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The *in vivo* effects of adenine-induced chronic kidney disease on some renal and hepatic function and CYP450 metabolizing enzymes

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Short title: Drug metabolism in adenine-induced chronic kidney disease

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Summary

Adenine-induced model of chronic kidney disease (CKD) is a widely used model especially in studies testing novel nephroprotective agents. We investigated the effects of adenine-induced CKD in rats on the activities of some xenobiotic metabolizing enzymes in liver and kidneys, and on some *in vivo* indicators of drug metabolism (viz pentobarbitone sleeping time, and plasma concentration of theophylline 90 min post administration). CKD was induced by orally feeding adenine (0.25% w/w) for 35 days. Adenine induced all the characteristics of CKD, which was confirmed by biochemical and histological findings. Glutathione concentration and activities of some enzymes involved in its metabolism were reduced in kidneys and livers of rats with CKD. Renal CYP450 1A1 activity was significantly inhibited by adenine, but other measured isoenzymes (1A2, 3A4 and 2E1) were not significantly affected. Adenine significantly prolonged pentobarbitone-sleeping time and increased plasma theophylline concentration 90 min post administration. Adenine also induced a moderate degree of hepatic damages as indicated histologically and by significant elevations in some plasma enzymes. The results suggest that adenine-induced CKD is associated with significant *in vivo* inhibitory activities on some drug-metabolizing enzymes, with most of the effect on the kidneys rather than the liver.

Key words: adenine, cytochrome P450, chronic kidney disease, drug metabolism.

Introduction

Metabolic clearance of many xenobiotics (especially via cytochrome P450) has been reported to be decreased in humans with chronic renal failure (CRF) and chronic kidney disease (CKD) (Michaud et al., 2008; Verbeeck & Musuamba, 2009; Philips et al., 2014; Velenosi & Urquhart, 2014) and experimental animals with CRF induced by subtotal nephrectomy (Naud et al., 2011; Naud et al., 2012; Dani et al., 2010; Guévin et al., 2002; Velenosi et al., 2012; Feere et al., 2015). Pharmacokinetic studies reported in patients or experimental animals with either acute renal failure (ARF) or CRF suggested that the nonrenal clearance of several drugs is decreased (McIntyre et al., 2002; Srinivas, 2010; Sun et al., 2006). Although the mechanism by which this occurs is not well known, several studies have shown that CRF affects the metabolism of drugs by inhibiting key enzymatic systems in the liver, intestine and kidneys (Guévin et al., 2002, Velenosi et al., 2012). It has been suggested that CRF is associated with downregulation of selected hepatic isoforms of cytochrome P450 (CYP450). The enzyme activities of CYP1A2, 2C6, 2C11, 2D1/2D2, 3A2 and 4A1/4A3 were significantly reduced, while CYP2E1 level was not significantly affected (Michaud et al., 2005; Yeung et al., 2014).

Renal dysfunction may affect the metabolism and/or pharmacokinetics of several compounds, and thus could have potentially therapeutic and safety consequences (Velenosi & Urquhart, 2014; Saito et al., 2014; Krens et al., 2014; Nolin et al., 2009).

The adenine-induced model of CKD was first reported in rats some thirty years ago (Yokozawa et al., 1986), but has recently gained renewed interest, especially in studies testing novel nephroprotective agents such as the prebiotic gum acacia (Ali et al., 2010, 2013a, 2013b, 2014). Therefore, the present work aim is to investigate the effect of

adenine-induced CKD on activities of some isoforms of CYP450 in the liver and kidneys rats.

Material and methods

Animals

Male Wistar rats (9-10 weeks old, weighing 249 ± 10 g) were housed at Sultan Qaboos University Small Animals Facility in a room kept at a controlled temperature of $22 \pm 2^\circ\text{C}$, relative humidity of about 60%, with a 12 h light/dark cycle (lights on 6:00), and free access to standard pellet chow diet containing 0.85% phosphorus, 1.12% calcium, 0.35% magnesium, 25.3% crude protein and 2.5 IU/g vitamin D3 (Oman Flour Mills, Muscat, Oman) and water. All procedures involving animals and their care were conducted according to international laws and policies (EEC Council directives 86/609, OJL 358, 1 December, 12, 1987; NIH Guide for the Care and Use of Laboratory Animals, NIH Publications No. 85-23, 1985). Ethical clearance was obtained from the Animal Research Ethics Committee of Sultan Qaboos University.

