EVALUATION OF THE NEPHROTOXICITY OF AROMATIC NITRO-AMINO COMPOUNDS BY URINARY ENZYME ACTIVITIES

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Accepted August 7, 1989

Abstract.....Nephrotoxicity of some aromatic nitro-amino compounds were evaluated by urinary enzyme activities and renal histopathological changes. Male Fischer 344 rats were intraperitoneally injected with aniline, *p*-aminophenol, acetyl-*p*-aminophenol, *p*-chloroaniline, *p*-chloronitrobenzene, *p*-anisidine, or *p*-nitroaniline at 1.0 mmol/kg. In the rats injected with *p*-aminophenol, necrosis of renal tubular epithelial cells and remarkable elevation of urinary N-acetyl- β -D-glucosaminidase (NAG) and γ -glutamyltranspeptidase (γ -GTP) activities were observed. Injection with *p*-chloroaniline caused significant elevation of the urinary NAG and γ -GTP activities. *p*-Anisidine and *p*-nitroaniline induced swelling of the tubular epithelial cells and a significant elevation in urinary NAG activities in rats, which was also caused by *p*-chloronitrobenzene. However, administration of aniline or acetyl-*p*-aminophenol did not change either the urinary enzymes or renal histopathology. These results indicate that *p*-aminophenol is a highly nephrotoxic substance, and that nephrotoxicity of *p*-chloroaniline, *p*-chloronitrobenzene, *p*-anisidine and *p*-nitroaniline exceed that of acetyl-*p*-aminophenol which has been known to cause a renal damage.

Key words: Aromatic nitro-amino compounds, nephrotoxicity, *p*-aminophenol, *p*-chloroaniline, *p*-chloronitrobenzene, *p*-anisidine, *p*-nitroaniline, urinary enzyme, methemoglobin.

INTRODUCTION

Aromatic nitro-amino (ANA) compounds are widely used as raw materials or intermediates for production of drugs, rubber, pesticides and dyes. A common

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toxicological reaction to ANA compounds is the formation of methemoglobin followed by cyanosis and anemia (Beard and Noe, 1981).

Some analgesics or antifebrics containing ANA compounds produce nephrotoxicity as a side effect (Stygles and Iuliucci, 1981). In particular, acetaminophen (acetyl-*p*-aminophenol), commonly used as an antipyretic analgesic, has been reported to cause the renal damage in rats (Newton et al., 1983) and in patients with overdoses of this compound (Cobden et al., 1982).

Recently, certain urinary enzyme activities have measured as an index of renal damage (Piperno, 1981). We observed the elevation of the activities of urinary N-acetyl- β -D-glucosaminidase (NAG) and β -galactosidase in workers with long-term exposure to ANA compounds including *p*-chloronitrobenzene, *p*-anisidine, *p*-nitroaniline or *p*-aminophenol (Yoshida et al., 1989). In animal experiments, renal damage caused by *p*-aminophenol (Crowe et al., 1979) and *p*-chloroaniline (Rankin et al., 1986) have been reported.

In the present study, to evaluate nephrotoxicity of some ANA compounds, we examined urinary NAG and γ -glutamyltranspeptidase (γ -GTP) activities and renal pathological changes in rats to whom aniline, *p*-aminophenol, acetyl-*p*-aminophenol, *p*-chloroaniline, *p*-chloronitrobenzene, *p*-nitroaniline or *p*-anisidine was administered.

MATERIALS AND METHODS

Animal treatment :

Male Fischer 344 rats, weighing 180 to 200 g, were individually housed in stainless metabolic cages in a temperature (22–24°C)-and moisture (60%)-controlled room with a 12 h light (8 AM to 8 PM)-dark cycle, and were allowed free access to a commercial stock diet (Type MF, Oriental Yeast Co., Tokyo) and drinking tap water. In Experiment 1, after a week of feeding, the rats were intraperitoneally injected with *p*-aminophenol (1.0 mmol/kg body weight). Urine was collected every 24 h for two days prior to and for five days following the injection. In Experiment 2, the rats were intraperitoneally injected with aniline, *p*-aminophenol, acetyl-*p*-aminophenol, *p*-chloroaniline, *p*-chloronitrobenzene, *p*-anisidine or *p*-nitroaniline at a level of 1.0 mmol/kg body weight. These ANA compounds were dissolved or suspended in olive oil and control rats were injected with olive oil. The urine of each rat was collected for 24 h after the injection. The blood was collected into heparinized tubes by heart puncture and the kidney was removed at 48 h after the injection. All injections were performed between 8 and 10 AM.

