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1 **Probenecid prevents acute tubular necrosis in a mouse model of**
2 **aristolochic acid nephropathy**

3

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17 **Running headline:** Protective effects of probenecid in AAN

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1 **ABSTRACT**

2 Experimental aristolochic acid nephropathy (AAN) is characterized by early tubulo-
3 interstitial (TI) injury (necrosis of proximal tubular epithelial cells (PTEC) and
4 inflammatory infiltrate). It also reproduces chronic lesions seen in humans (tubular
5 atrophy and interstitial fibrosis). *In vitro*, probenecid (PBN) inhibits AA entry through
6 organic anion transporters (OATs), reduces specific AA-DNA adduct formation and
7 preserves cellular viability. To confirm these results *in vivo*, we reproduced
8 experimental AAN in a mouse model. Plasma creatinine level (Pcr), tubulo-interstitial
9 (TI) lesions, DNA repair processes (proliferating cell nuclear antigen tissue expression)
10 and AA-DNA adduct formation were studied. AA induced severe TI injuries (necrosis of
11 PTEC followed by mononuclear cells infiltration, tubular atrophy and an interstitial
12 fibrosis) and transient acute kidney injury. Addition of PBN prevented Pcr increase, TI
13 injuries and reduced both the extent and the severity of ultrastructural lesions induced
14 by AA (loss of brush border, mitochondrial edema and disappearance of mitochondrial
15 crests). Further, PCNA positive cells count and total AA-DNA adduct levels were
16 significantly reduced in mice receiving AA+PBN compared to mice treated with AA
17 alone. The present data demonstrate *in vivo* the nephroprotective effect of PBN, an OATs
18 inhibitor, towards acute PTEC toxicity in a mouse model of AAN.

19

- 1 KEYWORDS
- 2 Aristolochic acid nephropathy
- 3 Proximal tubular epithelial cells
- 4 Probenecid
- 5 Acute tubular necrosis
- 6 Interstitial renal fibrosis
- 7 DNA adducts
- 8
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1 **ABBREVIATIONS**

2 AA; Aristolochic acid

3 AAN; Aristolochic acid nephropathy

4 TI; Tubulointerstitial

5 PTEC; Proximal tubular epithelial cell

6 α -SMA; α -Smooth Muscle Actin

7 Pcr; Plasma creatinine

8 Mn/M ϕ ; Monocytes/Macrophages

9 NEP; Neutral endopeptidase

10 OA; Organic anion

11 OAT; Organic anion transporter

12 PBN; Probenecid

13 PCNA; Proliferating Cell Nuclear Antigen

14 PEG; Polyethylene glycol

15

1 INTRODUCTION

2 Human aristolochic acid nephropathy (AAN) is a tubulointerstitial (TI) nephritis
3 reported after intake of herbal remedies containing aristolochic acid (AA). (1, 2) It is
4 histologically characterized by a typical corticomedullary gradient of interstitial fibrosis
5 and the progressive atrophy of proximal tubules, resulting in the rapid deterioration of
6 renal function to the end-stage. (3, 4) AA intoxication also leads to the formation of
7 specific AA-DNA adducts which are premutagenic lesions involved in the development
8 of AAN-associated urothelial cancer and their long-term presence in renal tissue is used
9 as a biomarker of AA exposure. (5, 6)

10 AA-induced TI nephritis was experimentally reproduced in rabbits, mice and rats
11 (7-10). A biphasic evolution of TI lesions was identified in our Wistar rat model. (11, 12)
12 In the early, so-called *acute phase*, a transient tubular necrosis located in the S3 segment
13 (proximal tubular epithelial cells (PTECs)) and a mononuclear cell infiltration are
14 observed; later, in the so-called *chronic phase*, tubular atrophy and interstitial fibrosis
15 are clearly the prominent features. In this step-by-step model, inflammatory cells were
16 proposed as the physiopathological link between both phases. (11) *In vitro* data early
17 confirmed that PTEC were the target of AA (13), suggesting the presence of specific
18 molecular mechanisms responsible for the accumulation of AA in PTECs. The excretion
19 of numerous organic anions (OAs) including endogenous metabolites through PTECs is
20 actually achieved via unidirectional transcellular transport, involving the uptake of OAs
21 from the blood across at the basolateral membrane and their extrusion across the apical
22 membrane into the tubular lumen. Organic anion transporters (OATs) play a key role in
23 this process. At least eleven isoforms of OATs have been identified; a majority of them
24 was found in the kidney. OATs are exchangers linked to two other transporters, the

1 sodium dicarboxylate cotransporter and the sodium-potassium ATPase. OA are taken up
2 by OAT 1 and/or 3 in the basolateral membrane of the proximal tubule. This uptake is
3 processed in parallel to the countertransport of α -ketoglutarate. The drug then crosses
4 the cell and is excreted in the lumen of the tubule. (14, 15) The activity of OATs has been
5 associated with proximal tubular injury due to the accumulation of toxics, such as
6 uremic toxins, drugs and mercuric species. (14-17) In embryonic kidney cells (HEK293)
7 as well as *Xenopus laevis* oocytes, three human isoforms (OAT1, OAT3 or OAT4) were
8 reported to play a role in intracellular accumulation of AA. (18, 19) Moreover,
9 probenecid (PBN) blocked AA entry by inhibition of human OATs, reducing the
10 formation of AA-DNA adduct (19), and preserved cell viability. (18)

11 We investigated this last aspect *in vivo* in a mouse model of AAN. We
12 hypothesized that PBN, by reducing AA entry through OATs, could protect PTECs against
13 lesions, preventing AA-DNA adduct formation and thus preserve cell viability.

14

15 **RESULTS**

16 Ninety-six mice C57BL/6 were randomly assigned to 4 groups of 24 animals each.
17 According to group, mice were injected with AA, AA+PBN or solvent (polyethylene-
18 glycol (PEG))+PBN. Control group was injected with PEG (Figure 1). AA (5 mg/kg body
19 weight) or PEG was injected once a day and PBN (150 mg/kg body weight) twice a day.
20 These dosing regimens of PBN have been shown to inhibit organic anion transporter
21 (20)

22 Plasma creatinine level (Pcr), TI lesions, DNA repair processes (proliferating cell
23 nuclear antigen tissue expression) and AA-DNA adduct formation were quantified in
24 each group after 2, 4, 5 and 8 days of AA injections.

25

1 ***Probenecid prevents AA-induced acute kidney injury***

2 A transient acute kidney injury, as reflected by significant increase in Pcr levels was
3 observed in mice receiving AA after 5 days of injections as compared to control animals
4 [PCr (mg/dl), median (min-max): 0.353 (0.222-0.504) vs 0.135 (0.112-0.211);
5 $p < 0.0022$]. Addition of PBN prevents Pcr increase in AA animals [PCr (mg/dl), median
6 (min-max): 0.125 (0.105-0.139) vs 0.353 (0.222-0.504); $p < 0.0022$]. No significant
7 change in Pcr levels was measured in PEG+PBN group as compared to controls (Figure
8 2).

9

10 ***Probenecid significantly reduces AA-induced TI injury***

11 As demonstrated in Figures 3-4a-d and 3-4e-h, the renal parenchyma from PEG and
12 PEG+PBN groups remained normal in optical microscopy analyses at all studied time
13 points of protocol. In contrast, early histological lesions were present in the AA group
14 (Figures 3-4i-l). As early as day 2, a swelling of PTEC was found in the medullary rays
15 (Figure 4i). In the same areas, prominent PTEC necrosis was observed at days 4 and 5
16 (Figures 3-4j-k). After 8 days of AA treatment, tubular atrophy was clearly widespread
17 as reflected by dilatation and flattening of PTECs as well as tubular basement membrane
18 thickening. In the surrounding interstitial areas, mononuclear cells infiltration was
19 observed at day 4 and progressively extended to day 8. At that time point, this
20 inflammatory process was associated with extracellular matrix deposition. In mice
21 receiving AA+PBN, swelling and necrosis of PTECs was limited to few tubules located in
22 the medullary rays only at day 4 without any interstitial inflammatory cells infiltration
23 (Figures 3-4n). Moreover, proximal tubules as well as the surrounding interstitial areas
24 appeared normal under optical microscopy analysis at days 5 and 8 (Figure 3-4o-p).

