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GENOTOXICITY AND CARCINOGENICITY

Ying Li · Zhihong Liu · Xiaohua Guo
Jian Shu · Zhaohong Chen · Leishi Li

Aristolochic acid I-induced DNA damage and cell cycle arrest in renal tubular epithelial cells in vitro

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Abstract DNA damage is a critical event preceding cellular apoptosis or necrosis. This study was carried out to investigate the effect of aristolochic acid I (AAI) on DNA damage and cell cycle in porcine proximal tubular epithelial cell lines (LLC-PK1 cells). LLC-PK1 cells were stimulated with AAI at the concentrations of 80, 320, and 1,280 ng/ml for 24 h. DNA damage was examined by comet assay and the cell cycle was assayed by flow cytometry (FCM), cellular apoptosis and lysis were examined simultaneously. Cellular nuclear changes were observed by electron microscopy and the expression of wild-type p53 protein and mRNA were measured by FCM and RT-PCR. We found that AAI-induced DNA damage prior to apoptosis and lysis in LLC-PK1 cells in a dose-dependent manner ($P < 0.01$). The percentage of cells in the G2/M phase that were treated with AAI (320 and 1,280 ng/ml) for 24 h increased significantly ($P < 0.01$). Electron micrographs showed the nuclear abnormalities in AAI-treated cells. The expression of p53 protein and mRNA did not change in the AAI-treated cells. AAI may cause DNA damage and cell cycle arrest in LLC-PK1 cells through a wild-type p53-independent pathway, prior to apoptosis or necrosis. This study on the molecular mechanism of AAI-induced toxicity may explain why tubular epithelial cells present limited proliferation and regeneration abilities in the clinical presentation of AAI-associated nephrotoxicity.

Y. Li · Z. Liu (✉) · X. Guo · J. Shu · Z. Chen · L. Li
Research Institute of Nephrology, Jinling Hospital,
Nanjing University School of Medicine,
210002 Nanjing, China
E-mail: zhihong@21cn.net
Tel.: +86-25-80860042
Fax: +86-25-84801992

Y. Li
Division of Nephrology, The Second Xiangya Hospital,
Central South University, 410011 Changsha, China
E-mail: liyingdoctor@yahoo.com.cn

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Introduction

Recently, evidence has demonstrated an unequivocal role of aristolochic acid in the so-called Chinese herb nephropathy (But and Ma 1999; Lord et al. 1999; Tanaka et al. 2001). Aristolochic acid, the plant extract of *Aristolochia* species, is nephrotoxic and carcinogenic. It can induce acute renal failure and involve the onset of progressive renal tubulointerstitial fibrosis in several organisms. Thus, there is a trend to refer to aristolochic acid nephropathy (AAN) rather than the Chinese herb nephropathy (Cosyns 2002, 2003). Both clinical and in vitro findings suggest that the proximal tubule is the direct target of aristolochic acid injury (Depierreux et al. 1994; Kabanda et al. 1995; Chang et al. 2001; Nortier et al. 1997). Studies have shown that the formation of aristolochic acid-related DNA adduct is toxic to tubular epithelium and carcinogenic to urethral epithelium (Schmeiser et al. 1996; Nortier et al. 2000; Arlt et al. 2001). Consequences of aristolochic acid attack on tubular epithelial cells include apoptosis, transdifferentiation, and necrosis (Gao et al. 2000; Zheng et al. 2000; Su et al. 2002). However, the pathogenesis that underlies aristolochic acid-induced toxicity in renal tubular epithelial cells has not completely been elucidated and is still a matter of debate.

In contrast to other tubulointerstitial diseases, tubular epithelial cells with AAN undergo limited regeneration following aristolochic acid injury, which usually cause tubular atrophy and extensive paucicellular interstitial fibrosis (Cosyns 2003; Li et al. 2001). Growth arrest, mediated by nephrotoxic agents, can inhibit tubular cell regeneration, thus impede the recovery from acute renal failure, and may also contribute to the overgrowth of other cell types, such as

fibroblasts, and lead to interstitial fibrosis (McCauley et al. 1991). DNA damage is a relatively common event in the life of a cell, and DNA damage can result in various cellular effects such as cell cycle arrest to allow for repair of the transcriptional response that may be beneficial to the cell, cellular apoptosis or gene mutation, cellular or organismic death, etc. (Sancar et al. 2004). It had been demonstrated that the DNA damage is an early event during CsA injury in renal epithelial cells (Lally et al. 1999). Pippin et al. (2003) indicated that DNA damage was a novel response to sublytic amounts of complement C5b-9-induced injury in podocytes, which might explain why podocyte proliferation can be limited following immune-mediated injury. We suggest that there is a mitotic defect in tubular epithelial cells caused by aristolochic acid exposure, before they suffer from apoptosis or necrosis. This study was carried out to investigate the effects of aristolochic acid I (AAI) on DNA damage and cell cycle prior to cellular apoptosis and lysis, with respect to the involvement of wild-type p53 pathway activation induced by AAI in proximal tubular epithelial cells.

