on this subject may now focus on the role played by tumour-suppressor genes and cytoskeletal proteins, known to be of importance in early human bladder lesions (Mallofré et al., 2003).

Acknowledgements

The authors gratefully acknowledge Prof. Adélio Mendes (LEPAE, Chemical Engineering Department, Engineering Faculty, University of Porto) for advice on adsorption processes, Fuji-Silysia Chemical Company for the generous gift of ODS. R. M. Gil da Costa was supported by the Portuguese Foundation for Science and Technology grant nº. SFRH/BD/37565/2007, co-financed by the Portuguese Ministry for Science and Technology and The Social European Fund.
References


COT - Committee on the Toxicity of Chemicals in Food, Consumer Products and the Environment. 2008. COT statement on the risk to consumers of eating foods derived from animals that have eaten bracken. London: Food Standards Agency.


Fig. 1-A Urinary bladder. Ptaquiloside-exposed (group 1) animal. Note two fibrovascular polyps. H&E 200x. Bar= 50 μm

Fig. 1-B Urinary bladder. Ptaquiloside-exposed (group 1) animal. Note two Ki-67-positive nuclei. IHC-Mayer’s haematoxylin 400x. Bar= 20 μm

Fig. 1-C Urinary bladder. Ptaquiloside-exposed (group 1) animal. Strong, membrane-associated staining for E-cadherin. IHC-Mayer’s haematoxylin 400x. Bar= 20 μm

Fig. 1-D Urinary bladder. Ptaquiloside-exposed (group 1) animal. Strong, membrane-associated staining for β-catenin. IHC-Mayer’s haematoxylin 400x. Bar= 20 μm
Fig. 2-A Urinary bladder. Control (group 2) animal. Normal urothelium. H&E 400x. Bar= 20 μm

Fig 2-B Urinary bladder. Control (group 2) animal. Immunohistochemical staining for Ki-67. Note absence of immunopositive nuclei. IHC-Mayer’s haematoxylin 400x. Bar= 20 μm

Fig 2-C Urinary bladder. Control (group 2) animal. Normal immunohistochemical staining for E-cadherin. IHC-Mayer’s haematoxylin 400x. Bar= 20 μm

Fig 2-D Urinary bladder. Control (group 2) animal. Normal immunohistochemical staining for E-cadherin. IHC-Mayer’s haematoxylin 400x. Bar= 20 μm
Fig. 3 Average Ki-67 labelling index values (%) for the urinary bladder urothelium of control and ptaquiloside-exposed CD-1 mice (p<0.05)
### TABLE I. Average body weight, food, and water intake for ICR mice exposed to ptaquiloside (group 1) and control mice (group 2)\(^a\)

<table>
<thead>
<tr>
<th>Group</th>
<th>Initial Body Weight (g)</th>
<th>Final Body Weight (g)</th>
<th>Initial Food Intake (g/mouse/day)</th>
<th>Final Food Intake (g/mouse/day)</th>
<th>Initial Water Intake (g/mouse/day)</th>
<th>Final Water Intake (g/mouse/day)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 (n = 10)</td>
<td>32.99 ± 3.09</td>
<td>48.23 ± 7.98</td>
<td>5.83 ± 0.09</td>
<td>4.98 ± 1.49</td>
<td>8.07 ± 0.77</td>
<td>6.30 ± 0.67</td>
</tr>
<tr>
<td>2 (n = 12)</td>
<td>32.47 ± 1.89</td>
<td>52.29 ± 5.90</td>
<td>5.59 ± 0.07</td>
<td>6.30 ± 0.67</td>
<td>7.69 ± 1.28</td>
<td>7.89 ± 0.59</td>
</tr>
</tbody>
</table>

\(^a\)Values are mean values ± SEM (standard error to the mean).