1 Throughout the protocol, no abnormality was detected within the glomeruli from all
2 groups under optical microscopy analysis.

3 As compared with controls, the semiquantitative score of TI injury obtained in
4 AA-treated mice revealed tubular necrosis from day 4 to 8 with an evident peak at day 5
5 (Figure 5a), lymphocytic infiltration from day 5 (Figure 5b), marked tubular atrophy at
6 day 5 accompanied by progressive interstitial fibrosis (Figure 5c and 5d, respectively).
7 In the AA+PBN group, a significant reduction of all the semiquantitative scores was
8 found: of tubular necrosis on days 5 ($p<0.0013$) and 8 ($p<0.0025$), of lymphocytic
9 infiltrate ($p<0.0013$) and of tubular atrophy ($p<0.0018$) (day 8) as well as of interstitial
10 fibrosis on days 5 ($p<0.0022$) and 8 ($p<0.0013$) (Figure 5a-d).

11

12 ***Necrosis of positive neutral endopeptidase tubules is prevented by PBN***

13 To further assess the distribution of necrotic tubules, an immunostaining of neutral
14 endopeptidase (NEP) was performed and evaluated. NEP is a specific marker for the
15 brush border of S3 segment of the proximal tubule in rat. (21) As shown in Figure 6,
16 immunostaining of NEP in control groups demonstrated that NEP positive cells were
17 mainly located in medullary rays and in the outer stripe of outer medulla, reproducing
18 the typical distribution of NEP positive cells in *pars recta* of proximal tubule observed
19 previously in our rat model (11, 22). No disappearance of NEP immunostaining was
20 observed in PEG or PEG+PBN groups. AA administration lead to a progressive necrosis
21 of PTECs, especially NEP positive as suggested by the progressive disappearance of NEP
22 staining in the medullary rays and the presence of intratubular necrotic NEP positive
23 cells on day 5 (Figure 6c). At day 8, NEP positive cells had completely disappeared from
24 medullary rays (Figure 6d). On the contrary, NEP immunostaining was maintained in
25 the AA+PBN group (Figure 6e,f).

1

2 Addition of PBN leads only to a mild reduction of AA-DNA adduct formation

3 As PBN administration was effective in significantly preventing acute kidney injury and
4 TI lesions induced by AA, we examined possible effect of PBN on AA-DNA adduct
5 formation in kidney cortex tissue samples. As shown in Figure 7a, the pattern of AA-DNA
6 adducts consisted of three major adduct spots: 7-(deoxyadenosin-*N*⁶-yl)-aristolactam I
7 (dA-AAI, spot 1), 7-(deoxyguanosin-*N*²-yl)-aristolactam I (dG-AAI, spot 2) and 7-
8 (deoxyadenosin-*N*⁶-yl) aristolactam II (dA-AAII, spot 3). This pattern is identical to those
9 observed previously in our rat model and in AA-exposed patients. (6, 22)

10 In the AA+PBN group, as compared to the AA group, there were no significant changes in
11 AA-DNA adducts at days 2,4 and 5, while a significant reduction of the total AA-DNA
12 adduct levels was observed at day 8 (Figure 7b). There was no correlation between the
13 Pcr levels or the TI scores and AA-DNA adduct formation (data not shown).

14 Previous in vivo studies showed that DNA adduct formation by AA reaches a steady-
15 state level which is likely the result of a balance between adduct formation and their loss
16 through either DNA-repair processes or apoptosis. (22, 23) Moreover, this level seems
17 to reached quickly, even 2 days after the first injection in a rat model. (22) This could
18 explain the only small differences in DNA adduct levels between the two groups after 8
19 days of treatment. Therefore, we conducted an additional experiment to investigate the
20 early time course and kinetic of AA-DNA adduct formation. Thirty-two mice were
21 injected with AA or AA+PBN as previously and 4 mice per group were sacrificed after 6,
22 12, 18 and 24 hours. At these time points, differences in AA-specific DNA adduct levels
23 between the two groups were clearly observed (Figure 7a) confirming that PBN
24 significantly inhibits AA-DNA adduct formation.

25

1
2 ***PBN significantly reduces AA-induced DNA damage repair processes and cell***
3 ***proliferation attested by PCNA immunostaining***

4 PCNA is a polymerase cofactor, involved in DNA damage repair processes and in the
5 stability of the DNA microsatellite region. (24) Only few tubular cells expressed PCNA in
6 controls (PEG and PEG+PBN groups) (Figure 8a-h). In AA-treated mice, typical nuclear
7 patterns of PCNA immunostaining were predominantly seen in PTECs and less
8 frequently in interstitial cells from the corticomedullary junction as soon as day 2 and
9 still increased until day 8 (Figure 8i-l). PBN administration resulted in a reduction of
10 PCNA expression induced by AA in mice as soon as day 4 (Figure 8m-p).

11 As compared to controls, the proportion of PCNA positively stained areas per
12 field was higher in AA-treated mice from day 5 ($p < 0.0043$) to day 8 ($p < 0.0152$) (Figure
13 8q). This proportion was significantly decreased in the AA+PBN group at day 5
14 ($p < 0.0043$) and day 8 ($p < 0.0022$) (Figure 8q).

15
16 ***PBN reduces the degree of ultrastructural lesions of PTEC induced by AA***

17 Control groups (PEG and PEG+PBN) exhibited only mild mitochondrial swelling in few
18 PTEC at day 8 (Figure 9a,b). In kidneys from AA-treated mice, considerable variation in
19 the degree of cellular damage may occur. Normal tubules were frequently admixed with
20 injured nephron showing extensive mitochondria disruption and altered brush borders
21 (Figure 9c,e). PBN administration reduced both the extent and the severity of cellular
22 damage induced by AA (Figure 9d,f).

23
24 **DISCUSSION**

25 Since the cluster outbreak of the so-called Chinese herbs nephropathy in 1993,

1 AAN is now recognized as a public health problem worldwide (25): it is identified as an
2 environmental kidney disease in the Balkan region and probably underestimated in
3 Asian countries where traditional Chinese medicine is widely used, as suggested by two
4 recent studies. (26, 27) Understanding its physiopathology may lead to effective
5 therapies preventing the progression of chronic kidney disease.

6 In the present study, we reproduced histopathological features of human AAN
7 (tubular necrosis, inflammatory interstitial infiltrate, tubular atrophy and interstitial
8 fibrosis) in a short-term mouse model. Male C57BL/6 mice were injected daily with a
9 mixture of AAI and AAI, the same as the one present in *Aristolochia sp.* and ingested by
10 our patients. After 4 days of injection, a massive necrosis of PTEC from the medullary
11 rays was observed, resulting in an acute kidney injury on day 5. This “acute” phase was
12 followed by a prominent atrophy and fibrosis on day 8. The normalization of creatinine
13 on day 8 is consistent with our observations of the acute phase in the AAN rat model in
14 which a transient creatinine increase on day 5 was followed by a normalization of
15 creatinine on day 8, contrasting with persistent histological lesions. (22) In addition,
16 such dissociation between plasma creatinine and histology during the recovery phase
17 has been described in other models of acute kidney injury like ischemia and reperfusion.
18 (28)

19 The addition of PBN prevented acute kidney injury and significantly reduced
20 tubular necrosis, lymphocytic infiltrate, atrophy and fibrosis. Moreover,
21 immunohistochemical study using PCNA staining confirmed the protective effect of PBN
22 from AA. Increase in PCNA staining reflects a proliferation process of PTECs secondary
23 to necrosis, which is in accordance with previous histological findings obtained in our
24 rat model using Ki67 immunostaining. (12) Finally, a reduction of AA-DNA adduct
25 formation was found in mice receiving AA+PBN as compared to mice treated with AA

1 alone.

