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Study of the Effect of Aristolochic Acid on Mice Kidney and the Effect of Withdrawal: Histological and Immunohistochemical Study

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Abstract

Aristolochic acid (AA), one of the commonly used herbal medicines was reported to cause harmful effect on kidney. In this work, we aimed to study the effect of AA on renal tissue and to investigate the effect of its withdrawal. Methods: Forty five adult male mice were randomly assigned to three groups. Control group, Group 1 where mices were treated with aristolochic acid (AA) i.p. in a dose of 3 mg/kg every 3 days for 6 weeks. In group 2 the (remodeling group), AA was administered i.p. in a dose of 3 mg/kg every 3 days for 6 weeks, followed by 6 weeks remodeling (withdrawal) time. Urine and blood samples were collected; creatinine, BUN and P/C ratio were estimated. Renal specimens were processed for histological examination by H&E, Masson trichrome and immunohistochemical stain for PCNA and for real time RT-PCR of PCNA, IL-8 and TGF- β1 gene expression. Results: in group 1 treated with AA, histological examination revealed that some tubules were atrophied collapsed while others were dilated. Tubular cells exhibited cytoplasmic vacuolation, flattening, necrosis and even shedding. The matrix between tubules was expanded. In the surrounding interstitial areas, mononuclear cell infiltration was observed. Tubulointerstitial cell proliferation was significantly increased as indicated by PCNA immunostaining. This correlates with worsening of renal parameters and significant increase in PCNA, IL-8 and TGF- β1gene expression in renal tissues. However, in group 2, withdrawal of AA led to marked improvement of renal parameters, preservation of renal tissue and reduction of tubular damage and cellular infiltrate. Also, the number of PCNA-positive cells was significantly reduced and the expression of PCNA, IL-8 and TGF- β1 significantly reduced. In conclusion, aristolochic acid is nephrotoxic and cessation of its administration leads to reduction of this toxicity as was evidenced by laboratory, histological and immunohistochemical methods.

Abbreviations ESRD: End Stage Renal Disease; AA: Aristolochic Acid; BUN: Blood Urea Nitrogen; BEN: Bulkan Endemic Nephropathy; PCNA: Proliferating Cell Nuclear Antigen; TIMPS: Tissue Inhibitors of Metalloproteinases, RT- PCR: reverse transcriptase polymerase chain reaction

Keywords: Aristolochic Acid; Inflammatory; Immunohistochemical; Immunostaining

Introduction

Aristolochic acid (AA) is found in plants of the Aristolochiaceae family, which have been used widely in random recipes for thousands of years to treat asthma, gout, arthritis, pain, hiccough, snake bites, weight loss and slimming [1-3]. Today, it is known that almost all species of the genus aristolochia were considered as human nephrotoxic and carcinogens. This first came to light when cases of nephritis and kidney failure were seen in a group of women in Belgium who had all taken a particular weight-loss product, which contained aristolochic acid [4,5]. After that, more cases have been described worldwide related to exposure to AA [6,7]. In 2001, the FDA issued warnings that herbal products are unsafe if they contain or are suspected to contain aristolochic acid (AA) [8,9].

AAN causes two main distinct health problems. In the upper urinary tract, it induces transitional cell carcinoma, whereas in the kidney it induces cortical tubular atrophy and extensive interstitial fibrosis [10]. However, the exact mechanisms that initiate renal toxicity appear to converge to apoptosis and oxidative stress [11]. In addition, AAN has been shown to present G2/M cell cycle arrest, a pathological feature closely linked to the development of fibrosis [12]. Profibrotic factors such as transforming growth factor-β1 (TGF-β1) and connective tissue growth factor (CTGF) are upregulated both in vitro and in vivo in the tubulointerstitial cells in aristolochic acid-treated mice, as determined by western blot analysis. These profibrogenic growth factors can stimulate the proliferation and collagen production of fibroblasts [13].

Therefore, this study was done to prove the effect of aristolochic acid on the kidney and to increase the awareness of its serious effects as a possible cause of ESRD. Also, this study was set up to test our hypothesis about the potential protective role of its withdrawal.