Materials and methods

Cell culture and preparation

Aristolochic acid is a mixture consisting of structurally related nitrophenanthrene carboxylic acids. AAI and AAII, the two major components of the carcinogenic plant extract of aristolochic acid (Schaneberg and Khan 2004), are known to be mutagenic and to form DNA adducts in vivo (Arlt et al. 2000). AAI was shown to be more toxic than AAII (Balachandran et al. 2005), and the extent of DNA modification by AAI was higher than that of AAII in all enzymatic in vitro systems (Schmeiser et al. 1997). Therefore, we chose AAI as the experimental material in this study. AAI (purity >95%) was purchased from China Pharmaceutical University (Nanjing, China). LLC-PK1 cells (i.e., porcine kidney cell lines analogous to the proximal tubular epithelial cells) were obtained from the American Type Culture Collection (ATCC Rockville, MD, USA). Cells were grown in six-well plates with RPMI-1640 medium (Gibco, USA) and supplemented with 10% fetal calf serum (FCS). At confluence, cells were starved and synchronized, then treated with different concentrations of AAI (80, 320, and 1,280 ng/ml) for 24 h. LLC-PK1 cells with solvent stimulation alone were set up as the control groups. All experiments were performed on the cells between passages 15 and 20. In the preliminary experiment, we found that the concentration of AAI greater than 1,280 ng/ml induced apoptosis and necrosis in LLC-PK1 cells, and therefore, we chose the concentrations of AAI less than 1,280 ng/ml in this experiment.

Single-cell gel electrophoresis (or comet assay) for DNA damage

After incubating LLC-PK1 cells for 24 h with AAI, cells were harvested using trypsin (0.5% w/v). DNA strand breakage was quantified using an alkaline version of the comet assay (Tice et al. 2000). An aliquot of the cell suspension (80 µl) was mixed with 80 µl of 0.75% low-melting point agarose in PBS (pH 7.4) at 37°C, giving a final concentration of approximately 1×10^7 cells/l. Immediately after mixing, 80 µl of the mixture was pipetted onto a frosted glass microscope slide that was precoated with an 80 µl layer of 0.3% normal melting point agarose. The agarose was placed on ice for 10 min. The slides were immersed in a lysis solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, 1% Nasarcosinate, 10% DMSO, and 1% Triton; pH 10.0) for 2 h at 4°C to remove non-nuclear cell components, then placed in electrophoresis buffer (0.3 M NaOH, 1 mM Na_2EDTA) for 40 min and electrophoresed for 20 min at 25 V and 300 mA. The above procedures allowed the DNA-containing strand break to unwind and the DNA fragments to move toward the anode, forming a comet-like tail during electrophoresis. The slides were then washed with distilled water, renatured in 0.4 M Tris–HCl (pH 7.5), stained with propidium iodide (PI 5 µg/ml) for 15 min in the dark, and examined under confocal microscopy (Zeiss LSM 510). Fifty cells were analyzed using the comet assay in each experiment. DNA damage was evaluated and quantified using an established system to detect different comet assay parameters, such as tail length and the fluorescence intensity of the tail under confocal microscopy. Such indices were considered proportional to the extent or severity of DNA damage.

Flow cytometry analysis for cell cycle distribution

After the LLC-PK1 cells were incubated with AAI for 24 h, cells were harvested using trypsin (0.5% w/v), centrifuged (140*g* for 12 min), washed with PBS, and fixed with 2 ml ice-cold ethanol (70%) overnight. Cells (1×10^6) were centrifuged at 140*g* for 10 min, and the pellet was resuspended in 500 µl lysis buffer and incubated for 10 min at room temperature. Cells were resuspended with 10 µg/ml PI and 100 µg/ml RNAase at 37°C for 30 min. The distribution of cells in the different cell cycle phases was studied by flow cytometry and percentages were determined using multicycle software (Phoenix Flow Systems, San Diego, CA, USA).

Cytotoxicity assay for cell necrosis

To ensure that the concentrations of AAI in the experiment did not induce significant cellular lysis, cells were treated with different concentrations of AAI for 24 h. Cytotoxicity was assessed using an automated biochemistry analyzer (Beckman Inc., USA). The amount

of lactate dehydrogenase (LDH) released in the supernatants was measured at 30°C using UV spectrophotometry at 340 nm.

FCM analysis for cell apoptosis

To ensure that the concentrations of AAI in the experiment did not induce cellular apoptosis, cells were treated with different concentrations of AAI for 24 h and two assays were performed to analyze biochemical evidence of apoptosis in LLC-PK1 cells induced by AAI with FCM.