2 Recently, two *in vitro* studies demonstrated that PBN inhibits AA entry in human
3 OAT-transfected HEK293 kidney cell lines (19) and in human OAT-transfected cell lines
4 derived from the second portion of the proximal tubule. (18) Further, the former study
5 indicated that PBN can reduce AA-DNA adduct formation and that addition of PBN to AA
6 preserved cellular viability. The present work brings significant *in vivo* results
7 confirming the protective effects of PBN against AA-induced TI lesions by blocking AA
8 entry into PTEC via OATs. Actually, these histomorphometric data can be related to a
9 recent pharmacological study focusing on the effects of PBN on AA liver and kidney
10 metabolism. (29) These authors reported a significantly reduced accumulation of renal
11 AAI in mice exposed to AA and PBN as well as an increase in AAI liver content and
12 biliary clearance.

13 Regarding the evident protective effect of PBN in terms of TI AA-induced lesions,
14 it could be surprising to measure a only slight, difference of AA-DNA adduct level
15 between AA and AA+PBN group. However, this discrepancy could be easily interpreted.
16 First of all, in AA+PBN group, AA may enter PTEC independently of OAT. Other
17 endogenous transmembrane transporters or passive diffusion may also be involved in
18 the uptake of AA, as suggested by a only partial blockade of AA entry by PBN in an *in*
19 *vitro* model of human OAT-transfected HEK293 cells (19). Secondly, DNA adduct
20 formation by AA often quickly reaches a steady-state level as seen in previous *in vivo*
21 studies and our present results. In addition, our data suggest that although AA-DNA
22 adducts are a clue biomarker of AA exposure, there is no correlation between AA-
23 specific DNA adduct levels and nephrotoxicity.

24 Dissociation between AA-mediated nephrotoxicity and adduct formation was
25 first suggested by a clinical case report of AA-induced tumor development without renal

1 impairment. (30) This observation was followed by two rodent studies showing that AA-
2 DNA adducts were the basis for the carcinogenic effect of AA but were unrelated to
3 nephrotoxic insult. Indeed, both AAI and AAI could cause similar types of DNA damage
4 (i.e. bulky DNA adducts) whereas only AAI is nephrotoxic *in vivo*. (9, 10) *In vitro* studies
5 confirmed that AAI is much more cytotoxic compared to AAI due to the presence of a
6 methoxy group in position 8. (31, 32) On the other hand, the carboxyl group rather than
7 the nitro group is important to facilitate AA entry into tubular cells via OATs. Finally,
8 nitroreduction results in *N*-hydroxy-aristolactam formation and these metabolites bind
9 covalently to the exocyclic amino groups of adenine or guanine forming AA-specific DNA
10 adducts. (33, 34) On the other hand, AAI seems to directly cause renal injury by
11 activating mitochondrial permeability transition (35), and reticulum endoplasmic stress.
12 (36) Further, AA is responsible for increased oxidative stress-related DNA lesions due to
13 glutathione depletions (37) and AA can block DNA replication causing cycle arrest
14 and/or apoptosis in renal epithelial cells *in vitro* and *in vivo*. (38-40)

15 In conclusion, we developed an *in vivo* model of AAN characterized by an early
16 episode of acute kidney injury induced by daily injections of AA. Despite highly
17 nephrotoxic effects of AA, we were able to demonstrate a sustained protective effect of
18 PBN by blocking AA entry into PTEC and then preventing acute tubular necrosis.

19
20

21 **METHODS**

22 **Experimental protocols**

23 All procedures were in accordance with the Ethical Committee for Animal Care (Faculty
24 of Medicine, Université Libre de Bruxelles). After one week of acclimatization, 10 weeks
25 old C57BL/6 male mice, n=96 (Elevage Janvier, Le Genest Saint-Isle, France) were

1 randomly assigned to 4 groups of 24 mice each and were injected ip with solvent
2 (polyethylene glycol (PEG group)) alone; PEG+PBN (4-
3 [(dipropylamino)sulfonyl]benzoic acid) (PEG group); AA (AA group) or AA+PBN
4 (AA+PBN group). AA (Acros Organics Co., Geel, Belgium; 40% AAI, 60% AAI_{II}) was
5 dissolved in PEG (Fluka Chemie, Buchs, Switzerland). PBN (Sigma-Aldrich, Bornem
6 Belgium) was solubilized in NaOH 0.5 M at 45°C for 10 min then diluted with PBS and
7 buffered to 7.4 with HCl. AA (5 mg/kg body weight) or equivalent volume of PEG were
8 given once a day and PBN (150 mg/kg body weight) twice a day. AA was given once a
9 day ip in 150 µl of solvent and PBN (150 mg/kg body weight) was injected with 150 µl of
10 PBS twice a day for a total of 8 days maximum. After 2, 4, 5 or 8 days of injection, 6 mice
11 per group were sacrificed. After intraperitoneal anesthesia with ketamine-HCl (Merial,
12 Brussels, Belgium) and 2% xylazine (Bayer, Brussels, Belgium), a blood specimen was
13 obtained by cardiac puncture and kidneys were harvested for analysis. Different
14 samples of kidneys were fixed. One part in alcohol-formalin-acetic for optical
15 microscopy, one in 4% buffered formaldehyde for immunohistochemistry, one in
16 glutaraldehyde sodium cacodylate buffer for electron microscopy analysis and one
17 frozen in liquid nitrogen and stored at -80°C for subsequent DNA adduct analysis.

18

19 **Renal histopathology**

20 TI injury semiquantification was evaluated on hematoxylin/eosin and Masson's
21 trichrome-stained paraffin-embedded sections. Complete kidney sections were analyzed
22 with a light microscope (Carl Zeiss, Oberkochen, Germany) using a 20× magnification
23 lens by two investigators (AAP and TB) blind to the group origin of the mice. The scoring
24 systems were defined as previously described (11, 12): tubular necrosis: 0, normal
25 tubules; 1, rare single necrotic tubule; 2, several clusters of necrotic tubules; 3,

1 confluence of necrotic clusters; tubular atrophy: 0, normal tubules; 1, rare single
2 atrophic tubule; 2, several clusters of atrophic tubules; 3, confluence of atrophic tubular
3 clusters; lymphocytic infiltrate: 0, absent; 1, few scattered cells; 2, group of lymphocytes;
4 3, widespread infiltrate; interstitial fibrosis: 0, absent; 1, minimal fibrosis; 2, moderate
5 fibrosis; 3, severe fibrosis. If differences in grading occurred, the appropriate sections
6 were re-examined until a consensus was obtained.