Materials and Methods

Experimental animals

This study was conducted on forty five male mice, eight-week-old and weighting (20 - 30 g) were used in this study. The animals were housed in hygienic plastic cages for urine collection before AAI treatment and kept in clean well-ventilated room, with food and water

ad-libitum. All animals' procedures were done according to the recommendation of El-Minia University Ethics committee for proper care and use of experimental animals. Mice were euthanized by ether and euthanized by decapitation, for blood collection.

Animals were randomly assigned to three groups

1. Control group: ten rats received only phosphate-buffered saline at a dose of 6 mg/kg body weight for 3 days according to Schaefer [14].

2. Aristolochic acid (AA) treated group: fifteen mices were treated with (AA) dissolved in phosphate-buffered saline (purchased from Sigma, St. Louis, MO). i.p in a dose of 3 mg/kg every 3 days for 6 weeks.

3. Remodeling group: fifteen mices were injected with AA i.p. in a dose of 3 mg/kg every 3 days for 6 weeks, followed by 6 weeks remodeling (withdrawal) time.

Laboratory investigations

1. Urine samples: The day before being sacrificed, the animals were placed in metabolic cages for 24 h urine collections. Samples were centrifuged at 4°C for creatinine and urine protein/creatinine ratio. All urine analysis kits were used following manufacturers' instructions.

2. Blood samples: were collected at sacrifice and were allowed to clot at room temperature, and the serum was separated by centrifugation at 1600 × g for 15 min at 4°C and stored at -20°C for estimation of serum creatinine and BUN.

Histological examination

Kidneys were quickly removed at sacrifice and fixed in10% formalin. After fixation, tissues were dehydrated in 96% ethanol and isopropyl alcohol, then embedded in paraffin and cut on a rotation microtome (Leica) in sections with a thickness of 5 to 6 μm, and then stained with hematoxylin & eosin and Masson trichrome stain.

Immunohistochemical examination

The formalin fixed paraffin embedded sections (5 mm) were attached to poly-L-lysine-pretreated slides (Sigma-Aldrich). After air-drying the paraffin from FFPE, tissue sections were removed (xylene solution). The sections were rehydrated and immersed in a retrieval solution, sodium citrate buffer (pH 6.0); the microwave oven technique was used (650 W, 1 5 min). Phosphate-buffered saline was used for all washing steps. Endogenous peroxidase activity was quenched with 0.3% hydrogen peroxide in a methanol solution (30 min). Nonspecific protein-binding sites (background staining due to the Fc receptor) were blocked with 20% normal serum (Vectastain Elite ABC kit IgG; Vector Laboratories, Labconsult, Brussels, Belgium) and then with avidin D and biotin solution (avidin/biotin blocking kit; Vector Laboratories, Labconsult). Subsequently, the sections were incubated overnight with rabbit anti-mouse PCNA (1/4000) monoclonal primary antibody (Abcam, Cambridge, England, UK, ab2426) or with rat monoclonal antibody anti-NEP (1/4000) (Santa Cruz Biotechnology, Boechout, Belgium, sc-80021) and diluted in the blocking buffer. Slides were then incubated with specific biotinylated secondary antibody (Vectastain Elite ABC kit; Vector Laboratories, Labconsult). The extent of the specifically bound primary antibodies was visualized by means of the avidin–biotin peroxidase complex method. The diaminobenzidine/hydrogen peroxide was used as the chromogene substrate, producing a brown end product. Counterstaining with hematoxylin completed the processing. The specificity of antibodies used was established by the producer. Normal serum (5% solution) instead ofthe primary antibody (used in order to exclude nonspecific staining of kit reagents) showed no staining.

Quantification of immunostainings

Quantifications were performed by Fields containing more than two glomeruli or large vessels were excluded.The patterns of PCNA expression were defined as nuclear staining and was analyzed (40 magnification lens) with a blinded method (AA versus control groups). The PCNA positively stained cells were counted in 30 fields and expressed as an average of positive cells per field.