Experimental Design

Three replicated experiments (with 24 rats in each) were conducted. After an acclimatization period of one week, the rats ($n=24$ in each experiment) were randomly divided into two equal groups and treated for five consecutive weeks. The first group continued to receive the same diet without treatment until the end of the study (control group). The second group was switched to a powder diet containing adenine (0.25% w/w in feed). During the treatment period, the rats were weighted weekly. For the collection

of urine, they were placed individually in metabolic cages for 24 h, after the 35 days of treatment.

a) In the first experiment, and on the morning after the metabolic sampling, the rats were anesthetized with an intraperitoneal injection (i.p.) of ketamine (75 mg/kg) and xylazine (5 mg/kg), and blood (about 3.5 mL) was collected from the anterior vena cava and placed into heparinized tubes. The blood and urine were centrifuged at 900 g at 4°C for 15 min. The plasma obtained, together with the urine specimens, was stored at -80°C to await analysis within 4 weeks after the end of the treatment. The left kidney and the same lobe of the liver were excised and immediately snap frozen in liquid nitrogen, and wrapped in aluminum foil and placed in a deep freezer at -80°C pending measurement of some drug metabolizing enzymes. Small pieces of liver and kidney were placed in formal-saline and processed for histopathology as described before (Ali et al., 2013b).

b) In the second experiment, 24 h after the last day of treatment, theophylline (10 mg/kg) was injected i.p. into each of the 24 rats, and 90 min thereafter, heparinized blood samples were collected from each rat and processed as above to collect plasma. The plasma collected was immediately frozen (-80°C) pending measurement of theophylline concentration using an automated analyzer Roche/Hitachi Cobas[®] systems (USA).

c) In the third experiment, 24 h after the last day of treatment, rats were injected i.p. pentobarbitone Na, and the onset of sleep and the sleeping time of animals were recorded as described before (Sharif & Ali, 1994).

Biochemical measurements

Renal function tests in plasma (creatinine, urea, creatinine clearance) were measured as described before (Ali et al., 2013b) and the urinary toxin indoxyl sulfate (IS) was measured in plasma by an HPLC method (Al Za'abi et al., 2013). Pieces of liver and kidney from control and treated rats were rinsed with ice-cold PBS (pH 7.4) before homogenization in 10 mM phosphate buffer, pH 7.4, containing 0.15 M KCl, 0.1 mM EDTA, 1 mM DTT and 0.1 mM phenylmethylsulfonylfluoride at 4°C. Postmitochondrial (PMS) fractions were prepared by centrifugation as described before (Bhagwat et al., 1999; Raza et al., 2004). Reactive oxygen species in the collected hepatic and renal tissues were measured using either DCFDA as a fluorescent probe or lucigenin based chemiluminescence methods, as described before (Raza et al., 2004; Raza & John, 2004). Glutathione (GSH) concentration and enzymes of GSH metabolism in the PMS fractions were measured by standard procedures, as described previously (Habig et al., 1974) (Raza et al., 2013). CYP 2E1, CYP3A4, CYP1A1 and CYP1A2 activities in the tissues from control and treated rat tissues were measured using standard substrates as fully described before (Matsubara et al., 1976).

The plasma L- γ -glutamyltransferase (GGT) and lactate dehydrogenase (LDH) were measured using kits from Human GmbH (Mannheim, Germany) and Sigma Chemical, St. Louis, MO, USA, respectively. The plasma concentrations of aspartate transaminase (AST), alanine transaminase (ALT), and creatine kinase (CK) were measured using standard laboratory methods by an LX20 multiple automated analyzer (Beckman Coulter, CA, USA).

Drugs and Chemicals

Adenine and substrates for enzymatic activities were obtained from Sigma (St. Louis, MO, USA). All other chemicals used were of analytical reagent grade.

Statistical analysis

Data are expressed as means \pm SEM. Comparisons between groups were performed by one-way analysis of variance (ANOVA), followed by Newman-Keuls multiple range tests. *P* values < 0.05 were considered significant.

Results

The results of the first experiment (Table 1) showed that, compared with their initial body weight, the control rats have increased their weight by 29% while the rats fed adenine alone lost about 5% of their body weight. The urine volume voided by the adenine-treated rats was about five times that of the controls. The renal function biomarkers (creatinine, urea, creatinine clearance, IS and cystatin C) all confirmed that adenine had damaged the renal tissues. The renal histology results (not shown) concurred with earlier data (Ali et al., 2010, 2014; Al Za'abi et al., 2015).

Adenine treatment caused significant and marked increases (about 2-4 -fold) in the plasma activities of ALT, AST, CK, GGT and LDH (Table 2).

Figures 1 and 2 depicts the renal and hepatic concentration of GSH and activities of some enzymes involved in GSH-dependent metabolism. Renal and hepatic GSH concentrations were significantly reduced by adenine treatment ($P < 0.01$ for renal data and $P < 0.0001$ for hepatic data). The activity of renal GSSH reductase was significantly

inhibited by adenine and the activities of the enzymes GST and GSSG were both insignificantly inhibited in renal (but not hepatic) tissues in adenine-treated rats.