7

8 **Biochemical evaluation of renal function**

9 Plasma creatinine (Pcr) excretion levels were determined as previously described using
10 an HPLC technique. (11, 12)

11

12 **Immunohistochemistry**

13 The FFPE sections (4 μm) were attached to poly-L-lysine pretreated slides (Sigma-
14 Aldrich, Bornem, Belgium). After air-drying the paraffin from FFPE tissue sections was
15 removed (xylene solution). The sections were rehydrated and immersed in a retrieval
16 solution, sodium citrate buffer (pH 6.0), the microwave oven technique was used (650
17 W, 1 \times 5 min). PBS was used for all washing steps. Endogenous peroxidase activity was
18 quenched with 0.3% hydrogen peroxide in a methanol solution (30 min). Non-specific
19 protein binding sites (background staining due to Fc receptor) were blocked with 20%
20 normal serum (Vectastain Elite_ABC kit IgG, Vector Laboratories, Labconsult, Brussels,
21 Belgium) then with avidin D solution and with biotin solution (Avidin/biotin blocking
22 kit, Vector Laboratories). Subsequently, the sections were incubated overnight with
23 rabbit anti-mouse PCNA (1/4000) monoclonal primary antibody (Abcam, ab2426) or
24 with rat monoclonal antibody anti NEP (1/4000) (Santacruz, sc-80021) diluted in the
25 blocking buffer. Slides were then incubated with specific biotinylated secondary

1 antibody (Vectastain Elite_ABC kit, Vector Laboratories, Labconsult, Brussels, Belgium).
2 The extent of the specifically bound primary antibodies was visualized by means of the
3 avidin-biotin peroxidase complex (ABC) method. The diaminobenzidine/hydrogen
4 peroxide was used as the chromogene substrate producing a brown end product.
5 Counterstaining with haematoxylin completed the processing. The specificity of
6 antibodies used was established by the producer. Normal serum (5% solution) instead
7 of the primary antibody (used in order to exclude non-specific staining of kit reagents)
8 showed no staining.

9

10 **Quantification of PCNA immunostainings**

11 Quantifications were performed by one investigator (TB) blind to the group origin of the
12 mice using ImageJ, a public domain Java image processing program (U.S. NIH) software
13 (available at <http://rsb.info.nih.gov/ij>) as detailed in Figure 10. Thresholding conditions
14 were set identically for all images. Finally the percentage of DAB positive surface
15 corresponding to DAB-positive cells were counted using ImageJ analyse particle
16 command.

17

18 **Ultrastructural analysis**

19 Analysis of cellular ultrastructure using transmission electron microscopy was
20 performed in the same period. Small pieces of renal tissue were fixed in 3%
21 glutaraldehyde in 0.1 mol/L phosphate buffer, pH 7.2, Fixation was performed with the
22 microwave oven technique, After rinsing, samples were post-fixed in 1% osmium
23 tetroxide in phosphate buffer 0.1 M for 1 h at 4°C, processed through a graded acetone
24 series, embedded in Araldite (TAAB Laboratories England UK), and polymerized
25 overnight at 60°C. Sections (50 nm) were then stained with uranyl acetate and lead

1 citrate and examined with a 10-10 JEOL electron microscopy (JEOL, Tokyo, Japan).

2

3 **AA-DNA adduct analysis**

4 DNA was extracted from frozen tissues using a standard phenol-chloroform extraction
5 method. ³²P-postlabelling analysis (41) nuclease P1 enrichment, chromatography on
6 polyethyleneimine-cellulose thin-layer plates (Machery and Nagel, Düren, Germany),
7 autoradiography using a Packard Instant Imager (Canberra Co., Dowers Grove, IL, USA)
8 and quantitation were essentially performed as described. (42) Results were expressed
9 as DNA adducts per 10⁸ normal nucleotides.

10

11 **Statistical analysis**

12 All the scores and data obtained from AA and control groups were compared for each
13 corresponding time point with Kruskal-Wallis test followed by Mann-Whitney U-test
14 and Bonferroni post-hoc test.

15

16 **DISCLOSURE**

17

18 All the authors declared no competing interests.

19

20

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2

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6 5-7, 2011, Bordeaux, France), and at the Annual Meeting of the American Society of
7 Nephrology (November 9-13, 2011, Philadelphia, USA).

8

1 **LEGENDS TO THE FIGURES**

2

3 **Figure 1 | Schematic representation of experimental protocols performed in the**
4 **mouse model of aristolochic acid nephropathy (AAN).**

5 C57BL/6 male mice ($n=96$) were randomized in 4 groups of 24 mice each. AA (5 mg/kg
6 body weight) was injected once a day and PBN (150 mg/kg body weight) twice a day.
7 After 2, 4, 5 and 8 days of injection, six mice/group were sacrificed and blood sample
8 and kidneys were harvested for further analysis.

9

10 **Figure 2 | Evolution of plasma creatinine levels.**

11 Plasma creatinine from AA (white columns), AA+PBN (grey columns) treated mice as
12 compared to PEG+PBN (dashed columns) and PEG (dotted columns) controls from days
13 2 to 8. Results are presented as the mean \pm SEM ; $n = 6$ mice/group. (** $P < 0.01$)

14

15 **Figure 3 | Histological analysis of tubulointerstitial injury in AA-treated mice**
16 **compared to mice receiving AA+PBN.**

17 Representative photomicrographs of renal cortex longitudinal sections at studied time
18 points in each group. No lesions were observed in controls: PEG (**a-d**) and PEG+PBN (**e-**
19 **h**). In AA group (**i-l**), tubular necrosis (arrow) was observed at days 4 and 5 in the outer
20 stripe of outer medulla. In AA+PBN group (**m-p**), only sparse proximal tubules exhibited
21 necrotic cells on day 4 (arrow) but lymphocytic infiltrate, tubular atrophy and
22 interstitial fibrosis were absent on day 8. Original magnification x400, hematoxyllin-
23 eosin stained kidney longitudinal sections.

24

25 **Figure 4 | Histological analysis of tubulointerstitial injury in AA-treated mice**

1 **compared to mice receiving AA+PBN.**

2 Representative photomicrographs of renal cortex longitudinal sections at studied time
3 points in each group. No lesions were observed in controls: PEG (**a-d**) and PEG+PBN (**e-
4 h**). In AA group (**i-l**), swelling of PTEC was observed after 2 days of injection (**j**),
5 followed by tubular necrosis at days 4 and 5 (arrow). Tubular atrophy (star) and
6 progressive interstitial fibrosis (arrowhead) were present after 8 days of injection. In
7 AA+PBN group (**m-p**), tubular atrophy and interstitial fibrosis were absent on day 8.
8 Original magnification x400, Goldner's trichrome stained kidney longitudinal sections.

9

10 **Figure 5 | Semiquantitative tubulointerstitial score.**

11 In control groups (PEG or PEG+PBN), no lesions were observed (data not shown).
12 However, a significant increase in the necrosis score was observed in AA group (white
13 columns) as soon as day 4 and was maximal on day 5 (**a**) as compared to controls.
14 Tubular necrosis phase was followed by a significant lymphocytic infiltrate (**b**), atrophy
15 (**c**) and fibrosis (**d**) respectively on day 8. A significant reduction of necrosis, atrophy
16 and fibrosis was observed in the AA+PBN group (grey columns). Results are presented
17 as the mean \pm SEM, n= 6 mice/group. Significant levels are ** P<0.01.

18

19 **Figure 6 | Representative photomicrographs of NEP staining in different groups
20 and time.**

21 In control groups (PEG (**a-b**)) a NEP (neutral endopeptidase) positive staining was
22 observed in medullary rays and in the outer stripe of outer medulla. An identical
23 distribution was observed in PEG+PBN group (data not shown). However, in the AA
24 group (**c-d**), a severe necrosis followed by a profound atrophy of PTECs was observed
25 mainly in NEP positive area corresponding to S3 segment of proximal tubule. In the

1 AA+PBN group (**e-f**), necrosis was limited. (Original magnification: x200)

2

3 **Figure 7 | Time course of AA-DNA adduct formation in renal tissue. Total AA-DNA**
4 adduct formation was determined by ^{32}P -postlabeling in AA (white columns) and
5 AA+PBN groups (grey columns) in two separate experiments from 6 to 24 hours and
6 from day 2 to day 8. As shown in (**a**), the pattern of AA-DNA adducts consisted of three
7 major adduct spots: 7-(deoxyadenosin- N^6 -yl)- aristolactam I (dA-AAI; spot 1); 7-
8 (deoxyguanosin- N^2 -yl)-aristolactam I (dG-AAI; spot 2); and 7-(deoxyadenosin- N^6 -yl)-
9 aristolactam II (dA-AAII; spot 3). Results (**b**) are presented as the mean \pm SEM n= 4
10 mice/group (6-24 hours) or n = 6 (2-8 days)/group. Significant levels are * $p < 0.05$ and
11 ** $P < 0.01$.

