Tumour induction in rats following exposure to short-term high dose aristolochic acid I

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The purpose of this study was to assess the carcinogenic activity of aristolochic acid I (AAI) in short-term high doses. Forty-four female Sprague–Dawley rats were randomly assigned to two groups. A dose of 50 mg/kg/day AAI was administrated to rats in the experimental group by gavage consecutively for 3 days, while the control group received only distilled water, after which renal function and pathological changes were assessed. At day 8 post-treatment AAI had induced elevations of both plasma urea and creatinine, coupled with increased urine production, urinary proteins, glucose and N-acetyl-β-glucosaminidase. At 1, 3 and 6 months post-treatment renal function and urinary parameters for the experimental group approached baseline values. However, tumours and preneoplastic proliferation were both observed at 6 months for the experimental group. The rate of occurrence of preneoplastic proliferation in the kidneys was 100% (14/14); the rate of occurrence of renal tumours was 28.6% (4/14), which included three mesenchymal tumours and one case of renal oncocytoma; the rate of occurrence of extrarenal tumours was 7.1% (1/14), which was a case of mammary duct carcinoma. Renal preneoplastic proliferation and renal tumours, as well as extrarenal tumours, were not observed in control rats during the 6 months. These results differ from previous reports in that tumours originating from both epithelial and mesenchymal tissues were found, which may be attributed to the duration of treatment and the dosage of the drug. These data indicate that AAI administered in an acute manner at high doses does in fact have carcinogenic properties.

Introduction

The plant extract aristolochic acid (AA) is associated with rapidly progressive renal interstitial fibrosis, which is also called Chinese herbs nephropathy (CHN). In the majority of cases, progression to end-stage renal failure occurs rapidly, despite discontinuation of AA ingestion, necessitating dialysis and subsequent renal transplantation (Vanherweghem et al., 1993). Subsequently it has been shown that a significant percentage of the patients affected by aristolochic acid nephropathy (AAN) go on to have urothelial malignancy. Recently, an increasing number of urothelial carcinomas have been reported in CHN patients, suggesting that AA also plays a role in the formation of these tumours (Cosyns et al., 1999; Nortier et al., 2000; Arlt et al., 2002).

Previous studies have demonstrated the carcinogenic action of AA in rats (Mengs et al., 1982; Mengs, 1993). In those studies AA was orally administrated over a period of 3–6 months in doses ranging from 0.1 to 10.0 mg/kg. As a result of the treatment, the rats developed metastasizing squamous cell carcinomas of the forestomach, as well as benign and malignant tumours of the kidneys and of the urinary tract. The same author reported that AA exerted a carcinogenic effect of similar strength in mice (Mengs, 1988). AA given to rats in a single oral dose of 10, 50 or 100 mg/kg could cause dose-dependent renal damage (Mengs and Stotzem, 1993); the work was carried out as a short-term test for nephrotoxicity, so the experimental period was too short to investigate carcinogenic effects and prognosis of renal injury was not observed.

AA is a mixture consisting of aristolochic acid I (AAI) and aristolochic acid II (AAII). Among the compounds of aristolochic acid the content of AAI is highest and AAII is the most representative substance in the compound of AA. In this study the carcinogenic effect of short-term high dose AAI was evaluated in conjunction with renal function and histopathological changes in the kidneys.

Materials and methods

Animals and reagents

Forty-four female Sprague–Dawley rats (Shanghai Experimental Animal Center, Chinese Academy of Sciences) with a body weight of 160–180 g were used in this study. A protocol approved by the Animal Care Committee (Institute of Science and Technology, Jiangsu Province) was followed for handling and care of the animals.

AAI (purity >95%) was extracted from AMK (Aristolochia manshuriensis Kom) by the Chinese Pharmaceutical University (Nanjing, China).

Experimental protocol

Rats were randomly divided into two groups according to their weight. AAI was dissolved in distilled water at a dose of 4 mg/ml and administrated to 24 rats in the experimental group by gavage at 50 mg/kg/day consecutively for 3 days while the remainder of the rats received distilled water only. All animals were allowed free access to food and water during the experiment.