Assays :

Immediately after the blood collection, 100 μ l of whole blood was hemolyzed in 8 ml of cold water. Methemoglobin levels in this hemolysate were determined spectrophotometrically within 3 h (Hashimoto, 1958). The remaining blood was centrifuged at 750 x g for 15 min and the plasma was obtained. Urea nitrogen and

transaminase (aspartate aminotransferase (GOT) and alanine aminotransferase (GPT)) activities in the plasma, and urinary NAG activity and creatinine were assayed by the use of commercial kits ; for urea nitrogen, Urea N B-Test Wako (Wako Pure Chemical Industries, Ltd., Osaka) ; for GOT and GPT, Transaminase C-II Test Wako (Wako Pure Chemical Industries) ; for NAG, NAG-Test SHIONOGI (Shionogi Pharmaceuticals Ltd., Osaka) ; for creatinine, Creatinine Test Wako (Wako Pure Chemical Industries). Urinary γ -GTP activities were assayed using *L*- γ -glutamyl-*p*-nitroanilide as a substrate (Orlo'wski and Meister, 1963).

Histopathology :

Kidneys were sectioned longitudinally through the hilum, and blocks were fixed in 4% formaldehyde solution. Tissues were embedded in paraffin, sectioned 5 μ m thick, and stained with hematoxylin and eosin.

The sections were evaluated by a pathologist without knowledge of the animal's treatment.

Statistical analysis :

Experimental data were examined by analysis of variance (ANOVA) followed by the least significant difference (LSD) test (Snedecor and Cochran, 1969).

RESULTS

Experiment 1 :

Fig. 1 shows the change in urine volume before and after the injection of *p*-aminophenol. Administration of the aminophenol caused a remarkable elevation in the urine volume. The volume returned to the pre-injection level 3 days after the administration.

Alteration of urinary NAG and γ -GTP activities are described in Fig. 2. Similar to the effect on urinary volume, the enzyme activities were remarkably elevated after aminophenol administration and returned to normal levels 3 days after the injection.

Experiment 2 :

Urine volume and urinary creatinine excretion for 24 h after the injection of each of the ANA compounds are shown in Table 1. The injection with *p*-aminophenol, *p*-chloroaniline, *p*-chloronitrobenzene or *p*-nitroaniline caused significant increases in urine volume. On the other hand, the creatinine excretion did not change after injection of any of these compounds.

Table 2 describes alteration of urinary NAG and γ -GTP activities in the rats injected with each of the ANA compounds. Similar to the results of Experiment 1, elevation of the urinary enzyme activities in the rats injected with *p*-aminophenol were remarkable. Injection of *p*-chloroaniline also caused significant increases in both enzymes. *p*-Chloronitrobenzene, *p*-anisidine and *p*-nitroaniline caused significant elevation of NAG activity but not of the γ -GTP activity. Aniline and acetyl-*p*-aminophenol had no effect on the urinary enzyme activity under the present

Fig. 1. Effect of injection of *p*-aminophenol on urine volume in rats.

Male Fischer 344 rats were intraperitoneally injected with *p*-aminophenol (1.0 mmol/kg body weight) on day 3 at 8 AM. Urine was collected every 24 h (8 AM to 8 AM) for 2 days prior to and for 5 days following the injection. Band heights and bars represent means $\pm$ SEM ($n=5$). Significant difference from pre-injection values at $P < 0.001$ (***) or at $P < 0.05$ (*).

experimental conditions.