RNA extraction and Real-Time RT-PCR

Total RNA was extracted from the renal tissue of experimented mice directly after removed from animals freshly by using RNeasy Plus Mini kit (Qiagen GmbH, Hilden, Germany) and their concentrations were measured by spectrophotometer (Nanodrop 2000, Thermo Scientific, USA) then stored for cDNA conversion in liquid nitrogen. 2 g of total RNA of each sample was converted into cDNA using RT First Strand kit (Qiagen Sciences, Maryland, USA). Gene expression was examined for PCNA, interleukin -8 (IL-8) and transforming growth factor –β1 (TGF- β1). Glyceraldehyde-3- dehydrogenase (GAPDH) was included as an internal control and for normalization. Amplifications were performed in 25u l reaction volume in each tube that contains 12.5 ul SYBR Green (SensiFast SYBR, Bioline, UK), 1 ul of cDNA template, 2 ul of 10 pM primers and 9.5 ul of nuclease free water. Cycling protocol of PCR amplification was done as follows: initial denaturation at 95°C for 2 min., followed by 40 cycles of denaturation (95°C for 15 sec), primer annealing (57°C for 30 sec), and primer extension (72°C for 30 sec) Reac¬tions were completed with a final extension at 72°C for 10 min. For each sample, the procedure was carried out in triplicate. Relative expression of PCNA, TGF- β1 and IL-8 genes was calculated using the comparative threshold cycle method (Ct). All values were normalized to the GADH gene, primers used table1.

Primer Reverse	Primer Forward	Gene primer
5-CACGTATATGCCGAGACCTTAGC-3	5-CACGTATATGCCGAGACCTTAGC-3	PCNA
5-CAGCAGAACTGAACTACCATCG-3	5-CACCTCAAGAACATCCAGAGCT-3	IL-8
5-GAA CAC TAC TAC ATG CCA TTA T-3	5-GCT TCA GAC AGA AAC TCA CT-3	TGF- β1
5-GACAACCTGGTCCTCAGTGTAGC-3	5-GGAGAAACCTGCCAAGTATGATG-3	GADH

Table 1: Primers used in RT-PCR.

Results

Biochemical results

Compared with control mice, the value of serum creatinine (Figure 1) and urea nitrogen (Figure 2) in AA group were significantly elevated. In the withdrawal group, they were significantly lowered than that of the AA treated group (Table 2).

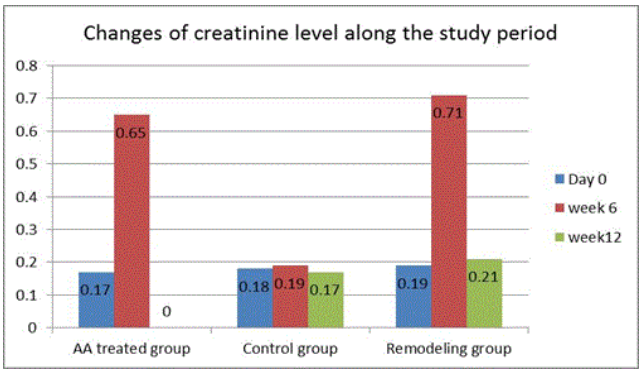

Figure 1: Changes in creatinine level throughout the experiment.

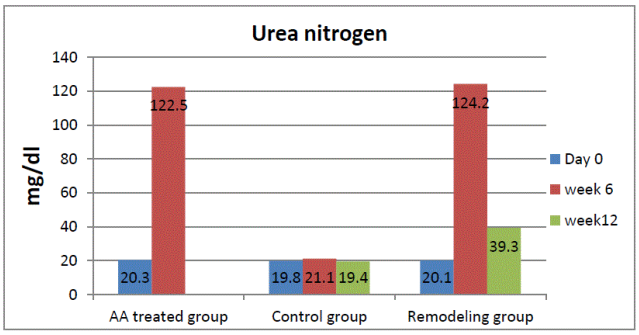

Figure 2: Changes in urea nitrogen level throughout the experiment.

Histological results

Hematoxylin and eosin stain

In this study, H&E stained sections of the normal control group showed renal corpuscles formed of tuft of glomeruli, Bowman's capsule including Bowman's space, proximal convoluted tubules with narrow lumen, rounded nuclei, apical brush border and basal striations and distal convoluted tubules. The DCTs showed wider lumen and were lined with cubical cells with rounded central nuclei and ill-defined basal striations (Figure 3a).