Evaluation of renal function

Blood and 24 h urine samples for renal function tests were collected from six rats randomly selected at day 8 and 1, 3 and 6 months of the experiment. Plasma urea and urinary activity of N-acetyl-β-glucosaminidase (NAG) were determined by enzymatic colorimetric methods, plasma creatinine levels were determined by the Jaffe method (Seaton and Ali, 1984), urinary protein was tested by the Biuret method (Layne, 1957), urinary glucose was tested by the hexokinase method and osmolality was measured with an osmometer.

Histopathology

At day 8 and at 1, 3 and 6 months, 4, 3, 3 and 14 rats were killed, respectively. After external inspection, each animal was autopsied. Samples of liver, bilateral kidneys, heart and brain, as well as any tissue with an abnormal appearance, were excised and fixed in 10% neutral formalin for histological examination. The sections (4 µm) were stained in hematoxylin and eosin (HE) and periodic acid–Schiff (PAS), and examined microscopically.

Statistical analysis

Data were analysed using Student’s t-test. Comparison of percentages was performed with Fisher’s exact test. All analyses were performed using SPSS, version 10.0 (SPSS Inc, Chicago, IL). P values of <0.05 with two-tailed
Control (day 8) 6 7.37 ± 1.00 68.00 ± 1.79 7.80 ± 6.13 17.35 ± 4.02 9.16 ± 4.33 13.28 ± 2.64
AAI (day 8) 6 21.42 ± 10.64 a 125.00 ± 76.28 b 19.72 ± 6.44 a 136.67 ± 33.93 a 71.11 ± 21.39 a 159.30 ± 47.90 a
AAI (3 months) 6 7.82 ± 0.33 65.40 ± 1.67 7.70 ± 1.83 17.90 ± 14.07 11.44 ± 2.91 13.41 ± 5.01
AAI (6 months) 6 7.61 ± 0.70 68.67 ± 4.58 8.13 ± 5.45 18.02 ± 9.93 10.02 ± 13.11 13.24 ± 5.72

*P < 0.01 versus control group.
**P < 0.05 versus control group.

Results

Survival rate

The survival rates of the experimental and control groups were 100% over 6 months. Three days after the administration of AAI the rats were sluggish and showed hypersomnia and anorexia. All rats recovered within 1 week.

Renal function

As shown in Table I, at day 8 there were rises in plasma urea and creatinine together with increases in urine volume and urinary glucose, protein and NAG in the experimental group. Statistically significant differences were observed between the experimental and control groups (P < 0.05).

After the first month there were no differences in renal function and urinary parameters in the experimental group compared with the control group. After 3 and 6 months there were no significant differences in renal function and urinary parameters between the experimental and control groups.

The occurrence of renal preneoplastic proliferation, renal tumours and extrarenal tumours

The occurrence of preneoplastic proliferation and tumours in kidney as well as extrarenal tumours are shown in Tables II and III. No significant signs of preneoplastic proliferation or tumours were observed in the kidneys at day 8 and 1 and 3 months in the experimental group. At 6 months, however, the occurrence of preneoplastic proliferation in the kidneys was 100% (14/14); the occurrence of renal tumours was 28.6% (4/14), including three cases of mesenchymal tumours and one case of oncocytoma; the occurrence of extrarenal tumours was 7.1% (1/14), which was a case of mammary duct carcinoma. The occurrence of renal preneoplastic proliferation and renal tumours as well as extrarenal tumours was 0% in the control rats after 6 months (0/20).

Histopathological features of preneoplastic proliferation in kidney

Most kidneys appeared to be normal, however, occasionally there was an extension of preneoplastic proliferation through the renal capsule. Lesion size varied, with small nodules measuring from 2 to 3 mm across. White granules were observed on the surface of nodules. In sections of kidney we found that the lesions were situated in the cortex as well as the corticomedullary junction, growing in an infiltrating or cystic manner (Figure 1A).

Histological examination revealed preneoplastic proliferation of renal interstitial cells, with the degree of hyperplasia varying greatly. Mild lesions were observed in focal areas, most situated in the corticomedullary junction. Infiltration of poorly differentiated, short spindle-shaped mesenchymal cells surrounding the renal tubules was observed, with little cytoplasm faintly stained and deeply staining nuclei. Nuclear atypia was not found. Severe lesions occurred in sheets, with diffuse infiltration of poorly differentiated spindle cells in the interstitium seen. Remnant tubules were observed in the interstitium (Figure 1B). Diffuse proliferation and denaturation of fibrous tissue was seen in the interstitium and edema was present in the focal area. Diffuse atrophy and loss of tubules were observed, with the remaining tubules expanded by cystic dilation and degeneration of tubular epithelial cells. Protein casts were present in the tubules and remnant glomeruli were occasionally seen. There was no boundary distinguishing the lesions from normal renal tissue.