Photo. 1 shows typical microscopic photographs of renal sections from the rats injected with *p*-aminophenol (3-a) or *p*-anisidine (3-b). Necrosis of the renal tubular epithelial cells was observed in the aminophenol-administered rat. In the anisidine-administered rat, swelling of the epithelial cells was observed.

A summary of the histopathological changes seen in renal sections of the rats injected with each of the ANA compounds is shown in Table 3. The necrosis was observed to a moderate or severe extent only in the rats injected with *p*-aminophenol. On the other hand, swelling to a moderate or severe extent was observed in the rats injected with *p*-anisidine or *p*-nitroaniline.

Table 4 shows transaminase activities and urea nitrogen levels in plasma of the treated rats. Neither GOT nor GPT activities were changed by the administration

Fig. 2. Alteration of urinary NAG and γ -GTP activities in rats injected with *p*-aminophenol. Experimental conditions are the same as described in Fig. 1. Circles and bars represent means $\pm$ SEM ($n=5$). Significant difference from pre-injection values at $P < 0.001$ (***) or $P < 0.05$ (*).

Table 1. Urine volume and urinary creatinine excretion in rats injected with aromatic nitro-amino compounds.

Compound administered	Urine volume (ml/24h)	Creatinine (mg/24h)
Control	8.3± 1.1	4.5±0.3
Aniline	9.3± 0.8	4.6±0.4
<i>p</i> -Aminophenol	16.3± 0.7*	4.7±0.2
Acetyl- <i>p</i> -aminophenol	8.3± 1.0	4.5±0.6
<i>p</i> -Chloroaniline	16.8± 1.4*	4.0±0.2
<i>p</i> -Chloronitrobenzene	19.6± 2.8**	4.7±0.3
<i>p</i> -Anisidine	9.7± 1.1	4.7±0.3
<i>p</i> -Nitroaniline	19.8± 3.0**	5.4±0.6

Rats were intraperitoneally injected with each compound at a level of 1.0 mmol/kg body weight. Urine samples were collected for 24 h after the injection. Values are means ± SEM (n=5). Asterisks indicate a significant difference from control group at P<0.01 (**) or P<0.05 (*).

Table 2. Alteration of urinary NAG and γ -GTP activities in rats injected with aromatic nitro-amino compounds.

Compound administered	NAG (mUnit/24h)	γ -GTP (Unit/24h)
Control	34± 9	5.9±1.0
Aniline	42± 7	5.6±0.6
<i>p</i> -Aminophenol	404±10***	48.7±2.9***
Acetyl- <i>p</i> -aminophenol	37±11	6.4±0.8
<i>p</i> -Chloroaniline	111±14**	11.3±1.3*
<i>p</i> -Chloronitrobenzene	76±15*	9.4±0.8
<i>p</i> -Anisidine	109±19**	7.5±0.9
<i>p</i> -Nitroaniline	79±17*	7.6±1.1

Sampling conditions were same as described in Table 1. Values are means ± SEM (n=5). Asterisks indicate a significant difference from control group at P<0.001 (***), P<0.01 (**), or P<0.05 (*).

Renal toxicity of aromatic nitro-amino compounds

Photo. 1. Microscopic photographs (x 200) of renal sections of rats injected with *p*-aminophenol (a) or *p*-anisidine (b).

Rats were intraperitoneally injected with each compound at 1.0 mmol/kg. Kidneys were removed 48 h after the injection. Sections were stained with hematoxylin and eosin.

Table 3. Histopathological changes in renal tubular epithelial cells in rats injected with aromatic nitro-amino compounds.

Compound administered	Necrosis				Swelling			
	—	±	+	>++	—	±	+	>++
Control	5	0	0	0	4	1	0	0
Aniline	5	0	0	0	0	4	1	0
<i>p</i> -Aminophenol	0	0	0	5	3	2	0	0
Acetyl- <i>p</i> -aminophenol	5	0	0	0	3	1	1	0
<i>p</i> -Chloroaniline	5	0	0	0	1	3	1	0
<i>p</i> -Chloronitrobenzene	5	0	0	0	1	4	0	0
<i>p</i> -Anisidine	5	0	0	0	0	0	3	2
<i>p</i> -Nitroaniline	5	0	0	0	1	1	2	1

Rats were intraperitoneally injected with each compounds at 1.0mmol/kg body weight. The kidney was removed 48 h after the injection. The sections were evaluated by a pathologist without knowledge of the animal's treatment. The severity of each pathological change was scored on an arbitrary scale of — to +++, with an intermediate half grade (±) according to the following criteria : —, normal ; +, mild ; ++, moderate ; +++, severe.