Figure 3 and 4 shows the renal and hepatic activities of some isoenzymes of CYP450. Renal CYP450 1A1 was significantly inhibited ($P < 0.001$) in renal (but not hepatic) tissues of rats with adenine CKD, while the rest of the isoforms of the enzymes in the renal and hepatic tissues were insignificantly affected by adenine.

Liver tissue from the control shows intact parenchymal tissue with no lobular inflammation (Figure 5A), and portal areas show either no or minimal inflammatory infiltrates (Figure 5B). The liver tissue treated with adenine shows infiltration of the liver tissue with inflammatory cells (lobular inflammation, Figure 5C). The portal areas show mild to moderate round cell inflammatory infiltrate (Figure 5D).

Table 3 shows the effect of adenine-induced CKD on the pentobarbitone-sleeping time and theophylline concentration 90 min post administration. Adenine treatment produced significant increases in both when compared with the values obtained from the control rats ($P < 0.05$).

Discussion

It is established that hepatic CYP450 enzymes contribute directly to the metabolism of a wide array of xenobiotics via oxidation, reduction and hydrolysis. These enzymes are also present in the renal tissues in significant amounts (Feere et al., 2015). Therefore, it was of interest to find out whether adenine-induced CKD would affect the CYP450 enzymes in both the liver and the kidneys. It has long been known that extensive

renal damage resulting in end-stage renal failure would also result in hepatic dysfunction, an action probably mediated by the generation of uremic toxins (Pichette & Leblond, 2004). Also, some hepatic CYP450 isoenzymes are downregulated in rats with moderate CKD (Velenosi et al., 2012).

In a recent review article on drug disposition in several models of acute renal failure (ARF), it was suggested that this condition can lead to decreased renal elimination of the drug and associated metabolites (Srinivas, 2010). However, its influence on the overall body clearance depends on the propensity of the contribution of renal versus nonrenal mechanisms of elimination. More recently, it was reported that the influence of CKD on nonrenal drug clearance is increasingly apparent, and is now deemed an important factor by the Food and Drug Administration in the recommendation of drug dosage in CKD patients (Velenosi & Urquhart, 2014). CRF and even moderate CKD in rats have been suggested to downregulate some hepatic CYP450 isoenzymes, possibly via mediation of some uremic toxins (Saito et al., 2014).

In the present work, we have shown that, on the whole, the GSH and the enzymes involved in its metabolism, as well as CYP1A1 were much more affected by the inhibitory action of adenine in the kidneys than in the liver. This may suggest that the kidneys are the predilection site of adenine, in which this agent is metabolized into a toxic compound that accumulates in the renal tissues. Velenosi *et al.* have reported that the activity and expression of hepatic CYP3A and CYP2C are both decreased in both moderate and severe surgically-induced CKD (Velenosi et al., 2012). Their data suggest that hepatic drug metabolism is significantly decreased in the earlier stages of CKD. In our results we did not show any significant change in the activity of CYP3A in the liver

or kidneys of rats with a rather moderate CKD. Hepatic CYP3A is suggested to mediate nonrenal clearance of many drugs, and is involved in the oxidative metabolism of ~ 30% (Thomson et al., 2015) or up to 50% of all drugs on the market (Velenosi & Urquhart, 2014). The reason for the discrepancy is not certain, especially when we have found that, functionally, adenine-induced CKD actually significantly lessened the metabolism of pentobarbitone. The plasma concentration of theophylline, which is a probe drug metabolized in the liver and kidneys by CYP1A, 90 min post administration was found to be significantly increased. This may suggest a lack of correlation between these functional parameters and the activities of drug metabolizing enzymes.

Interestingly, Feere *et al.* have shown that the liver histology was normal in an adenine-induced model of CKD, yet their data on the activity of two plasma enzymes, ALT and AST (measured as hepatic functions tests), indicated that the activities of these enzymes had been significantly inhibited four- and two-fold, respectively, while the hepatic total CYP450 content was halved (Feere et al., 2015). Contrary to these data we have found, as well as others, that the activities of these plasma enzymes are elevated and not inhibited (Al Za'abi et al., 2015; Kim et al., 2013; Manivannan et al., 2013). This probably indicates some tissue damage in the liver in adenine-treated rats, which is at variance with results obtained by Feere *et al.* (Feere et al., 2015). In addition, our hepatic histology showed inflammatory infiltration of the portal and hepatic area (Figure 3) suggesting some degree of tissue damage. However, we are not certain whether the degree and the extent of this damage explain the discrepancy in our results to Feere *et al.*'s findings (Feere et al., 2015). Furthermore, it is not possible at this stage to conclude that the observed effects on liver are either due adenine-induced CKD or direct toxic

effect of adenine on the hepatic cells. Further histological and other specific hepatic function tests are required to ascertain the status of the liver in adenine-induced CKD. It is also worth mentioning that the liver-specific enzymes are known to be elevated more in acute rather than chronic kidney damage (Srinivas, 2010).