Table I. Effect of AAI on renal function

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Serum urea (mmol/l)</th>
<th>Serum creatinine (umol/l)</th>
<th>Urinary volume (ml)</th>
<th>Urinary NAG (U/g creatinine)</th>
<th>Urinary protein (mg/24 h)</th>
<th>Urinary glucose (umol/24 h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (day 8)</td>
<td>6</td>
<td>7.37 ± 1.00</td>
<td>68.00 ± 1.79</td>
<td>7.80 ± 6.13</td>
<td>17.35 ± 4.02</td>
<td>9.16 ± 4.33</td>
<td>13.28 ± 2.64</td>
</tr>
<tr>
<td>AAI (day 8)</td>
<td>6</td>
<td>21.42 ± 10.64 a</td>
<td>125.00 ± 76.28 b</td>
<td>19.72 ± 6.44 a</td>
<td>136.67 ± 33.93 a</td>
<td>71.11 ± 21.39 a</td>
<td>159.30 ± 47.90 a</td>
</tr>
<tr>
<td>AAI (3 months)</td>
<td>6</td>
<td>7.82 ± 0.33</td>
<td>65.40 ± 1.67</td>
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<td>13.41 ± 5.01</td>
</tr>
<tr>
<td>AAI (6 months)</td>
<td>6</td>
<td>7.61 ± 0.70</td>
<td>68.67 ± 4.58</td>
<td>8.13 ± 5.45</td>
<td>18.02 ± 9.93</td>
<td>10.02 ± 13.11</td>
<td>13.24 ± 5.72</td>
</tr>
</tbody>
</table>

*a*P < 0.01 versus control group.

Table II. Tumour occurrence in the AAI-treated group at 6 months post-treatment

<table>
<thead>
<tr>
<th>No.</th>
<th>Left kidney</th>
<th>Right kidney</th>
<th>Extrarenal organs</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>–</td>
<td>++</td>
<td>–</td>
</tr>
<tr>
<td>2</td>
<td>++</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>3</td>
<td>RMT</td>
<td>+ +</td>
<td>–</td>
</tr>
<tr>
<td>4</td>
<td>+</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>5</td>
<td>+</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>6</td>
<td>RMT</td>
<td>+ +</td>
<td>–</td>
</tr>
<tr>
<td>7</td>
<td>–</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>8</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>9</td>
<td>+</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>10</td>
<td>+</td>
<td>–</td>
<td>Mammary duct carcinoma</td>
</tr>
<tr>
<td>11</td>
<td>+</td>
<td>+ +</td>
<td>–</td>
</tr>
<tr>
<td>12</td>
<td>+ +</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>13</td>
<td>+ +</td>
<td>RMT</td>
<td>–</td>
</tr>
<tr>
<td>14</td>
<td>Oncocytoma</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

–, preneoplastic proliferation or tumours were not observed; +, focal preneoplastic proliferation; + +, diffused preneoplastic proliferation.

Table III. Occurrence of renal preneoplastic proliferation, renal tumours and extrarenal tumours in the AAI-treated group at 6 months post-treatment

<table>
<thead>
<tr>
<th>Group</th>
<th>n (%)</th>
<th>Renal preneoplastic proliferation (%)</th>
<th>Renal tumours (%)</th>
<th>Extrarenal tumour (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AAI-treated</td>
<td>14 (100)</td>
<td>14 (100) a</td>
<td>4 (28.6) b</td>
<td>1 (7.1)</td>
</tr>
<tr>
<td>Control</td>
<td>10 (100)</td>
<td>0 (0) c</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
</tbody>
</table>

*a*P < 0.01 versus control group.

**P < 0.05 versus control group.**
Histopathological features of renal mesenchymal tumours

Renal mesenchymal tumours (RMT) were observed in unilateral kidney of three rats (Table II). Grossly, the tumours were solitary and large, measuring 2.0 × 1.8 × 1.4 cm, 3.0 × 2.8 × 1.9 cm and 2.5 × 2.3 × 1.0 cm in size, respectively. The tumours presented as white or grey-white in colour. All tumours were located at one pole of the kidney with the renal capsules bulging outward. The tumours occupied ~1/6--1/2 of the whole kidney. In sections the new growth was white, tough and had small cysts in it (Figure 1C).