12

13 **Figure 8 | Representative photomicrographs of PCNA staining in different groups**
14 **and time points with quantification.**

15 In control groups (PEG (**a-d**) and PEG+PBN (**e-h**)) only scattered PCNA positive PTEC
16 were observed as compared to AA group (**i-l**) where numerous PCNA positive PTEC
17 were present. Coadministration of PBN and AA (**m-p**) resulted in a substantial reduction
18 of PCNA-positive PTEC on day 8. (Original magnification: x400) Quantification of
19 positive PCNA cells (**q**) in AA (white columns), AA+PBN (grey columns), PEG+PBN
20 (dashed columns) and PEG groups (dotted columns). Results are presented as the mean
21 \pm SEM; n= 6 mice/group. Significant levels are * $P < 0.05$, ** $P < 0.01$.

22

23 **Figure 9 | Representative electron photomicrographs from different groups on**
24 **day 8.**

25 Kidneys from PEG (Magnification x3000) (**a**) and PEG+PBN (Magnification x5000) (**b**)

1 groups : proximal tubules (E) are lined by tall columnar cells with acidophilic cytoplasm
2 rich in structures necessary for active fluid transport : densely packed microvilli forming
3 brush border, basal ondulations, endocytic vacuoles and mitochondria, often elongated
4 and tortuous. In the AA group (Magnification x3000) (c), PTEC exhibited severe injury
5 with disruption of brush border and cell detachment (\$). In kidneys from the AA+PBN
6 group (Magnification x3000) (d) PTEC displayed extensive cytoplasmic vacuolization
7 without necrotic changes (**). Kidneys from the AA group, (x 30,000) (e) normal (E) and
8 injured tubules were frequently admixed with injured nephron showing extensive
9 mitochondria disruption (arrow head) and altered brush borders. AA+PBN group
10 (Magnification x30,000) (f) displayed mitochondria vacualoziation (arrow head).

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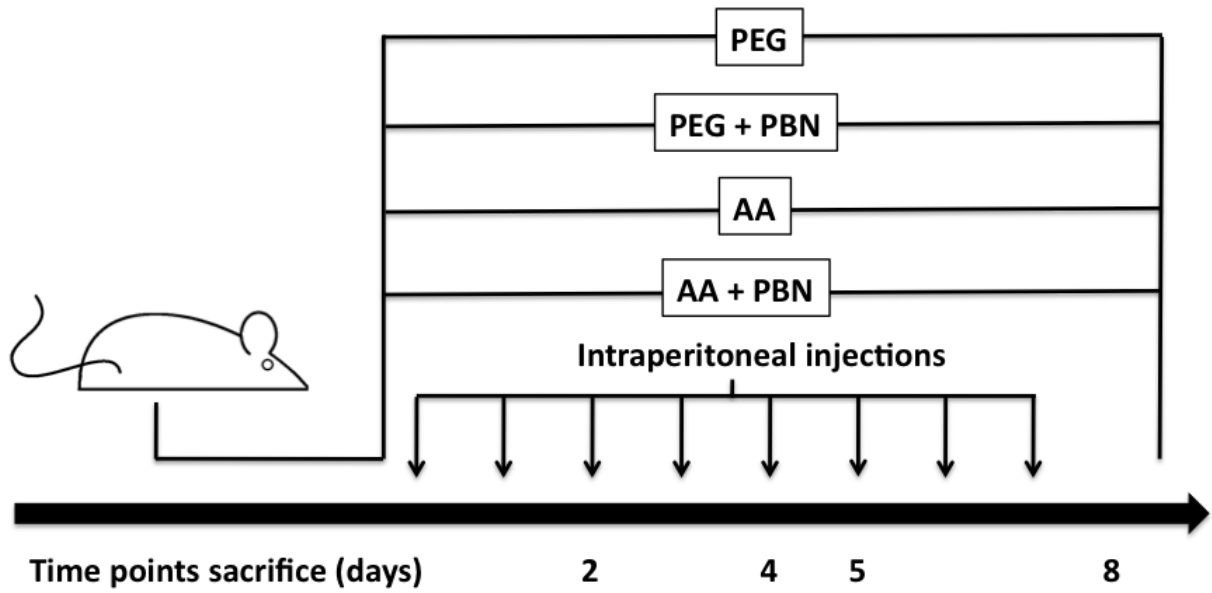
12 **Figure 10 | Quantitative analysis of PCNA staining.**

13 Twenty non-overlapping high power fields were photographed per section at a 400x
14 magnification. Identical imaging conditions, including illumination intensity and camera
15 exposure time, were applied to all photographs. A blankfield image was used to correct
16 uneven illumination and color balance with the calculator Plus plugin. Then, brown-
17 colored images specific for DAB stain and blue-colored images specific for hematoxylin
18 stain were extracted by color deconvolution plugin. Nuclei generated from DAB images
19 were isolated using specific threshold ImageJ internal commands followed by
20 conversion to a binary image.