Conclusion

Our data have suggested that adenine-induced CKD inhibited the activities of some drug-metabolizing enzymes, with most of the effect on the kidneys, rather than the liver. However, the condition is associated with significant *in vivo* effects related to hepatic and renal metabolism.

Acknowledgments

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Disclosure Statement

There are no conflicts of interest to disclose.

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Table 1. Effect of adenine-induced chronic renal failure on body weight change, urine volume and renal function tests in plasma of rats.

Parameters/ Groups	Control	Adenine
Initial body weight (g)	245.5±3.4	242.6±6.3
Final body weight (g)	304.5±5.6	233.0±6.9
% difference between initial & final body weight	28.5 ± 2.3	-5.1 ± 1.9***
Urine voided (ml/24h)	9.6 ± 1.2	47.2 ± 3.0***
Plasma creatinine (mmol/L)	32.2 ± 1.7	107.9 ± 17.3***
Plasma urea (mmol/L)	8.0 ± 0.5	34.9 ± 2.6***
Creatinine clearance (ml/min)	0.03 ± 0.003	0.002 ± 0.0005***
Plasma Indoxyl sulfate (µmol)	2.15 ± 0.8	72.2 ± 3.3***
Plasma Cystatin C (mg/ ml)	0.82 ± 0.06	1.74 ± 0.24***

Values in the table are means ± SEM (n = 6).

Rats were given adenine in the feed at a concentration of 0.25% for 35 days.

**P* less than 0.05 (compared with controls in each parameter)

***P* less than 0.001 (compared with controls in each parameter)

****P* less than 0.0001 (compared with controls in each parameter)

Table 2. Effect of adenine-induced chronic kidney disease on the plasma activities of some hepatic enzymes.

Enzymes	Control	Adenine
ALT (IU/L)	28.0 ± 3.9	113.2 ± 24.9 ^{***}
AST (IU/L)	40.2 ± 3.9	126.3 ± 21.6 ^{***}
CK (U/L)	265.0 ± 24.3	453.3 ± 13.5 ^{**}
GGT (U/L)	1.5 ± 0.3	5.3 ± 0.6 ^{***}
LDH (IU/L)	189.0 ± 14.2	358.7 ± 16.9 ^{***}

Data in the table are means ±SEM (n=6).

Rats were given either adenine (0.25% w/v in feed, 5 weeks).

ALT: alanine transaminase, AST: aspartate transaminase, CK: creatine kinase, GGT: L-γ-glutamyltransferase, LDH: lactate dehydrogenase

* $P < 0.05$, ** $P < 0.001$, *** $P < 0.0001$. (Compared with control)

Table 3. Pentobarbitone sleeping time and theophylline plasma concentration in rats treated with adenine.

Group	Treatment	Pentobarbitone sleeping time (min)	Theophylline concentration ($\mu\text{mol/L}$)
1	Control	30.5 ± 0.5	62.5 ± 2.8
2	Adenine	$70.5 \pm 4.1^*$	$74.4 \pm 1.1^*$

Data in the table are means \pm SEM (n = 6).

Rats were given adenine (0.25% w/v in feed, 5 weeks).

In one experiment, rats were anesthetized by pentobarbitone (40 mg/kg, intraperitoneally) and the sleeping time was measured. In another experiment, the animals were injected intraperitoneally with theophylline (10 mg/kg) 24 h before sacrifice, and blood was collected 90 min later.

**P* less than 0.05 (compared with control)

Legends to figures

Figure 1. The effect of adenine treatment on the renal activities of reduced glutathione (GSH) and enzymes involved in its metabolism in control rats (C, open white column), and rats treated with adenine (0.25% w/w, 5 weeks) (A, closed black column).

Figure 2. The effect of adenine treatment on the hepatic activities of reduced glutathione (GSH) and enzymes involved in its metabolism in control rats (C, open white column), and rats treated with adenine (0.25% w/w, 5 weeks) (A, closed black column).

Figure 3. The effect of adenine treatment on the renal activities of isoenzymes of cytochrome P-450 in control rats (C, open white column), and rats treated with adenine (0.25% w/w, 5 weeks) (A, closed black column).

Figure 4. The effect of adenine treatment on the hepatic activities of isoenzymes of cytochrome P-450 in control rats (C, open white column), and rats treated with adenine (0.25% w/w, 5 weeks) (A, closed black column).

Figure 5. A representative photomicrograph showing liver tissue from control with intact parenchyma (A) and portal area without inflammatory infiltrate (B). Liver tissue treated with adenine shows inflammatory infiltration in the parenchyma (C) and portal areas (D).