	AA treated group No.			Control group			Remodelling group No.		
	Day 0	Week6	Week 12	Day 0	Week6	Week 12	Day 0	Week6	Week 12
Creatinine mg/dl	0.17	0.65*	------	0.18	0.19	0.18	0.19	0.71	0.21**
BUN mg/dl	12	70*	------	15	16	15	13	82	22**
P/C ratio mg/g creatinine	13	852*	------	15	17	18	16	954	98**

BUN: Blood urea nitrogen. P/C ratio: protein/creatinine ratio mg/g creatinine.

*significantly different from the baseline at day 0, Also was significantly different from the control group at week 6. **significantly different at week 12 after 6 weeks of withdrawal.

Table 2: Laboratory Data of the groups throughout the study period.

On the other hand, examination of sections of AA treated mice showed that some tubules were atrophied collapsed while others were dilated. However, tubular cells exhibited cytoplasmic vacuolation, flattening, necrosis and even shedding. The matrix between tubules was expanded. In the surrounding interstitial areas, mononuclear cell infiltration was observed (Figure 3b). However, withdrawal of AA led to preservation of renal tissue and reduction of tubular damage and cellular infiltrate (Figure 3c).

Masson's trichrome stain

AA administration in Group 2 led to a marked extracellular matrix deposition around the glomeruli, blood vessels as well as between the tubules (Figure 4b1 and Figure 4b2). The expansion of matrix was significantly decreased after AA withdrawal (Figure 4c).

Immunohistochemical results

By PCNA immunostaining only few tubular cells expressed PCNA in the control group while in the aristolochic acid (AA) treated mice, the number of immunostained nuclei with PCNA were markedly

increased. However, withdrawal of AA resulted in a substantial reduction in number of PCNA-positive cells (Figure 5) (Figure 6).

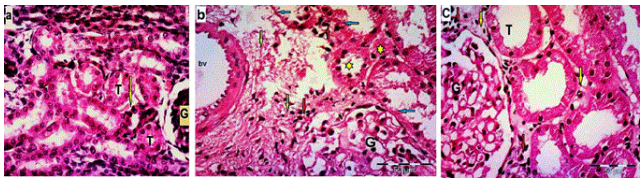

Figure 3: Photomicrographs of renal tissues showing: a) control group showing: (G), tubules (T) and interstitial matrix (yellow arrow). b) aristolochic acid treated group, there are wide areas of matrix (yellow arrows) with interstitial cellular infiltrate (red arrows). Notice also that some tubules are shrunken and atrophied (yellow astric), others are dilated (yellow pyramid). Tubular cells showed vacuolation, flattening and necrosis (blue arrows). c) AA withdrawal group, the amount of matrix is only slightly more than that in the control group (H&E x400).

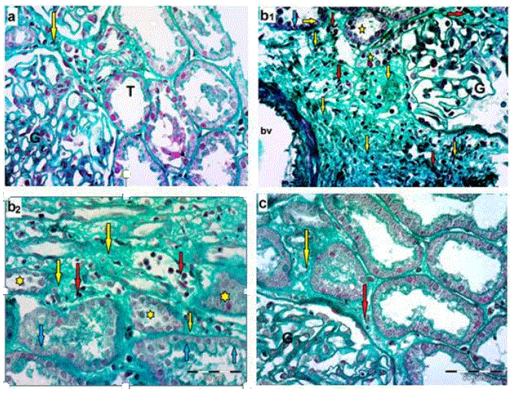

Figure 4: Photomicrographs of kidneys stained with the Masson trichrome stain showing: A) control group showing normal renal tissue: glomeruli (G), tubules (T) and interstitial matrix (yellow arrow). B) aristolochic acid treated group. Notice the presence of wide areas of matrix (yellow arrows) located periglomerular and perivascular in (b1) & between the tubules in (b2) with interstitial cellular infiltrate (red arrows). Notice also that some tubules are atrophied tubules (yellow star), others are dilated (yellow pyramid) with necrotic cells (blue arrows). C) AA withdrawal group, the amount of interstitial collagen is only slightly more than that in the control group (Masson trichrome x 400).