21

1

FIGURES



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Figure 1

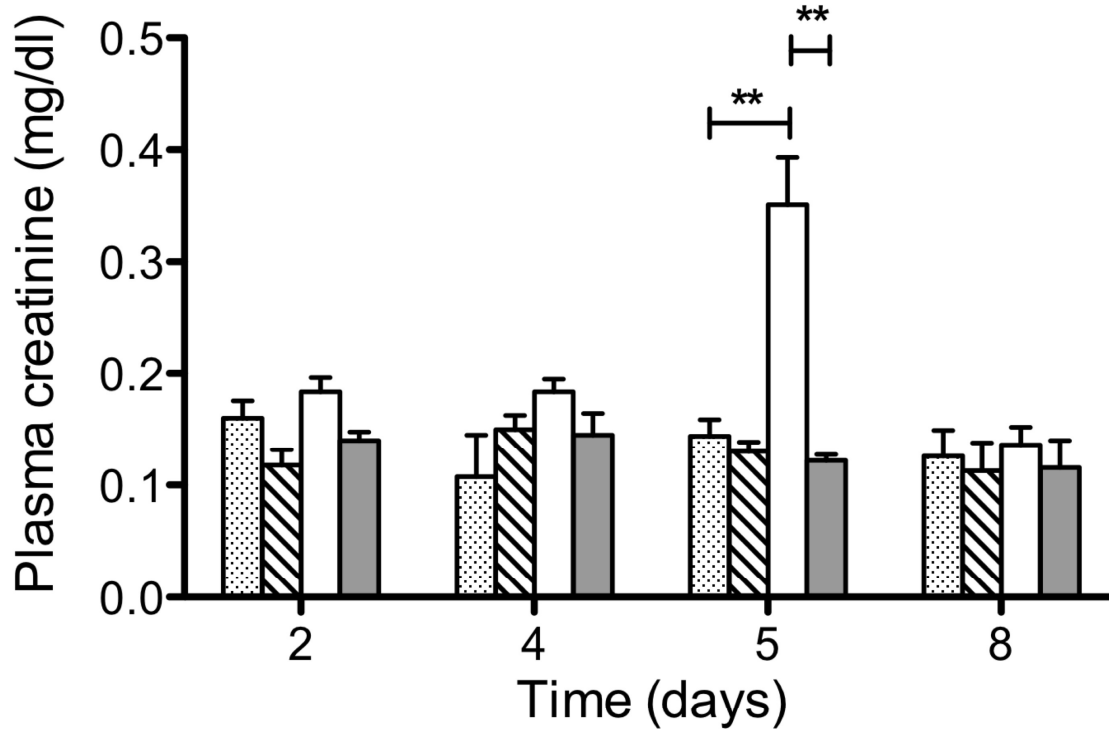


Figure 2

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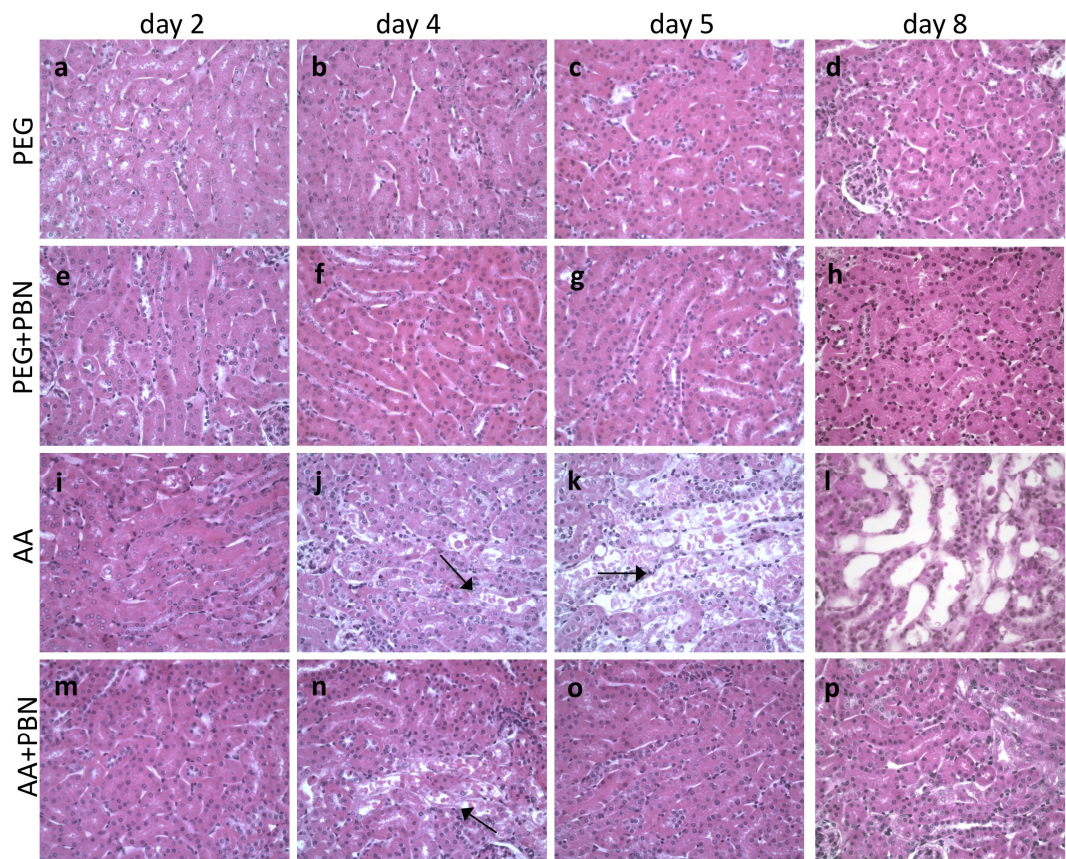


Figure 3

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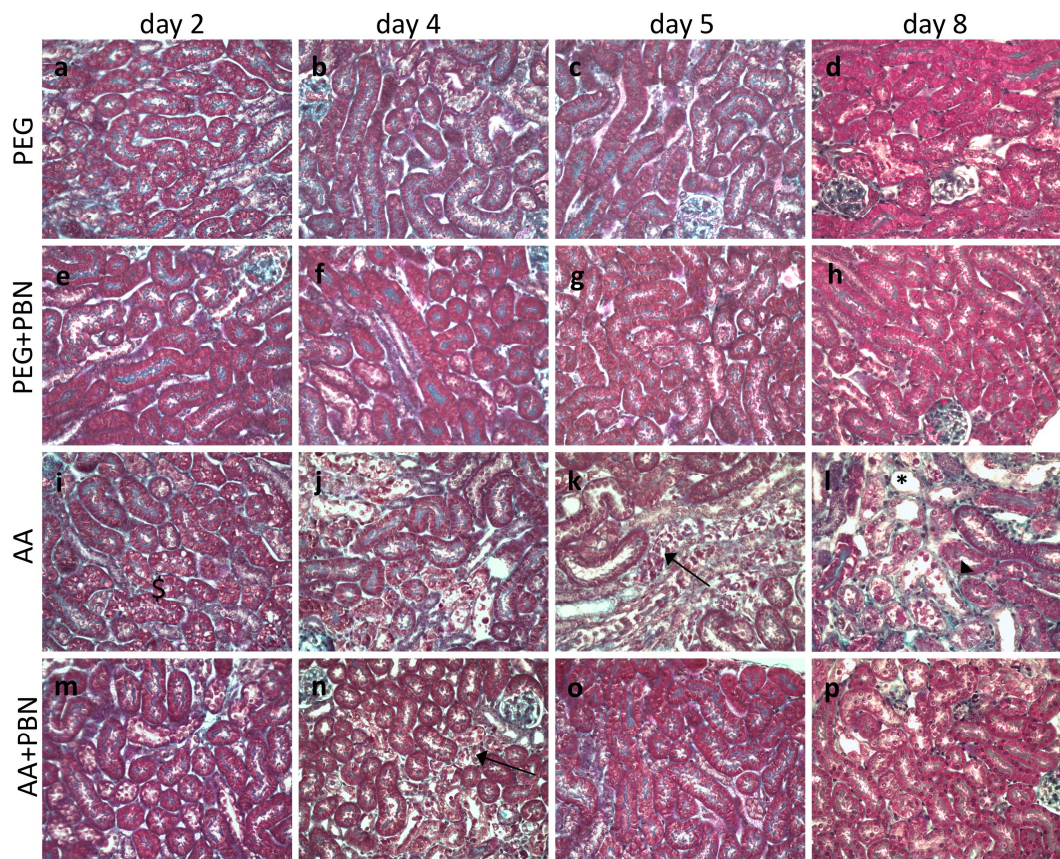