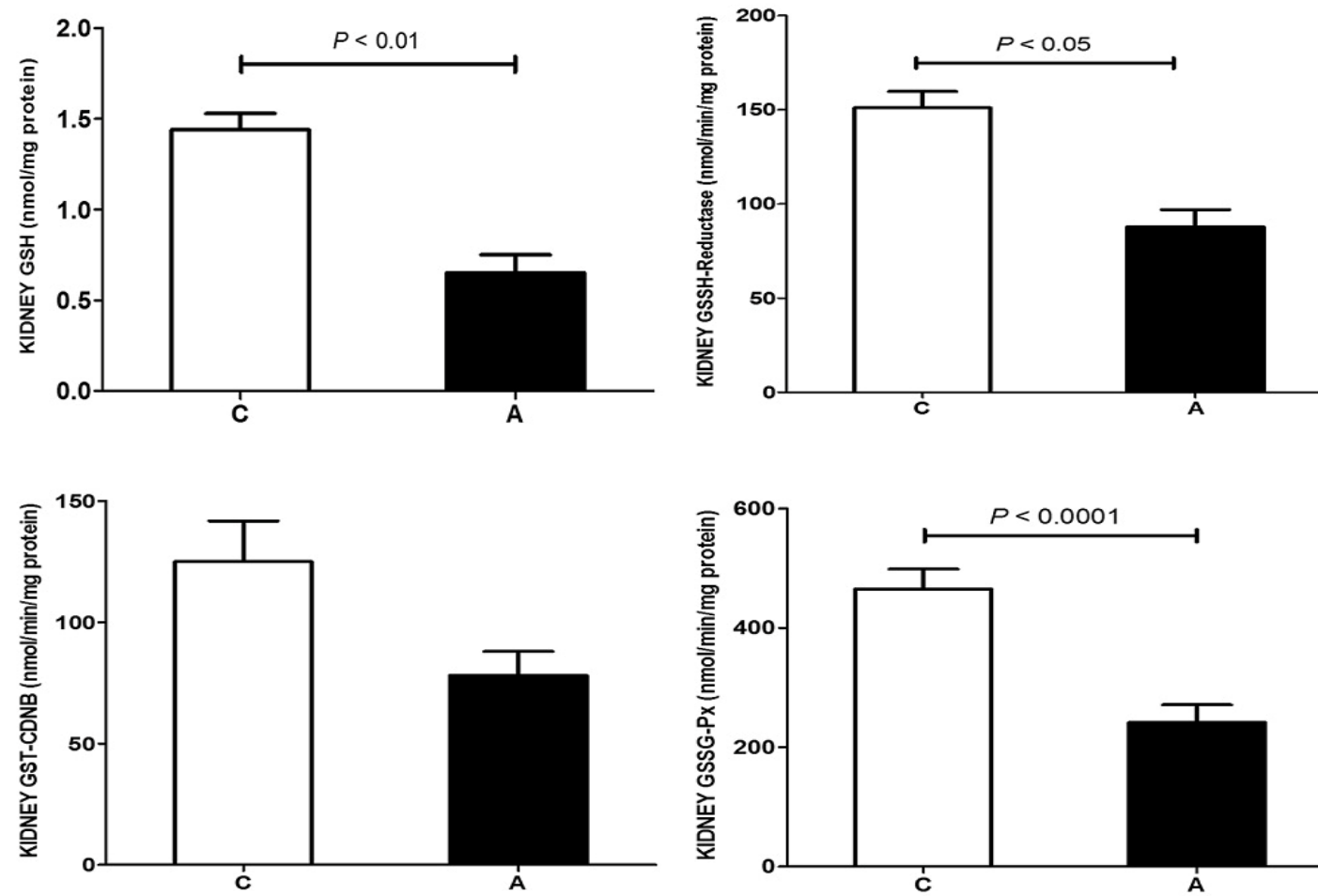


Fig 1

