Chromosome fragility in cattle with chronic enzootic haematuria

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Chronic enzootic haematuria (CEH) is a severe syndrome due to prolonged ingestion of toxic principles of bracken fern, such as quercetin and ptaquiloside. Little information is available on chromosomal instability of cattle with access to bracken fern and suffering from CEH. In the present study, 45 cattle, aged from 7 to 12 years and pastured in the south of Italy, were cytogenetically investigated for the first time in search of both chromosomal aberrations (aneuploidy, gaps, chromatid breaks, chromosome breaks and fragments) and sister chromatid exchanges (SCEs). Of these animals, 30 (group 1) had access to bracken fern and showed signs of CEH, and 15 (group 2; control) did not. Percentage of abnormal cells (aneuploidy, chromatid breaks, chromosome breaks and fragments) was higher in animals affected by CEH (34.7%, group 1) than that (24.3%) reached in the control (group 2). The same results were achieved when including gaps. Indeed, the mean number of cells with structural aberrations excluding gaps (chromatid breaks, chromosome breaks and fragments) per cell was higher ($P < 0.001$) in animals affected by CEH ($0.16 \pm 0.36$) than that ($0.09 \pm 0.29$) found in the control. Chromosome fragility in cells of animals affected by CEH was also confirmed when applying the SCE test: statistically higher levels ($P < 0.001$) of SCEs were observed in animals with CEH ($7.35 \pm 3.59$ SCE/cell, group 1) than those in the control ($5.40 \pm 2.68$ SCE/cell).

Introduction

Chronic enzootic haematuria (CEH) is a severe syndrome due to prolonged ingestion of bracken fern (Pteridium aquilinum), the only plant proven to cause cancer in animals (1). This syndrome occurs in several areas worldwide and is very common in southern Italy where bracken is widespread (2). Pinto et al. (3) in an epidemiological study demonstrated an association between CEH and the level of pasture infestation with bracken fern. It is well known that this plant contains toxic compounds like ptaquiloside (PT) and quercetin, which have mutagenic and carcinogenic effects, mainly in the bladder (2). PT is a sesquiterpenoid glycoside capable of inducing clastogenicity in cell cultures and also with mutagenic and carcinogenic activity (1,4). Shahin et al. (5) proposed a PT cancer model to explain bracken-induced carcinogenesis and supposed that it occurs at cellular and molecular levels. At the molecular level, PT is converted into a conjugated diene (APT) in alkaline conditions, which then alkylates DNA at N3 of adenine within 24 h of exposure (6). At the cellular level, affected cells have the capacity to repair such lesions in a short period of time, but a few of these lesions lead to erroneous repair which culminates in mutations in key genes, initiating cancer. Before any histopathological signs of neoplasia could be observed, mutations are detected in H-ras, an oncogene associated with several types of cancer including those occurring in colon, urinary bladder and mammary glands. Sardon et al. (7) confirmed the presence of DNA changes in bladder lesions of cattle exposed to bracken fern, H-ras mutations being significantly increased in chronic cystitis and tumours. The role of quercetin remains ill defined (5).

A strong relationship between bovine papillomavirus (BPV)-2 and bracken fern in both experimental and naturally occurring bovine bladder cancer has been established (8–10). Although the synergism between the virus and bracken is still poorly understood, it is likely that BPV-2 infects the bladder mucosa, producing an abortive latent infection, and the latent BPV is activated by bracken-induced immunosuppression, thus initiating progression to malignancy (10).

Chromosomal aberrations, especially chromatid breaks, are also known to occur in humans suffering from cancer (reviewed in ref. 11). There is little information on chromosomal instability in cattle with access to bracken fern and suffering from CEH. More recently, Lioi et al. (12) observed an increase in the number of structural chromosomal aberrations in 56 cattle with CEH raised on pastures giving access to bracken fern with the highest clastogenic effect observed in cattle with urinary bladder cancer (27 animals). However, these authors did not investigate for the presence of aneuploid cells and did not use the sister chromatid exchange (SCE) test.

The SCE test has been used to detect genome stability in humans (13) and in the main livestock species such as cattle (14–16), river buffalo (17), goat (18), sheep (19) and pig (20,21), as well as to discover DNA damage caused by a variety of natural and artificial chemical compounds (22–24).

In the present study, we report the results of our investigation on chromosome stability of a representative sample of cattle with CEH using for the first time cytogenetic tests that detect both chromosomal aberrations (aneuploidy, gaps, chromatid breaks, chromosome breaks and fragments) and SCEs.