Figure 4

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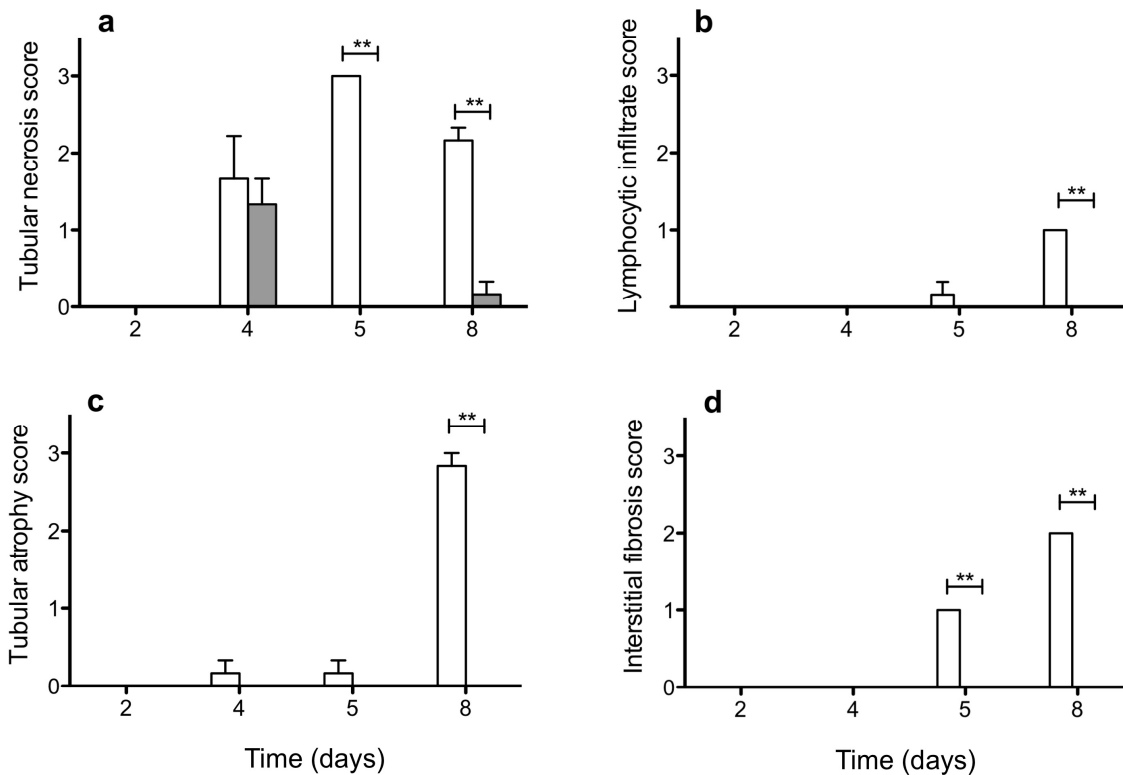
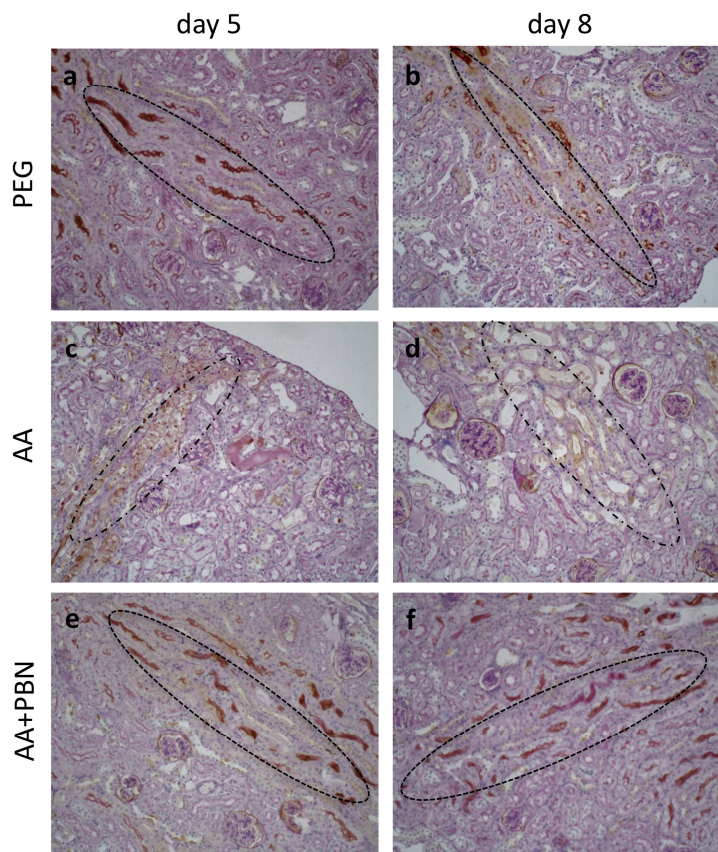


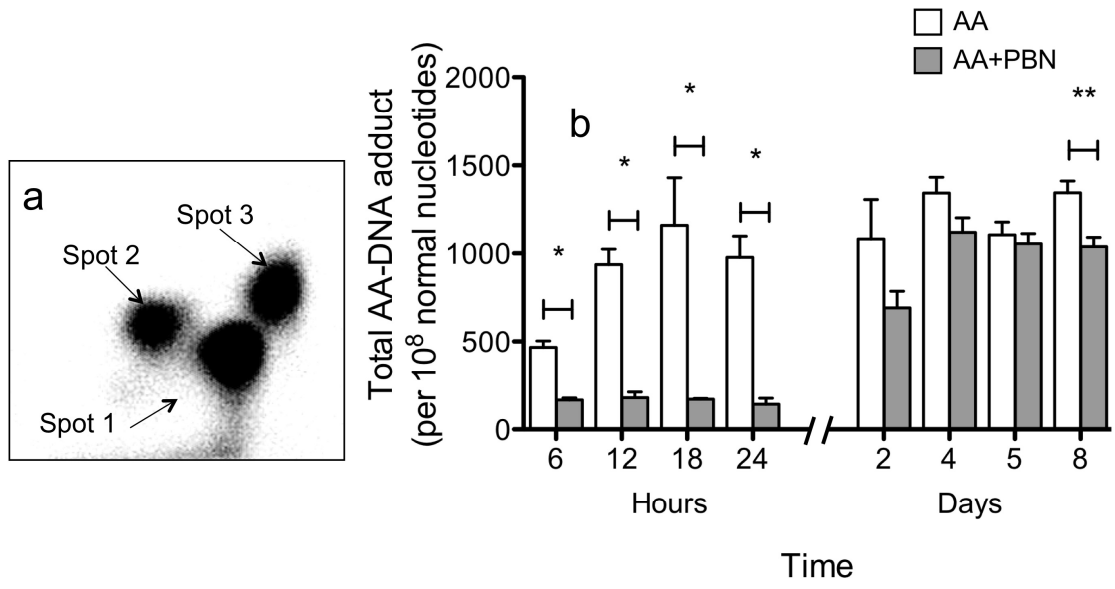
Figure 5

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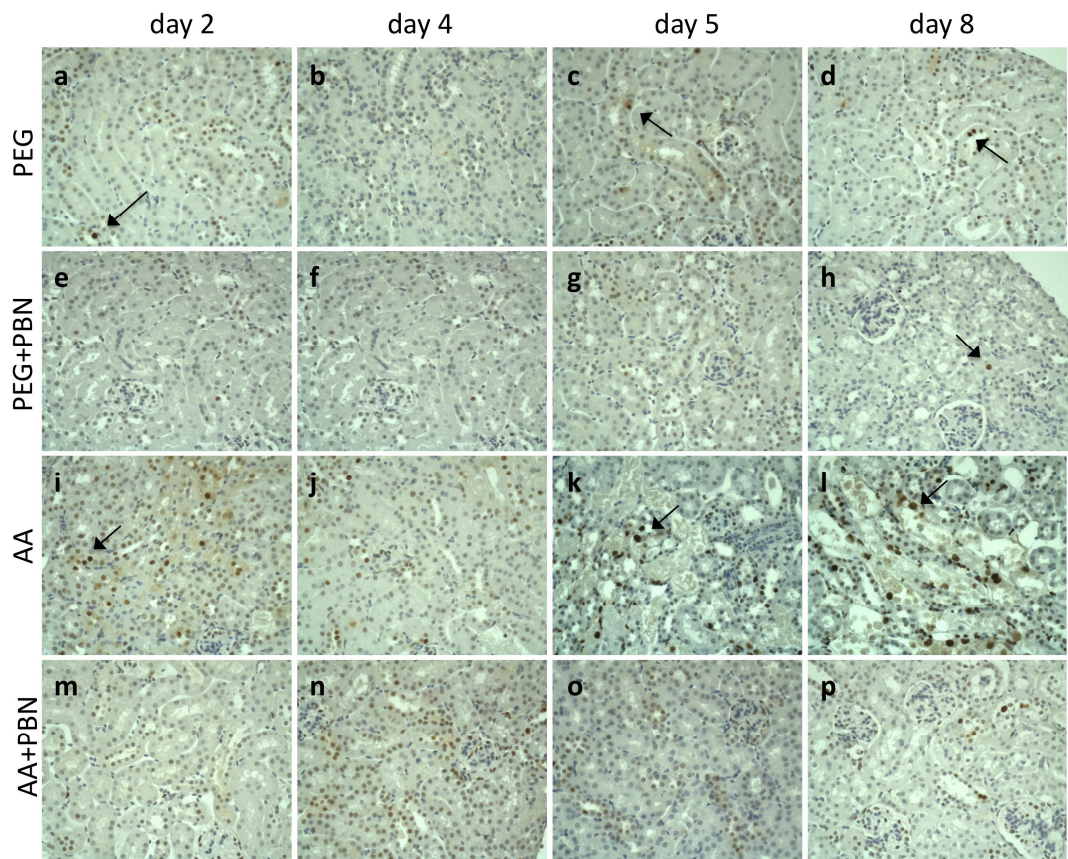
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Figure 6