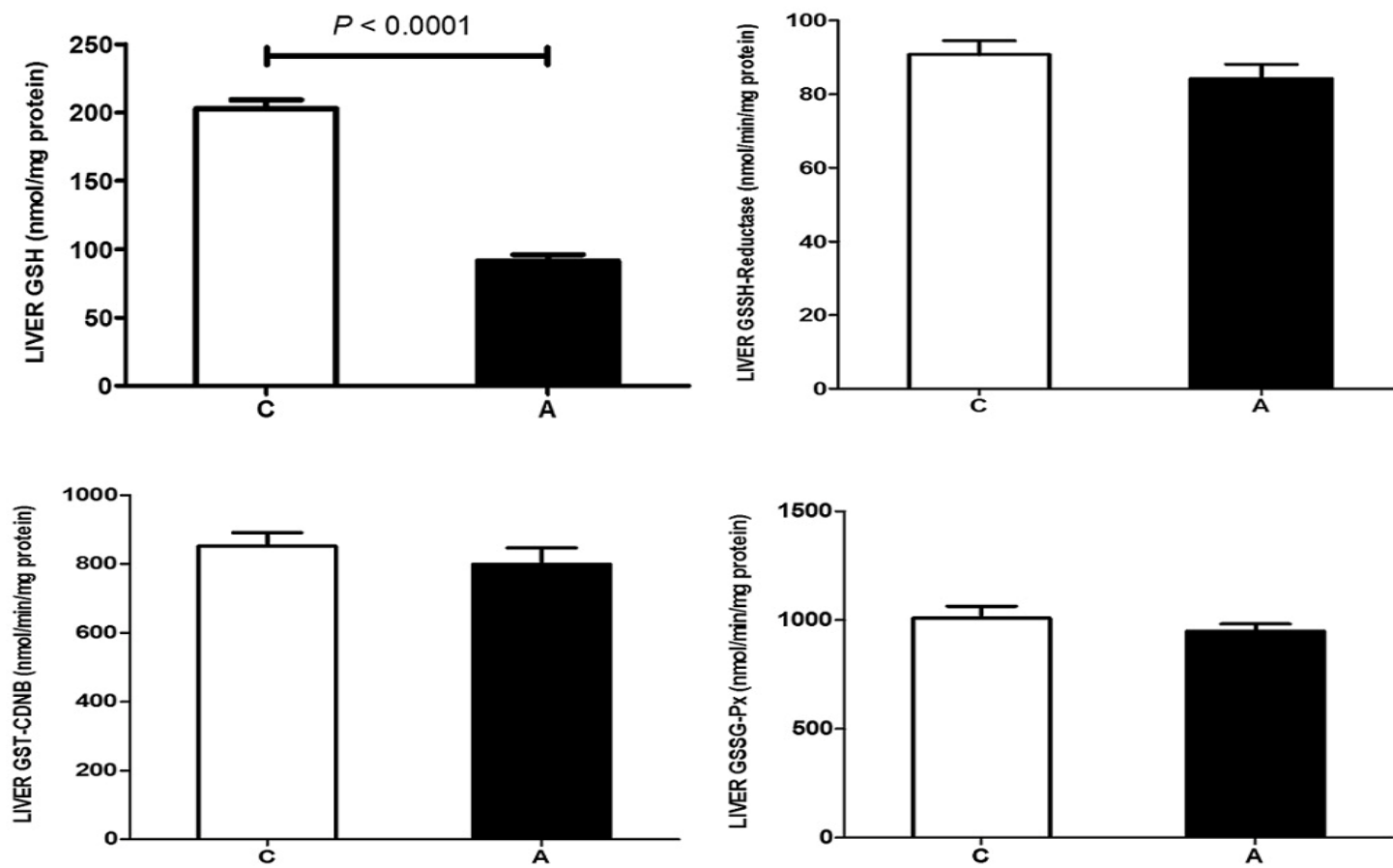


Fig 2

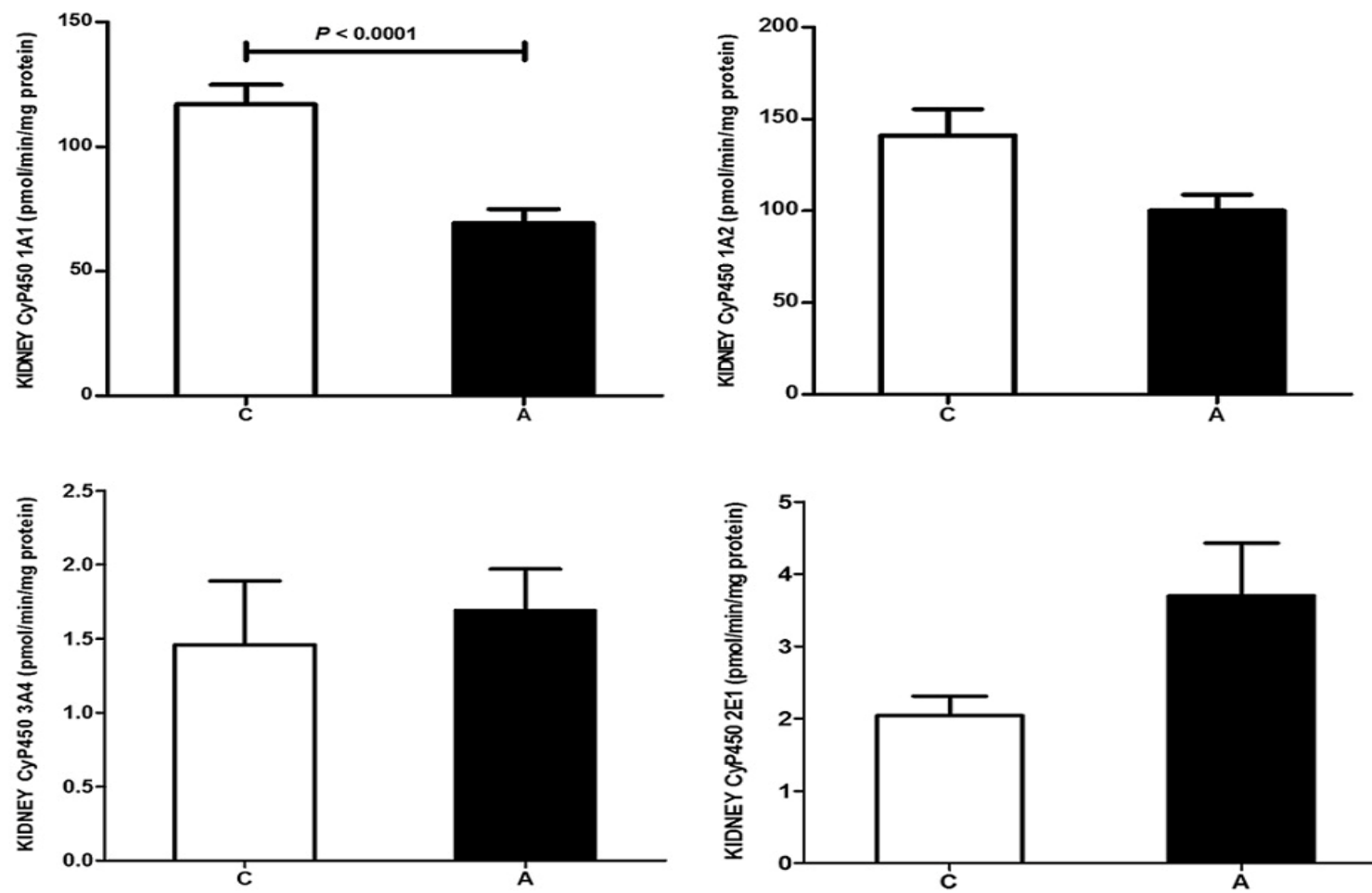