First, the apoptotic peak (sub-G_1) of cells was measured using FCM. Second, annexin-V assay (Jinmei Company, China) offers the possibility of detecting the early phases of apoptosis. Apoptosis was detected by monitoring the expression of phosphatidylserine on the outer leaflet, an early marker of apoptotic cell death. Phosphatidylserine was stained with fluorescein isothiocyanate (FITC)-labeled annexin V. Loss of membrane integrity as a consequence of necrosis was detected using PI staining of DNA. After 24 h incubation with AAI, LLC-PK1 cells were harvested using trypsin (0.5% w/v), centrifuged (140*g*, for 12 min), resuspended in 2 ml medium containing 10% FCS, kept on ice for 1 h, and recentrifuged (140*g*, for 12 min). The cell pellet was rinsed with PBS and resuspended in 100 µl binding buffer, and 5 µl of annexin-V/FITC solution, 2.5 µl PI solution were added. The suspension was then incubated on ice for 15 min in the dark and measured using FCS. Cells were separated into four subpopulations: annexin-V and PI negative (normal viable cells), annexin-negative and PI-positive (necrotic cells), annexin-positive and PI-negative (early apoptotic cells) and annexin- and PI-positive (secondary necrotic cells). Secondary necrotic cells are the consequence of an apoptotic process in culture (Van Engeland et al. 1996). Cells belonging to this subgroup were quantified and the results are provided as a percentage of the total cell count (Jagus et al. 1999).

Scanning electron microscopy for cell nucleus

After 24 h incubation of LLC-PK1 cells with AAI, cells were fixed with ice-cold 3.75% glutaraldehyde in phosphate buffer (pH 7.4) at 4°C overnight, and postfixed with 1% osmium tetroxide in phosphate buffer at 4°C for 2 h, then dehydrated in a graded ethanol series (15, 35, 70, 95, and 100%) for 10 min in each step and embedded in an Epon mixture. Ultrathin sections were cut with a glass knife, stained with a lead citrate–uranyl acetate solution, and observed under electron microscopy.

FCM analysis for p53 protein expression

After 24 h incubation of LLC-PK1 cells with AAI, cells (1×10^6) were collected with PBS containing 0.02% EDTA, fixed in 1% paraformaldehyde containing 0.1% saponin for 10 min, and washed twice in cold PBS containing 0.1% BSA. Cells were treated for 30 min at 37°C with monse anti-pig p53 antibody (1:250) (Caltag Company, USA). After washing, 1:10 dilution of FITC-labeled goat anti-monse IgG1 antibody (Caltag Company, USA) was added and the cells were incubated for another 30 min in the dark. To correct for nonspecific binding, PBS was added to the negative control tube. Cells were assayed by using FCM. Quantitative changes were assessed by mean fluorescence intensity (MFI).

Reverse transcription PCR for p53mRNA expression

After 24 h incubation of LLC-PK1 cells with AAI, total cellular RNA was extracted using the Trizol Reagent kit (Gibco, USA) according to the manufacturer's instructions. RNA samples were quantified by optical absorbance at 260 nm in a spectrophotometer the A260/A280 ratio between 1.8 and 2.0. RNA was reverse transcribed using a commercial kit (Promega, Wisconsin, USA). Porcine p53 primers (Sheng Gong Company, Shanghai, China) were chosen to yield an expected product of 168 bp. The sense primer sequence was 5′-ATCCAGATGACGCC TCCAG-3′, and the antisense primer sequence was 5′-CTGGCAAAACAGCTT ATTGAG-3′. One of the housekeeping genes of *β*-actin (450 bp) was co-amplified with p53 as internal control and specific primers were designed according to the published sequence (Guh et al. 1996). The sense primer sequence was 5′-TACAATGAGCTG CGTGTGG-3′ and the antisense primer sequence was 5′-TAGCTCTTCT CCAGGGAGGA-3′. PCR conditions were 35 cycles with denaturation at 94°C (30 s), annealing at 56°C (30 s), and extension at 72°C (30 s). Aliquots of PCR products were run on 2.5% agarose gels and relative densities of the bands (p53 over *β*-actin) were calculated.

Statistical analyses

Data are shown as the mean values ± SD. Results of two groups were analyzed using ''unpaired'' student's *t* tests. Multiple comparisons were analyzed using one-way analysis of variance (ANOVAs). Statistical significance was defined as $P < 0.05$ and $P < 0.01$.

Results

AAI exposure caused DNA damage in LLC-PK1 cells

DNA damage was not detected in either the control or the AAI (80 ng/ml) treatment groups. However, the comet assay was positive, i.e., the tail of damaged DNA migrated away from the nucleus, in LLC-PK1

cells exposed to AAI (320 and 1,280 ng/ml) for 24 h. The DNA damage was quantified by measuring the tail length and the fluorescence intensity of each tail. We found that AAI induced dose-dependent DNA damage in LLC-PK1 cells at concentrations of 320 and 1,280 ng/ml ($P < 0.01$ vs. control) (Fig. 1 and Table 1). Overall, these results showed that AAI exposure induced dose-dependent DNA damage in LLC-PK1 cells.

AAI exposure causes LLC-PK1 cell cycle arrest in G2/M phase

To determine the effect of AAI on the cell cycle, the cellular DNA content in G0/G1, S and G2/M stages were calculated using FCM analysis. There were no differences in the percentages of G_1 and S phase of cells between the controls and AAI exposure groups. However, the relative percentage of G2/M phase significantly increased in a dose-dependent manner after treatment with 320 and 1,280 ng/ml AAI for 24 h ($P < 0.05$ and 0.01, respectively) (Fig. 2).