Materials and methods

Animals

The study was performed on 45 cattle, aged from 7 to 12 years and naturally pastured in southern Italy. Of these, 30 (group 1) had access to bracken fern and 15 (group 2; control) did not. Animals from group 1 showing signs of CEH were included in the study, while the 15 animals from group 2 did not show any clinical signs of CEH. All other cattle were used for food production and were raised on pastures without access to bracken fern. The study was performed on 45 cattle, aged from 7 to 12 years and naturally pastured in southern Italy. Of these, 30 (group 1) had access to bracken fern and 15 (group 2; control) did not. Animals from group 1 showing signs of CEH were included in the study, while the 15 animals from group 2 did not show any clinical signs of CEH. All other cattle were used for food production and were raised on pastures without access to bracken fern.
were monitored during life and studied after slaughter at an abattoir. All animals from group 2 were healthy and did not show signs of CEH.

The control group animals were selected from those with the same age as animals affected by CEH (from 7 to 12 years old) because cytogenetic studies performed in humans (25) and animals (20) report a relationship between the increase in SCEs and age.

Clinical and histological evaluation
CEH (group 1) was diagnosed on the basis of clinical history or clinical examination. The neoplastic lesions at the urinary bladder were detected by gross examination (Figure 1). Tumour samples were fixed in 10% formalin, embedded in paraffin wax and stained with haematoxylin and eosin for routine histological examination. The following microscopic patterns were observed in the animals: inflammation (2 animals), dysplasia (1 animal), vascular cancer (6 animals), carcinoma in situ (7 animals), and carcinoma (14 animals).

Cytogenetic analysis
Peripheral blood (1 ml) was cultured in RPMI medium, enriched with foetal calf serum (10%), L-glutamine (1%) and Concanavalin A (1.5%) (as mitogen) for 48 h at 37.8 °C. Two types of cell cultures, without (normal cultures) and with addition of 5-bromodeoxyuridine (BrdU) (10 μg/ml) were performed. In the latter, BrdU was added 30 h before harvesting to allow its incorporation into DNA during the last two cell cycles (SCE test). Cells from both types of cell cultures were harvested after Colcemid (0.3 μg/ml) treatment for 1 h and given hypotonic treatment (KCl 0.5%) and three fixations in methanol–acetic acid (3:1), the third overnight. Three drops of cell suspension were air-dried on cleaned and wet slides which were stained with acridine orange (0.01% in a phosphate buffer, pH 7.0) for 10 min, washed in tap and distilled water and mounted in the same phosphate buffer. Slides were observed 24 h after staining or later (1 week).

At least 50 cells per animal were examined from slides of normal cultures to detect aneuploidy and structural aberrations, i.e. gaps, chromatid breaks, chromosome breaks and fragments (Figure 2) that were classified according to the criteria suggested by Savage (26) and Carrano and Natarajan (27). For the SCE test, 35 cells at the second cycle of replication (Figure 3) in the presence of BrdU and with a complete chromosome set (2n = 60) were considered. All metaphases plates were observed under a fluorescence Nikon microscope, captured with a digital camera Nikon DS-U1, transferred onto a PC and later processed by image analysis software of two cytogeneticists.

Statistical analysis
An independent two-sample Student’s t-test was performed and a confidence interval generated to compare the two groups of animals (with and without CEH), determining whether or not there is any evidence that the differences between the three cytogenetic analyses we applied: aneuploidy, chromosome abnormalities (gaps, chromatid breaks, chromosome breaks and fragments) and SCE were other than 0.

Results
The percentage of abnormal cells (chromatid breaks, chromosome breaks and fragments) was higher in the animals with CEH (16.1%, group 1) than that (9.7%) in the control. When including aneuploidy and gaps, percentages of abnormal cells were very high in both animals affected by CEH (92.6%, group 1) and the control (74.1%) (Table I), although percentages of

<table>
<thead>
<tr>
<th>Groups</th>
<th>Cells examined (n)</th>
<th>Abnormal cells with gaps and aneuploidy</th>
<th>Abnormal cells without gaps and aneuploidy</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>%</td>
<td>n</td>
</tr>
<tr>
<td>1 (30)</td>
<td>1500</td>
<td>1389</td>
<td>92.6</td>
</tr>
<tr>
<td>2 (15)</td>
<td>750</td>
<td>556</td>
<td>74.1</td>
</tr>
</tbody>
</table>
Chromosome fragility in cattle

Table II. Mean and SD of aneuploid cells, cells with structural aberrations excluding gaps, chromatid breaks, chromosome breaks, fragments and gaps in cells of animals with CEH (group 1) and control (group 2)