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Figure 7



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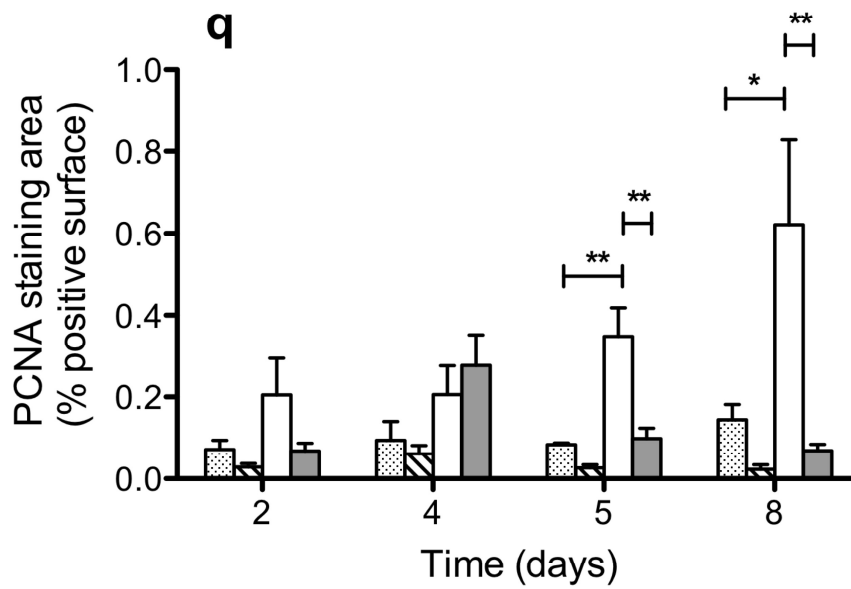


Figure 8

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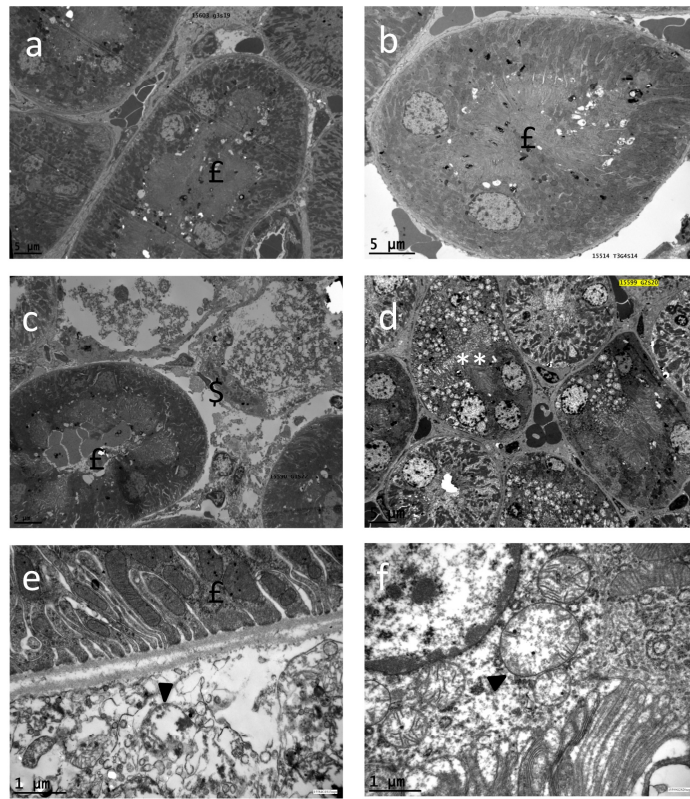
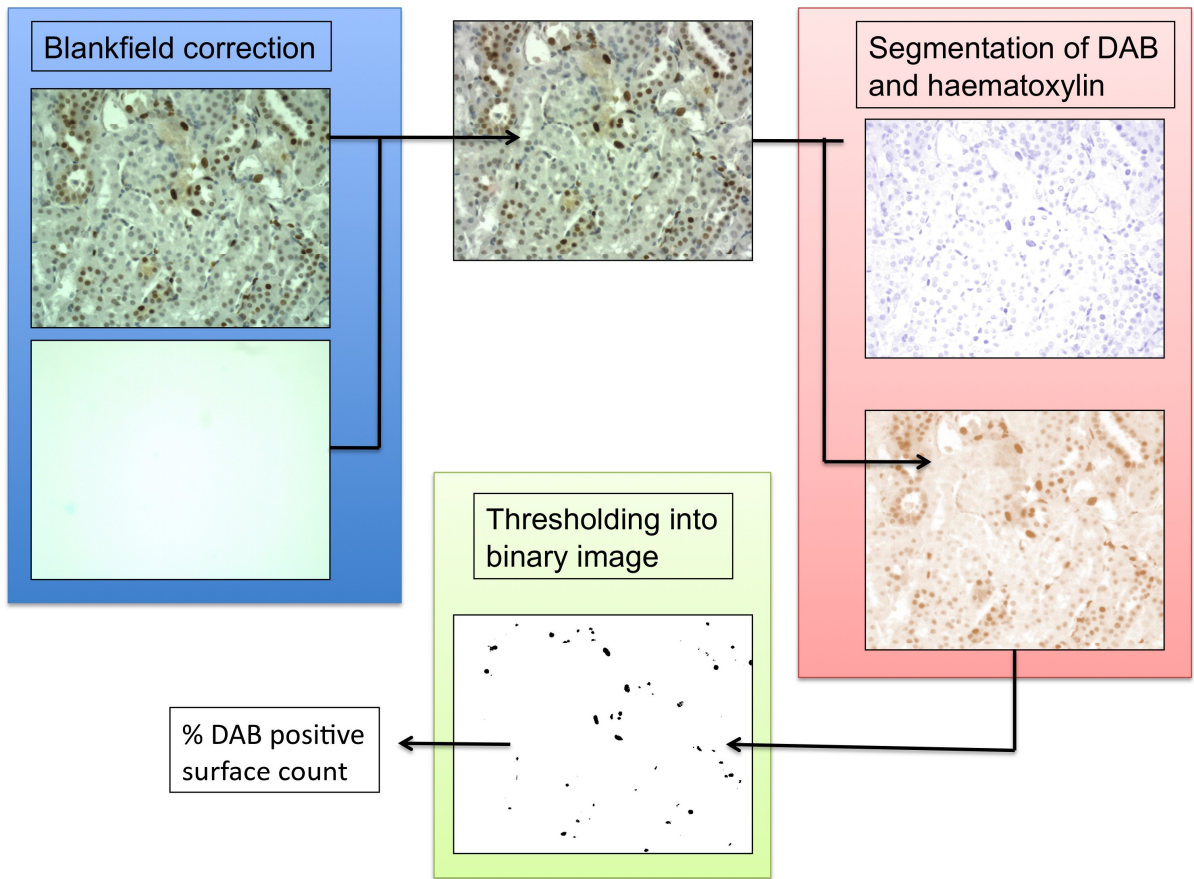


Figure 9

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Figure 10