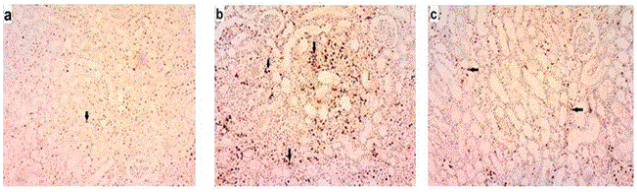

Figure 5: Representative photomicrographs of proliferating cell nuclear antigen (PCNA) staining in different groups with quantification. In control group, Only few tubular cells expressed PCNA while in the aristolochic acid (AA) treated mice, typical nuclear patterns of PCNA immunostaining were predominantly seen in peritubular areas interstitial cells (black arrows). Withdrawal of AA in (c) resulted in a substantial reduction of PCNA-positive cells (original magnification x 400).

PCNA-positive nuclei were quantified by counting three high-powered fields at 40x magnification. *Represents statistically significant difference ($p < 0.05$) compared with control mice, (**) represents statistically significant difference ($p < 0.05$) from AA treated group (Table 3).

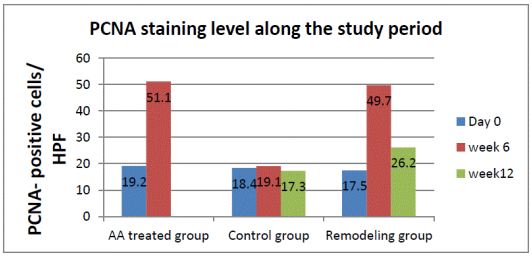

Figure 6: PCNA staining level throughout the experimental period.

	AA treated group No.			Control group			Remodelling group No.	
	Day 0	Week 6	Week 12	Day 0	Week 6	Week 12	Day 0	Week 6	Week 12
PCNA positive cells/ HPV	19.2	51.1*	------	18.4	19.1	17.3	17.5	49.7	26.2**

HPF: high power field. *Significantly different from the baseline at day 0 ($p < 0.05$). , Also was significantly different from the control group at week 6 ($p < 0.05$). **Significantly different at week 12 after 6 weeks of withdrawal

Table 3: Changes in PCNA level throughout the experiment.

Real time RT-PCR results

- PCNA gene expression significantly elevated at 6th day of AA treatment compared to control group ($p = 0.003$), and significantly reduced at 12th day compared to 6th day ($p = 0.05$), Figure 7.
- IL-8 gene expression significantly elevated at 6th day of AA treatment compared to control group ($p < 0.001$), and significantly reduced at 12th day compared to 6th day ($p = 0.008$), Figure 8.
- TGF- β gene expression significantly elevated at 6th day of AA treatment compared to control group ($p = 0.003$), and significantly reduced at 12th day compared to 6th day ($p = 0.05$), Figure 9.

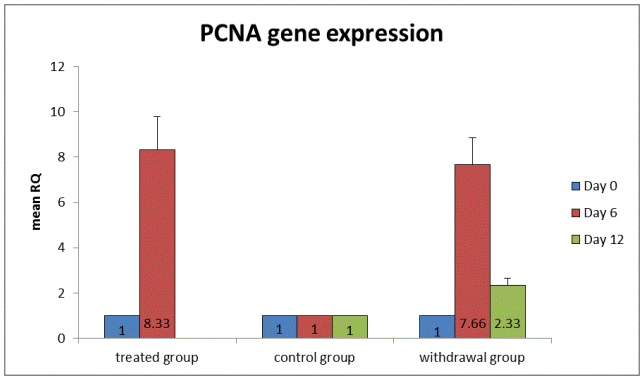

Figure 7: Bar graph representing mean±SE of RQ for PCNA gene expression in renal tissue in different groups.

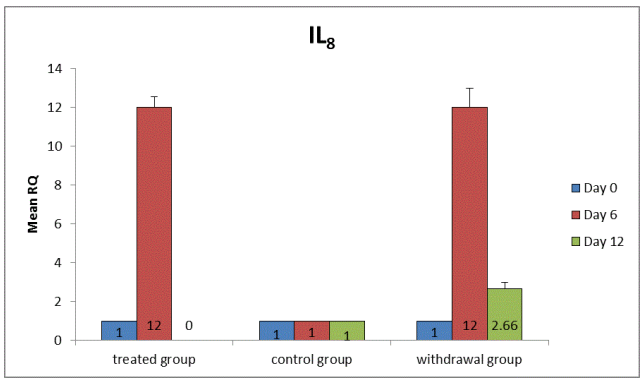

Figure 8: Bar graph representing mean±SE of RQ for IL-8 gene expression in renal tissue in different groups.