AAI exposure did not induce lysis in LLC-PK1 cells

Release of LDH by cells in culture is an evidence of cell toxicity and loss of cell membrane integrity. There was no significant difference in LDH release in LLC-PK1 cells exposed to AAI for 24 h compared with control cells ($P > 0.05$). Thus, the concentrations of AAI in our experiment did not produce lysis in LLC-PK1 cells (Fig. 3).

AAI exposure did not induce apoptosis in LLC-PK1 cells

Both PI and annexin V-FITC/PI double staining methods were used to test for apoptotic cells treated by AAI for 24 h using FCM. We did not measure the apoptotic peak (sub-G1) in this experiment with PI (Fig. 4). The annexin-V assay showed the areas representing different cell groups, including the secondary necrotic cells (annexin V^+/PI^+), early apoptotic cells (annexin V^+/PI^-), and the unlabeled area representing viable cells (annexin V^-/PI^-). Our results demonstrated that the percentage of the apoptotic cells in AAI groups (i.e., 80, 320 and 1,280 ng/ml) was nearly the same as that of the control group (0.90 ± 0.06, 1.27 ± 0.09, and $1.34 \pm 0.12\%$ vs. control $0.79 \pm 0.1\%$, respectively) ($P > 0.05$). Therefore, we concluded that the concentrations of AAI in our experiment did not induce apoptosis in LLC-PK1 cells.

Ultrastructural changes of LLC-PK1 cells stimulated with AAI

LLC-PK1 cells treated with AAI showed severe nuclear abnormalities, including nuclear malformation, giant nuclei, assembled chromosome at the edge of nucleus, cleaved nuclei, and rolled and thickened nuclear membranes. The severity of the nuclear and membranous

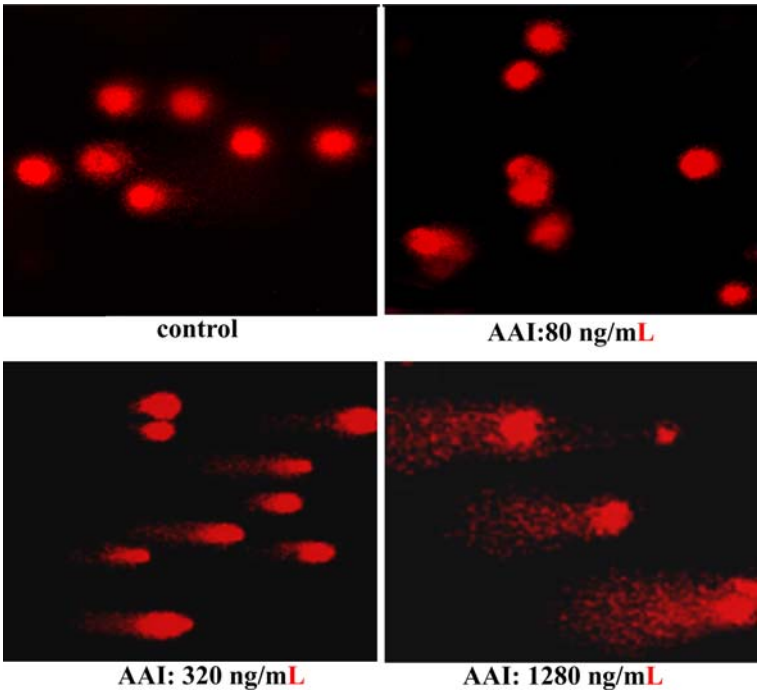

Fig. 1 DNA damage was detected in LLC-PK1 cells exposed to AAI for 24 h using the comet assay (×400)/ml. DNA damage was not detected in control cells and LLC-PK1 cells treated with AAI (80 ng/ml). However, comet assay was positive in LLC-PK1 cells exposed to AAI (320 and 1,280 ng/ml)

Table 1 Effect of AAI on DNA damage in LLC-PK1 cells

AAI (ng/ml)	Tail length (μm)	Fluorescence index of tail (OD)
0	0	0
80	0	0
320	30.77 ± 5.35**	128.07 ± 8.96**
1,280	80.43 ± 7.91**	148.50 ± 14.82**

**$P < 0.01$ versus cells without AAI exposure

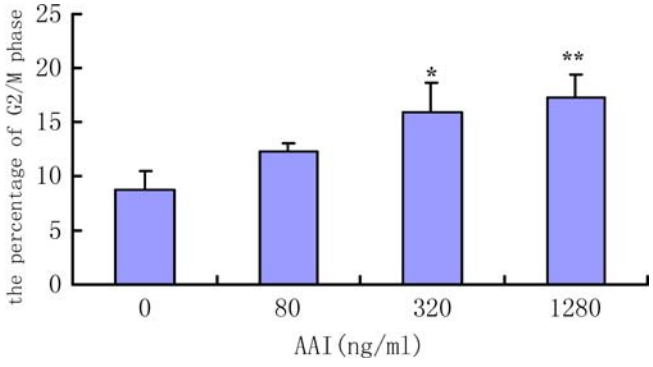

Fig. 2 Effect of AAI exposure on the percentage of LLC-PK1 cells in G2/M phase by FCM (%, mean ± SD). *$P < 0.05$ and **$P < 0.01$ versus cells without AAI exposure

lesions caused by AAI correlated significantly to drug concentration (Fig. 5).