Fig 3

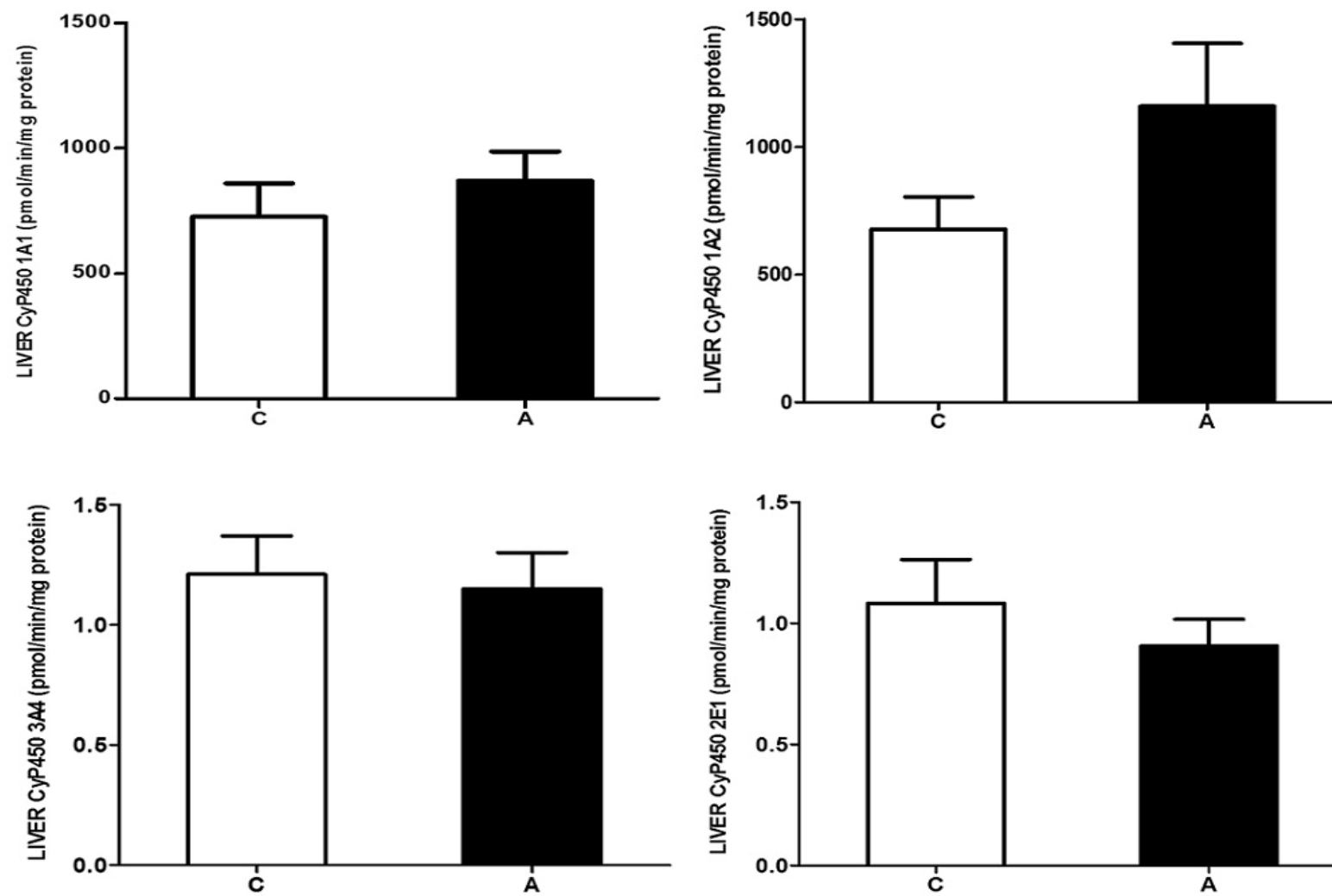