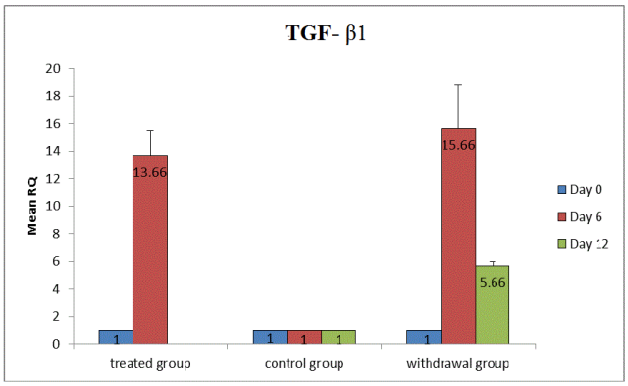

Figure 9: Bar graph representing mean±SE of RQ for TGF-β1gene expression in renal tissue in different groups.

Discussion

There is growing evidence that AA plant extracts which is used for curing many diseases, play a causal role in nephropathy. The kidney is the most vulnerable due to its highest blood flow and the high concentration of chemicals in tubular fluid adjacent to tubular cells [15,16].

In this study, we used male laboratory mice because many studies reported that female mice seem to be less sensitive to kidney damage [17] as female hormone 17β-estradiol (E2) was found to attenuate renal injury [18].

We found that, the value of serum creatinine and urea nitrogen in AA treated group were significantly elevated compared with control mice. This agrees with other investigators [19] who recorded increased levels of serum creatinine and BUN, and an elevated urine albumin/creatinine ratio. In the withdrawal group, after 12 weeks, serum creatinine and BUN were significantly lowered than that of the AA treated group. Other studies recorded that it took a longer period for functional recovery of the kidney to occur, and this is probably due to the fact that recovery from acute kidney injury depends on the dose and duration of exposure [20-22].

Microscopic examination revealed that administration of AA led to tubular atrophy, tubular cells showed necrosis and even detachment leaving a bare basement membrane. The same was observed by other investigators who found that tubular injury leads to tubular atrophy, and eventually end-stage renal failure [23-27].

In this regard, necrosis has been considered one of the mechanisms for aristolochic acid-induced renal injury [28,29] and this was confirmed by the ultrastructural study of Qi, et al. [30] who found that the epithelium losses many features of its terminally differentiated phenotype where the apical microvilli become effaced; cell height is reduced; the tight junctions lose many of their properties and functions.

Acute renal injury enhanced lysosomal proliferation and induced the autophagy pathway in proximal tubule cells in both animals and human beings [31]. In human kidney biopsies, electron microscopy showed autophagosomes containing organelles such as mitochondria [32]. Autophagy has been shown to be present in both cell-protective

and cell death mechanisms to get rid of damaged mitochondria and prevent apoptosis. Several triggers may lead to of protein kinase 3 (PK3) activation, which is the key mediator of necroptosis [33] as it phosphorylates pseudokinase, which induces plasma membrane rupture [34].

Moreover, AA can enter the cell via organic anion transporters (OATs) resulting in defective activation of anti-oxidative enzymes, mitochondrial damage [35,36] and impaired regeneration of proximal tubular epithelial cells [15].

In this research, there was a marked cellular infiltration in the interstitium in AA treated group. Normally, the medulla is completely devoid of leukocytes [28]. In AKI, the tubular epithelial cells generate pro-inflammatory cytokines and molecules responsible for macrophage chemotactic activity. Among them are monocyte chemoattractant peptide-1 (MCP-1) and interleukin-8 (IL-8) and transforming growth factor beta (TGF-β) [37]. Macrophages in turn can produce inflammatory cytokines and enhance type 1 T helper cells immune response [38,39]. Also, tubular epithelial cells express Toll-like receptors, which regulate T-cell activity. In addition to macrophage and T lymphocytes, neutrophils are important contributors to ischemic injury where they enhance production of ROS which in turn directly damage DNA of proximal tubules and augment apoptosis [40].

Our data indicated that also that kidneys subjected to AA had expanded matrix as demonstrated by massons trichrome stain compared to normal control group. However, in the remodelling group, the amount of collagen in the interstitium was markedly reduced.