Effect of AAI on p53 protein expression in LLC-PK1 cells

LLC-PK1 cells were cultured with the medium containing AAI for 24 h. FCM analysis was used to confirm the presence of p53 protein in LLC-PK1 cells with positive staining for p53 antibody. The MFI in different groups (AAI 80, 320 and 1,280 ng/ml) were 1.85 ± 0.29, 1.92 ± 0.12 and 1.88 ± 0.20 versus 2.11 ± 0.08, respectively ($P > 0.05$). Our study demonstrated that there were no changes of p53 protein expression between control and AAI treatment groups. As shown in Fig. 6, AAI stimulation did not increase p53 protein expression in LLC-PK1 cells.

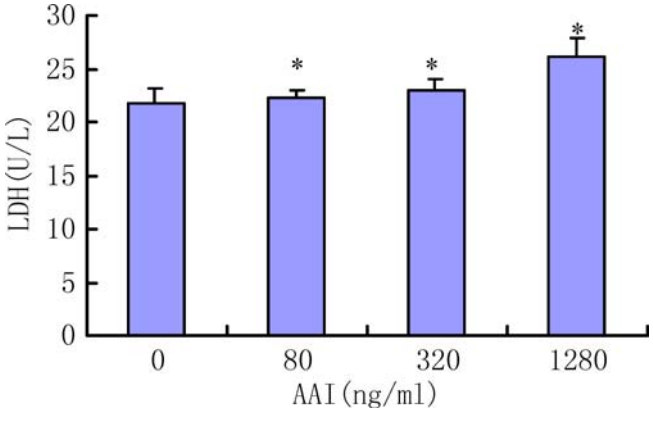

Fig. 3 The numerical results of LDH assay in LLC-PK1 cells with AAI exposure for 24 h (mean ± SD). *$P > 0.05$ versus cells without AAI exposure

Effect of AAI on p53 mRNA expression in LLC-PK1 cells

LLC-PK1 cells were cultured with the medium containing AAI for 24 h. p53 mRNA expression in LLC-PK1 cells was detected using RT-PCR. The results showed that the p53 mRNA expression in the AAI treatment group was similar to that in the control group. AAI stimulation did not increase p53 mRNA expression in LLC-PK1 cells (Fig. 7).

Discussion

Aristolochic acid is well known for its nephrotoxic effects, as well as for its carcinogenic and mutagenic properties in humans and animal models. Lebeau et al. (2001) showed that aristolochic acid impeded endocytosis and induced DNA adducts in proximal tubule cells, which supports the involvement of aristolochic acid in early proximal tubule dysfunction in patients suffering from AAN (Kabanda et al. 1995). Aristolochic acid–DNA adducts are of primary importance not only in the initiation of tumor development, but also in the progression of renal interstitial fibrosis because the adducts impair physiological processes at the transcriptional level (Arlt et al. 2001; Schmeiser et al. 1996; Nortier et al. 2000). Recently, there have been many clinical and experimental studies about AAN, especially regarding its mechanism. High dose of aristolochic acid could induce acute necrosis or lysis in tubular epithelial cells, whereas its chronic administration could lead to irreversible renal failure with hypocellular interstitial fibrosis by inducing cellular apoptosis and transdifferentiation or by directly activating interstitial myofibrotic cells. However, we wanted to know what effect aristolochic acid had on tubular epithelial cells when the dosage and duration of aristolochic acid ingestion were insufficient to induce apoptosis and lysis in tubular cells.

Kaufmann and Paules (1996) reported that cellular DNA damage and cell cycle arrest induced by UV irradiation represented different clinical phenomena. Cell responses to DNA damage, induced by physiological or pathophysiological stress, include cell cycle arrest, activation of genes and gene products involving in DNA repair, and programmed cell death under certain circumstances. Whether DNA damage is the result of AAI exposure has not previously been elucidated. We demonstrated for the first time that AAI can