Histological examination revealed that poorly differentiated mesenchymal tumours consisted of closely aggregated short spindle cells, with little cytoplasm faintly stained, a thick nuclear membrane and nuclear hyperchromasia (Figure 1D). Multifocal areas with haemorrhage and necrosis were seen in the stroma. In highly differentiated mesenchymal tumours large numbers of spindle cells were distributed diffusely in the tumours. The shape of the tumour cells was similar to that of the fibrous cells, with the cytoplasm stained red, a thick nuclear membrane and oval-shaped hyperchromatic nuclei. Nuclear atypia and mitosis were frequently seen (Figure 1E).

Histopathological features of renal oncocytoma

An oncocytoma was observed in the kidney of rat 14 in the experimental group. The tumour was 0.5 × 0.5 × 0.4 cm in size. It was situated in the upper pole and occupied 1/8 of the kidney. In section the tumour tissue was firm and the cut surface of the growth was evenly white and had small cysts in it.

Histological examination revealed that the cellular elements were spherical, oval or irregular in shape and arranged in a tubulocystic pattern, with eosinophilic cytoplasm and round or oval nuclei. Nuclear mitosis was occasionally observed and necrosis was not seen (Figure 2A). The tumour was non-encapsulated, but clearly differentiated from normal renal tissues. Sparse infiltration of lymphocytes was seen in the interstitium.

Histopathological features of tumours in extrarenal organs

A case of mammary duct carcinoma was observed in experimental animal 10, the tumour was found on the right side of the abdomen, 5.0 × 4.9 × 2.5 cm in size. The cut surface of the tumour was soft and evenly white.

Histological examination revealed that the tumour cells were arranged in a tubulocystic, trabecular or nested pattern as well as in solid sheets. The tumour cells were oval or cubic, with densely stained large nuclei and nuclear atypia. Malignant mitotic figures were frequently seen. Multifocal necrosis was present in the interstitium. The excretion in the tubular acini...
stained with HE. Fibrous cells, proliferation, partial dilation and congestion of the blood vessels and sheets of necrosis were easily seen in the stroma.

**Renal histopathological changes**

On day 8 the histopathological changes were similar among the four rats in the experimental group. Acute tubular necrosis (ATN) was found. Focal loss of brush borders and desquamation of tubular epithelial cells were observed, predominantly in the corticomedullary junction. The tubular basement membranes remained intact. Protein casts and cellular debris were present in some tubules. Congestion was observed in the peritubular capillaries. Interstitial oedema, widening and cellular infiltration were not found.

After the first month the histopathological changes were similar among the three rats in the experimental group. Focal vacuolation and necrosis of tubular epithelial cells were seen, predominantly in the corticomedullary junction. The tubular basement membranes remained intact and part of them was naked. Occasionally protein casts were observed in tubules. Interstitial fibrosis or widening was not seen.

After 3 months the histopathological changes were similar among the three rats in the experimental group. Tubular lesions in the corticomedullary junction gradually recovered. A few tubular epithelial cells were necrotized or lost (Figure 2B). Focal interstitial infiltration of small spindle-shaped cells was observed occasionally, with little cytoplasm staining faintly. The nuclei were spindle or oval-shaped, staining uniformly and densely. Nuclear atypia was not observed (Figure 2C). Interstitial fibrosis or widening was not seen.

After 6 months the histopathological changes were similar among the 14 rats in the experimental group. Tubular lesions in the corticomedullary junction had nearly recovered. Vacuolation or necrosis of tubular epithelial cells was rarely seen. Interstitial fibrosis or widening was not observed.

No abnormality was observed in kidneys of control rats during the 6 months.

**Discussion**

In the past we reported that AMK, a herb used in China with the main toxic component being AA, could trigger acute renal failure and tumour formation in the kidneys as well as extrarenal organs of rats (Qiu et al., 2000). The present study suggests that AA (50 mg/kg/day for 3 days) administered orally to rats has similar carcinogenic properties and corroborates the results reported by Mengs and Stotzem (1993) on the nephrotoxic properties of AA in rats. While both renal dysfunction and histopathological changes in the kidney recovered spontaneously, preneoplastic proliferation occurred and tumours developed in the kidney and extrarenal organs. The predominant neoplasm found was RMT; however, renal oncocytoma and mammary duct carcinoma were also found.