The same was observed by Ballhause et al and by Gruia et al [41,42] who stated that the epithelial cells, as a result of injury, can move into the cell cycle and produce dedifferentiated daughter cells that express increased production of extracellular matrix. They can even undergo epithelial-mesenchymal trans differentiation, migrate from the epithelium, and contribute to the pool of interstitial myofibroblasts [30,40].

Several cytokines secreted by infiltrating macrophages and T-lymphocytes stimulate fibroblast proliferation, and that interstitial fibroblasts produce collagen types I, III, and IV [21]. Moreover, active TGF-β1 is a critical factor involved in tubulointerstitial fibrosis. It increases matrix protein synthesis, inhibits matrix protein degradation, and upregulates integrin matrix adhesion factors [43]. Transforming growth factor-β1 inhibits matrix degradation by increasing the activity of tissue inhibitors of metalloproteinases (TIMPS); and decreasing the activity of metalloproteinases (MMPs) [44]. It also stimulates the synthesis of receptors for extracellular matrix proteins. Furthermore, TGF-β1 is a chemoattractant for fibroblasts [38] and stimulates their proliferation [45]. However, fibrosis likely develops due to an imbalance between extracellular matrix synthesis and degradation [14].

In our study, by using real time RT-PCR ;we found that the expression of TGF- β 1 and IL-8 is significantly elevated in AA treated group more than control group and significantly decrease after cessation of AA at 12th day compared to the 6th day. Both are proinflammtory cytokines which responsible for macrophage chemotactic activity, macrophages in turn can produce inflammatory cytokines [38,39].

By using PCNA immunostaining, we found that the number of PCNA positive cells was observed more frequently in AA treated than in normal control kidneys. Also by using real time RT-PCR we found PCNA gene expression elevated significantly at 6th day of AA treated group compared to control group, and reduced significantly at 12th day (withdrawal) compared to 6th day group. According to the literature, glomerular and tubular epithelial cells in normal renal tissue are resting cells lacking the expression of cell proliferation antigen PCNA. After initial injury, histologic indicators of cell proliferation, such as mitosis, hyperchromatic nuclei, and a high nuclear-cytoplasmic ratio are seen. Most injured tubule cells may be replaced through extensive proliferation of neighboring cells, which may be the predominant mechanism of tubular cell injury repair [27]. The increased PCNA expression in tubular epithelial cells and interstitial infiltrates indicates upregulation of their proliferative rate and may be correlated with their proposed role in progression of renal disease [46,47] .

Moreover, under certain conditions like renal injury, tubular epithelial cells may be recapturing their fetal proliferation rates which is a property lost during differentiation [48]. However, there was a significant difference between PCNA expression in the remodeling and the control group.

After cessation of AA administration in this study, there was a significant improvement of renal condition as demonstrated by creatinine level, histological and immunohistochemical study. This disagrees with the results of [16] who found that even after cessation of the exposure, progression of renal failure is generally relentless. However, acute AAN may progress to ESRD within 1 month in patients having continuously ingesting a high dose during a short period of time [49] or in cases of either a consistent or intermittent intake of a low dose of AA for a long period of time [50]. Anyhow, we are concerned that this form of nephropathy may become more common in the future because of the continuous online trading of random therapeutic recipes containing AA. Furthermore, they are often bundled and sold as formulations rather than individually. To complicate matters, these products are sometimes not registered and improperly labeled, making it difficult for suppliers and consumers to be certain of its constituents [4,51]. Indeed, the web is a marketing tool with low barriers to entry and the disaster is that anyone can set up a web site inexpensively [52].

Conclusion

This study confirmed that aristolochic acid is nephrotoxic as evidenced by laboratory, histological and immunohistochemical methods. It also confirmed that cessation of administration of AA leads to reduction of its toxic effect on kidney.

Recommendations: We recommend that this thesis should be translated to Arabic language to increase awareness of people who use slimming herbs and to draw the attention of doctors to collect histories of herbal medication from patients as a routine clinical practice. We also recommend that herbal substances should be subject to the same strict scrutiny and controls as drugs. Lastly, we appeal to health officials to take the necessary measures to prevent trading random therapeutic recipes through social media to prevent kidney disease. Otherwise, it may cause serious complications to users that may reach death.

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