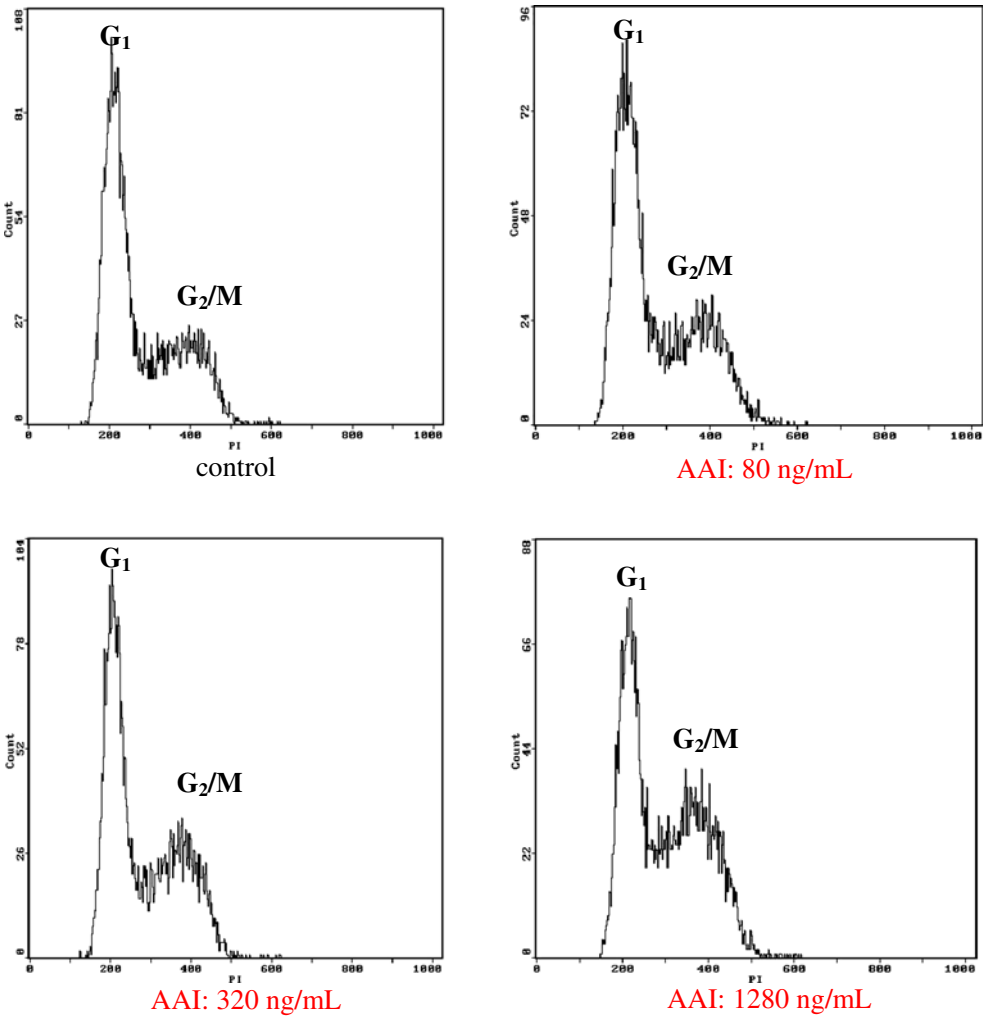

Fig. 4 Apoptotic peak (sub-G_1) could not be measured by FCM in LLC-PK1 cells with AAI exposure for 24 h

induce DNA damage and cell cycle arrest in LLC-PK1 cells.

Our study has showed that AAI caused a dose-dependent increase in the size of the comet tail, which was paralleled by an increase of fluorescence intensity of the tail. However, the amount of LDH released in the cell supernatant stimulated by AAI was similar to that of control cells when the comet assay was performed, and apoptosis was not detected during these experiments. In response to aristolochic acid-mediated injury, renal tubular epithelial cells may undergo lysis, apoptosis, or carcinogenesis. However, our assays showed that the range of AAI concentrations that induce injury of LLC-PK1 cells did not cause apoptosis or necrosis, instead resulting in DNA damage. The explanation for this phenomenon is a dose effect. In addition, the appearance in LLC-PK1 cells treated with AAI under electron microscopy in this study showed severe nuclear alterations, which added new evidence to cellular DNA damage. In this experiment, we reported that AAI causes DNA damage in tubular epithelial cells in vitro prior to cellular apoptosis or necrosis, which demonstrated that DNA damage is another consequence of cell exposure to AAI and could be an early event during aristolochic acid injury in tubular epithelial cells. In contrast to other toxic medicines, tubular epithelial cells do not readily proliferate in response to AAI-induced-DNA damage. This inadequate regenerative response by tubular epithelial cells may contribute to the development of renal interstitial fibrosis.

Cell cycle arrest is one of the most common means by which cells avoid injury. Cell cycle progression is regulated through major checkpoints located in the G1/S or G2/M phases. When cells receive an anti-proliferative signal, such as those caused by DNA damaging agents, feedback control is activated that acts as a brake to inhibit progression through these checkpoints. The induction of cell cycle arrest facilitates the repair of damaged DNA by preventing the damaged DNA from continuously replicating, and it stops the cell cycle from amplifying genetic damage (Stark and Taylor 2004). Coincident with AAI-induced DNA damage in LLC-PK1 cells, the cell cycle analysis in this study using FCM indicated that AAI treatments produced a dose-depen-