<table>
<thead>
<tr>
<th>Groups</th>
<th>Number of animals</th>
<th>Cells examined (n)</th>
<th>Aneuploid cells (mean/cell ± SD)</th>
<th>Cells with structural aberrations excluding gaps (mean/cell ± SD)</th>
<th>Chromatid breaks (mean/cell ± SD)</th>
<th>Chromosome breaks (mean/cell ± SD)</th>
<th>Fragments (Mean/cell ± SD)</th>
<th>Gaps (Mean/cell ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>30</td>
<td>1500</td>
<td>0.21 ± 0.41*</td>
<td>0.16 ± 0.36*</td>
<td>0.12 ± 0.36*</td>
<td>0.05 ± 0.27***</td>
<td>0.02 ± 0.16**</td>
<td>0.005 ± 0.09</td>
</tr>
<tr>
<td>2</td>
<td>15</td>
<td>750</td>
<td>0.15 ± 0.36</td>
<td>0.09 ± 0.29</td>
<td>0.08 ± 0.29</td>
<td>0.02 ± 0.16</td>
<td>1.71 ± 1.49</td>
<td></td>
</tr>
</tbody>
</table>

*P < 0.01; **P < 0.001; ***P < 0.05.

Table III. Animals, cells studied and mean SCE values in cells of animals with CEH (group 1) and control (group 2)

<table>
<thead>
<tr>
<th>Groups</th>
<th>Number of animals</th>
<th>Cells examined (n)</th>
<th>SCE/cell (mean/cell ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>30</td>
<td>1050</td>
<td>7717 7.35 ± 3.59**</td>
</tr>
<tr>
<td>2</td>
<td>15</td>
<td>525</td>
<td>2825 5.40 ± 2.68</td>
</tr>
</tbody>
</table>

**P < 0.001.

abnormal cells still remain higher in animals affected by CEH than those reached in the control.

Mean values of aneuploid cells (79% 58 < 2n < 60; 21% 50 < 2n < 58) were, respectively, 0.21 ± 0.41 in the animals with CEH and 0.15 ± 0.36 in the control group. The mean number of abnormal cells without gaps was higher in animals affected by CEH (0.16 ± 0.36) than that (0.09 ± 0.29) found in the control (P < 0.001).

Considering all structural aberrations, mean values of gaps are higher in cells of animals affected by CEH (2.83 ± 1.90) than those found in the control (1.71 ± 1.49), and similar results were obtained in chromatid breaks (0.12 ± 0.36, 0.08 ± 0.29, respectively), chromosome breaks (0.05 ± 0.27, 0.02 ± 0.16, respectively) and fragments (0.02 ± 0.16, 0.005 ± 0.09, respectively) (Table II).

Significant increases (P < 0.001) in the mean number of SCE/cell were observed in the group of animals with CEH (7.35 ± 3.59 SCE/cell), compared with that in the control (5.40 ± 2.68 SCE/cell) (Table III).

Discussion

Prolonged ingestion of toxic principles in bracken fern, such as quercetin and PT, is the cause of CEH in cattle (1). It is frequently accompanied by mesenchymal and urothelial neoplastic lesions of the urinary bladder (28; Figure 1). The animals affected by CEH and studied herein showed variable patterns under microscopic observation such as inflammation, dysplasia, vascular cancer, carcinoma in situ and carcinoma.

Cytogenetic analysis revealed pronounced chromosome fragility in cells of animals affected by CEH since a significantly higher (P < 0.001) frequency of abnormal cells without gaps was detected in the animals affected by CEH than in the control (Table II). After gaps, aneuploidy was the most common abnormality found in the animals, followed by chromatid breaks, chromosome breaks and fragments. Our results differed from those reported by Lioi et al. (12), who found a higher number of chromatid breaks than gaps, and are in agreement with previous studies where gaps were reported to be the most frequent chromosome abnormality found in cells of animals naturally exposed to dioxin and control (23,24).

Mean values of aneuploid cells were high not only in the animals affected by CEH (0.21 ± 0.41) but also in the control (0.15 ± 0.36) (Table II), as previously found in cells of sheep exposed and unexposed (control) to dioxins (23,24). It is difficult to establish whether this phenomenon is due to the presence of high percentages of chromosomal abnormality which can give rise to chromosome loss in subsequent cell division, or it is simply due to technical problems occurring during chromosome treatments. Indeed, all aneuploid cells were hypoploid (2n < 60). However, only now (use of image processing and analysis) can we easily count the number of chromosomes for each metaphase, compared to previous studies where aneuploid cells were difficult to detect by using only microscope observation, especially in cattle.

The SCE test (Table III) confirmed chromosome fragility in cells of animals affected by CEH when compared with the control. Indeed, a significantly higher mean number of SCE/cell was found in animals affected by CEH (7.35 ± 3.59 SCE/cell), compared with that of the control (5.40 ± 2.68 SCE/cell).

Our study also showed that animals, especially grazing cattle, may be used as environmental biological indicators of natural toxic compounds by using cytogenetic tests, such as those applied in the present study or, more generally, using biomarkers of DNA damage. Such tests can measure the biological effects of natural toxic compounds before overt disease develops.

Acknowledgements

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References


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