The oncogenicity of AAI is not clearly understood, however, it has been reported that AA is metabolized to reactive intermediates which bind covalently to DNA (Pfau et al., 1990; Schmeiser et al., 1996; Lebeau et al., 2001). AA–DNA adducts have been found in the tissues of both humans and experimental animals. These adducts can accumulate and are highly persistent in the tissues, which could possibly be responsible for the carcinogenic effect of AA. Mutations of the p53 and ras genes have been detected in the tissues of humans and animals that had been intoxicated with AA (Schmeiser et al., 1990; Arlt et al., 2001a,b; Lord et al., 2004) and a specific AA-associated p53 mutation was observed in primary mouse cells after exposure to AAI (Liu et al., 2004). The most prominent adduct found in all AAN patients analysed to date is the da–AAI adduct. Concerning formation of AA–DNA adducts in kidney cells, it is not surprising that the short-term administration of AAI could lead to formation of tumours. However, our findings differ from previous work in two respects. Firstly, all AAI-induced tumours previously reported (Mengs et al., 1982; Schmeiser et al., 1990; Cosyns et al., 1998) originated from epithelial tissues, whereas we found tumours in both epithelial and mesenchymal tissues. Secondly, in previous studies the tumourigenic effect of AAI was widespread, as opposed to our study, where all preneoplastic proliferation was found in the kidney and the only extrarenal tumour discovered was a case of mammary duct carcinoma. Differences in duration of treatment and dosage may be responsible for the different tumour locations. Further investigations detecting AAI–DNA adducts in specific components of the kidneys, including tubular cells and mesenchyma, may help in clarifying the underlying mechanism.

RMT is the designation proposed by Hard and Butler (1970) for a neoplasm of the rat referred to in the past under different terms, the one most used being nephroblastoma. The tumour type is characterized by a wide spectrum of connective tissue cell types occurring within a single tumour and is interpreted as a neoplasm of the secondary mesenchyma. Its spontaneous occurrence is rare, but it can be induced by various chemicals, including dimethylnitrosamine, cycasin, streptozotocin and methylthiourea (Nogueira et al., 1993). The equivalent in humans was suggested to be mesoblastic nephroma, a rare mesenchymal tumour of childhood and unusually present in adults (Bisceglia et al., 2000; Daniel et al., 2000).

Renal oncocytoma is a rare benign tumour which accounts for about 3–7% of all renal tumours. Because of its benign nature, most patients are asymptomatic and the lesion is discovered accidentally (Kuroda et al., 2003; Seyedzadeh et al., 2003). Although it is reported as a benign tumour with a good prognosis, some cases of oncocytoma have metastasized or resulted in death. Diagnosis of oncocytoma could only be firmly established by careful histological evaluation to exclude renal cell carcinoma. Klein and Valensi (1976) first reported 13 cases of renal oncocytoma and proved that it derived from the epithelial cells of the renal distal tubule.

As indicated in our study, short-term, high dose AA administered in rats could induce renal dysfunction and mild ATN which could pass undetected, however, renal neoplasms as well as extrarenal tumours developed subsequently. Lord et al. (2004) reported a patient that had urothelial malignancy 6 years after presenting with AAN and later had a breast carcinoma that metastasized to the liver, and their study suggested that her urothelial tumour was related to AA exposure while the breast and liver tumours were unlikely to have been induced by AA. Nortier et al. (2003) studied the prevalence of urothelial carcinoma among 39 patients with end-stage Chinese herb nephropathy (caused by *Aristolochia* spp.) and found 18 cases of urothelial carcinoma, however, whether patients who ingested the herbs but failed to develop renal disease are also at risk of urothelial malignancies remains to be verified. Recently, Nortier et al. (2003) reported a case of *Aristolochia*-related urinary tract cancer without significant renal failure, suggesting a dissociation between the carcinogenicity and nephrotoxicity of AA. The prevalence of urinary tract cancer...
is still unknown in patients exposed to AA when their renal function is preserved. Although the responses to AA may vary greatly between different species, our results suggest that a systematic anamnestic investigation of the use of herbal medicine is required when patients have urothelial tumours of unknown origin and normal renal function.

References


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