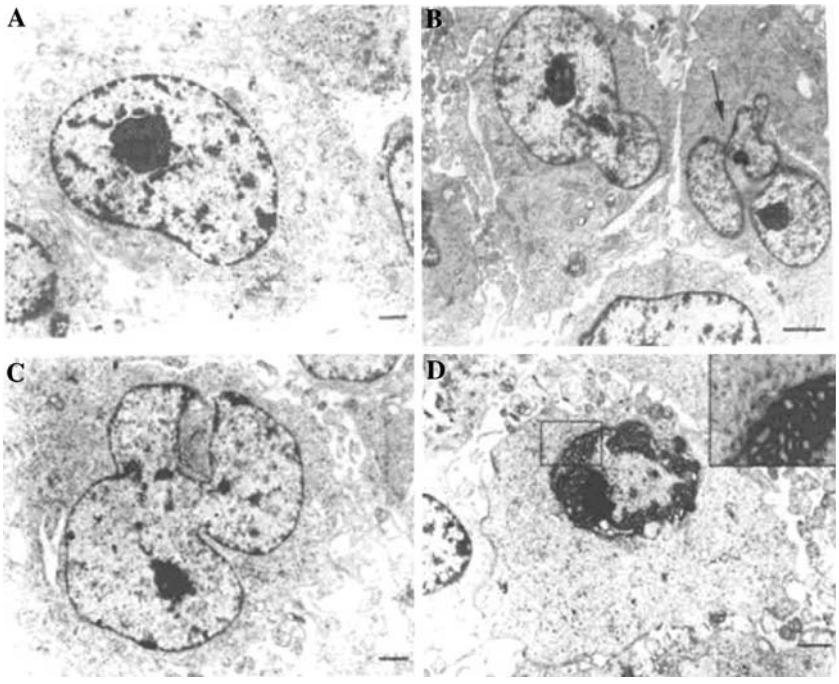

Fig. 5 Ultrastructural changes of LLC-PK1 cells incubated with AAI for 24 h. **a** Control group. **b** Split nuclei (*arrow*) in AAI group (320 ng/ml). **c** Malformed nucleus in AAI group (1,280 ng/ml). **d** Unique nuclear membrane changes in AAI group: nuclear membrane rolled and thickened forms numerous small cells (1,280 ng/ml)

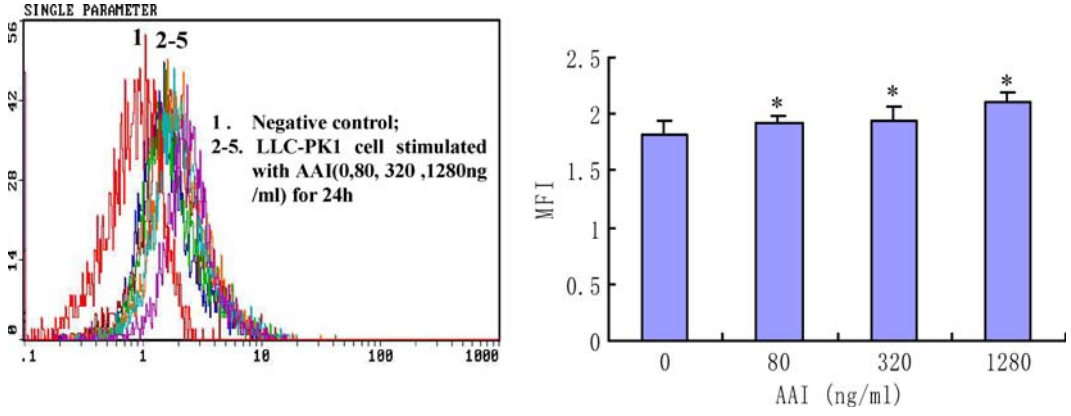

Fig. 6 Expression of wild-type p53 protein in LLC-PK1 cell stimulated with AAI for 24 h by FCM. $*P > 0.05$ versus cells without AAI exposure

dent increase the percentage of cells in the G2/M phase. Thus, we suggest that AAI delayed or inhibited the G2/M phase in tubular epithelial cells.

Various cellular effects induced by AAs ingestion might be closely associated with difference of individual's ability to repair damaged DNA. If damaged DNA is completely repaired, cells re-enter a normal cell cycle. If damaged DNA is continuously propagated from insult for a long duration, and renal epithelial cell cycle will entirely cease and cells will stop proliferation, which may explain why tubular epithelial cells present limited proliferation and regeneration in the clinical presentation of AAI-associated nephrotoxicity. It is clear that cell cycle arrest is an important component that triggers cellular responses to DNA damage. However, it is difficult to determine why AAI delayed LLC-PK1 cells only in G2/M but not G1/S and what is the possible pathway that underlies G2/M phase arrest in tubular epithelial cells following AAI-induced injury.