Table 4. Transaminase activities and urea nitrogen levels in plasma of rats injected with aromatic nitro-amino compounds.

Compound administered	Transaminase (Karmen unit/ml)		Urea nitrogen (mg/dl)
	GOT	GPT	
Control	53± 3	25± 7	22± 1
Aniline	50± 6	28± 3	21± 1
<i>p</i> -Aminophenol	45± 4	18± 1	44± 8***
Acetyl- <i>p</i> -aminophenol	59± 3	28± 2	19± 2
<i>p</i> -Chloroaniline	48± 4	21± 4	18± 2
<i>p</i> -Chloronitrobenzene	68± 9	21± 2	18± 1
<i>p</i> -Anisidine	94±38	44±25	17± 1
<i>p</i> -Nitroaniline	71±17	34± 8	19± 1

Rats were intraperitoneally injected with each compound at 1.0 mmol/kg body weight. Blood was collected by heart puncture 48 h after the injection. Values are means ± SEM (n=5). Asterisks indicate a significant difference from control group at P<0.001.

of ANA compounds. The urea nitrogen levels were significantly elevated only in the aminophenol-administered rats.

Table 5 shows methemoglobin levels formed by the administration of ANA compounds. The methemoglobin production in the rats injected with *p*-chloroaniline or *p*-chloronitrobenzene was high (about 5%), but the levels in the rats injected with aniline, *p*-aminophenol, acetyl-*p*-aminophenol, *p*-anisidine or *p*-nitroaniline were about 1% the same as the control rats.

Table 5. Increase in methemoglobin level by administration of aromatic nitro-amino compounds in rats.

Compound administered	Methemoglobin (%)
Control	1.1±0.2
Aniline	1.9±0.2
<i>p</i> -Aminophenol	1.4±0.2
Acetyl- <i>p</i> -aminophenol	1.2±0.2
<i>p</i> -Chloroaniline	4.9±0.2***
<i>p</i> -Chloronitrobenzene	5.4±0.5***
<i>p</i> -Anisidine	0.9±0.2
<i>p</i> -Nitroaniline	1.8±0.1

Experimental conditions were same as described in Table 4. Values are means ± SEM (n=5). Asterisks indicate a significant difference from control group at $P < 0.001$.

DISCUSSION

In the present study, we evaluated the nephrotoxicity of some ANA compounds using several indices; urine volumes, urinary NAG and γ -GTP activities, plasma nitrogen levels and histopathological changes. Among these indices, an increase in urine volume (polyuria) often signifies impaired renal concentrating ability, the elevation of the urinary enzymes indicates an impairment of renal tubules (Piperno, 1981), and the urea nitrogen level is used as an index of renal damage in clinical medicine and reflects the glomerular function.

In Table 6, results of the evaluation are summarized. Renal necrosis, the increased plasma urea nitrogen levels and the highest urinary enzyme activities were observed in the rats with administration of *p*-aminophenol. This led us to conclude that *p*-aminophenol is the most highly nephrotoxic substance among the ANA compounds presently evaluated. The nephrotoxicity of *p*-aminophenol has been reported by several researchers (Calder et al., 1979; Itoh et al., 1987). *p*-Aminophenol is metabolized to a reactive intermediate and is covalently bound to renal macromolecules followed by the necrosis of tubular epithelial cells (Crowe et

Table 6. Summary of evaluation of nephrotoxicity of aromatic nitro-amino compounds.