Fig 4

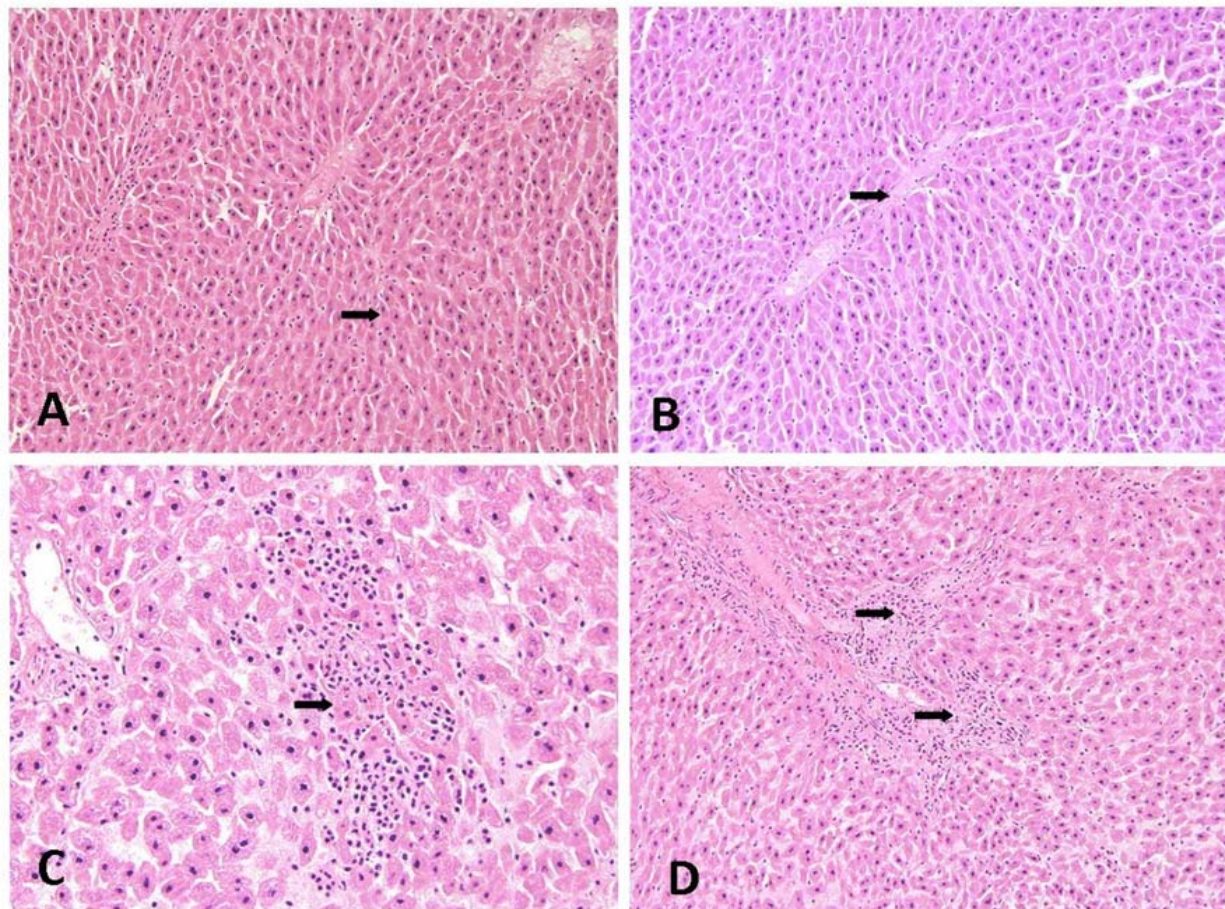


Fig 5