The tumor suppressor protein wild-type p53 is now firmly established as a key negative regulator of cell proliferation. It plays an important role in the G1/S and G2/M checkpoints of the cell cycle. Wild-type p53 has multiple functions, including cell cycle control in response to DNA damage, DNA repair and induction of apoptosis. It has been postulated that p53 has a regulatory role at cell cycle G2/M checkpoints (Hattori et al. 2004; Clifford et al. 2003). However, it has also been documented that the G2/M transition may be regulated independently of p53 (Minemoto et al. 2003; Saldeen et al. 2003). Growth arrest can occur by p53-dependent and p53-independent pathway or two pathways simul-

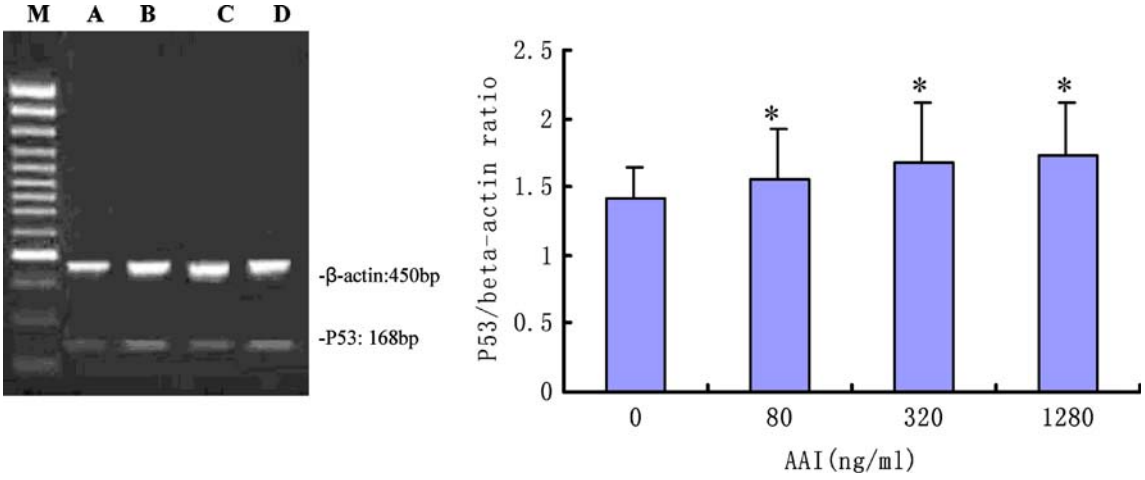

Fig. 7 Expression of wild-type p53 mRNA in LLC-PK1 cells stimulated with AAI for 24 h. *A* control group, *B* LLC-PK1 cell stimulated with AAI (80 ng/ml) for 24 h, *C* LLC-PK1 cell stimulated with AAI (320 ng/ml) for 24 h and *D* LLC-PK1 cell stimulated with AAI (1,280 ng/ml) for 24 h. $*P > 0.05$ versus cells without AAI exposure

taneously (Taylor and Stark 2001; Bache et al. 1999; Vincent et al. 1999). The p53-independent pathway involves a phosphorylation cascade that activates the chk1 effector kinase and induces G2 arrest through inhibitory tyrosine phosphorylation of Cdc2. Our study demonstrated that the expressions of wild-type p53 protein and mRNA in LLC-PK1 cells after 24 h of AAI treatment nearly similar to that in the control group. We concluded that in LLC-PK1 cells, the induction of cell cycle arrest was transduced by AAI through p53-independent pathway in response to DNA injury. Our result that transcriptional up-regulation of p53 is not expected is inconsistent with published data that carcinogenesis is associated with the overexpression of p53 (Cosyns et al. 1999). It is unclear whether wild-type p53 will be overexpressed in human cells treated with AAI under similar experimental conditions. It would be rather important to look at the different phosphorylation sites of p53 in order to understand whether it is activated. Moreover, other pathways that induce cell cycle arrest in LLC-PK1 cells after exposure to AAI, such as the cyclin pathways, need to be investigated in the future.

Presently, several health institutions, including the U.S. Food and Drug Administration (USFDA), have recently published safety information related to the presence of AAs in plant materials to prevent further cases of intoxication. Interestingly and fortunately, only 2–3% of persons treated with weight-reducing pills containing aristolochic acid are known to have suffered from renal injury in Belgium (Arlt et al. 2002). Furthermore, in Germany, aristolochic acid has been used by thousands of patients as an immunomodulatory agent for 25 years without any reports of chronic interstitial nephritis (De Broe 1999). The genetic heterogeneity of aristolochic acid metabolism and the individual or racial susceptibility should not be ignored. However, studies related to the effects of aristolochic acid use are limited.

Different from other drugs, once patients take herbs containing AAs, their renal function will be impaired even if they cease their administration and eventually these patients will develop permanent chronic renal failure regardless if the onset feature was chronic or acute. Our results demonstrated that AAI-induces DNA damage and cell cycle arrest in the G2/M phase through p53-independent pathway in LLC-PK1 cells, prior to cellular apoptosis or necrosis. This study of the molecular mechanism of AAI-induced toxicity in LLC-PK1 cells might explain paucicellular interstitial fibrosis and limited tubular regeneration in the clinical presentation of AAI-associated nephrotoxicity.

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