Compounds administered	Index of nephrotoxicity					
	Urine volume	NAG	γ -GTP	Urea nitrogen	Necrosis	Swelling
Control	—	—	—	—	—	—
Aniline	—	—	—	—	—	—
<i>p</i> -Aminophenol	+	+	+	+	+	—
Acetyl- <i>p</i> -aminophenol	—	—	—	—	—	—
<i>p</i> -Chloroaniline	+	+	+	—	—	—
<i>p</i> -Chloronitrobenzene	+	+	—	—	—	—
<i>p</i> -Anisidine	—	+	—	—	—	+
<i>p</i> -Nitroaniline	+	+	—	—	—	+

In the case of urine volume, NAG, γ -GTP and urea nitrogen, "+" indicates that a significant difference was observed from control group. In the case of necrosis and swelling, "+" indicates that 3 or more number of rats were scored on +, ++ or +++ in the histopathological evaluation.

al., 1979).

p-Chloroaniline caused polyuria and the elevation of both NAG and γ -GTP activities. Rankin et al. (1986) also reported that polyuria was induced by the intraperitoneal injection of 1.0 mmol/kg of *p*-chloroaniline in Fischer rats. *p*-Chloroaniline can be ranked as a nephrotoxic substance next to *p*-aminophenol among the compounds evaluated. Although the two urinary enzyme activities were elevated, renal tubular necrosis was not induced by *p*-chloroaniline. This indicates that the elevation of urinary enzymes precede renal histopathological changes.

The significant elevation of urinary NAG activities was also observed in the rats injected with *p*-chloronitrobenzene, *p*-anisidine and *p*-nitroaniline. Polyuria was also observed in the rats with *p*-chloronitrobenzene or *p*-nitroaniline. Additionally, the swelling of the tubular epithelial cells was induced by *p*-anisidine or *p*-nitroaniline. On the other hand, the indices of nephrotoxicity did not change after aniline or acetyl-*p*-aminophenol administration. These results led us to conclude that *p*-chloronitrobenzene, *p*-anisidine and *p*-nitroaniline have higher nephrotoxicity than acetyl-*p*-aminophenol whose overdose has been known to induce a renal damage (Cobden et al., 1982).

As shown in Table 5, *p*-chloroaniline and *p*-chloronitrobenzene strongly induce methemoglobin production. Methemoglobin formation can lead to anoxia in the renal cells, which may partly play a role in the manifestation of renal toxicity of *p*-chloroaniline and *p*-chloronitrobenzene. *p*-Anisidine is thought to be metabolized to *p*-aminophenol by hepatic microsomal O-demethylase (Wislocki et al., 1980; Dybing et al., 1979). The elevation of urinary NAG activities by

p-anisidine may be caused by *p*-aminophenol produced *in vivo*. However, the swelling of renal epithelial cells, which was observed in the rats injected with *p*-anisidine, was not induced by *p*-aminophenol. Accordingly, an unknown mechanism may play a role in the manifestation of the renal toxicity of *p*-anisidine.

In spite of the increased NAG activities, the γ -GTP activities were not altered by the *p*-chloronitrobenzene, *p*-anisidine or *p*-nitroaniline. γ -GTP localized in the plasma membrane of tubular epithelial cells in the kidney, and NAG is abundant in the lysosomes. The lysosomal enzymes may be more sensitive indicators of renal tubular damage than the membrane enzymes. In relation to this point, we observed that workers with long-term exposure to ANA compounds showed the elevation in urinary NAG activities but not in γ -GTP activities (Yoshida et al., 1989).

In the present study, the plasma transaminase activities were not altered by the injection of the ANA compounds at a level of 1.0 mmol/kg. Similarly, in our field study on workers exposed to ANA compounds (Yoshida et al., 1989), the workers with increased urinary NAG showed normal values of serum GOT, GPT, γ -GTP and alkaline phosphatase activities. These results indicate that several ANA compounds are more toxic to the kidney than to the liver. Therefore, examinations of renal function should be important in workers handling ANA compounds.

ACKNOWLEDGEMENTS

The authors are grateful to Dr. Shikata, N. in the histopathological evaluation of renal sections. This study was supported by Grant-in-Aid 63770368 for M. Y. from the Ministry of Education, Science and Culture of Japan.

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