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Letter

Scanning electron microscopic analysis of the bladder epithelium in terephthalic acid administered rats: a case in acute toxicity study

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ABSTRACT — Terephthalic acid (TPA) was observed to have induced tumors due to the physical cytotoxicity of urinary calculi in the urinary bladders of rats. In the acute toxicity study, the extrapolation to humans was evaluated hastily by observation of the urinary crystals and cytotoxicity on the bladder epithelium. We examined whether it was possible to observe urine crystals and corresponding alterations on the epithelium in the urinary bladders in the acute toxicity study of TPA. TPA at a dose level of 2,000 mg/kg body weight, were administered twice by oral gavage at a 21-hr interval and the bladder mucosal epithelium was observed at 3, 6 and 9 hr after the second administration using scanning electron microscope (SEM). As a result, micro crystals were observed in the bladder surface at 6 and 9 hr after the second administration, and small raised ridges on the bladder surface, which were considered to be the effects of cytotoxicity, were observed at 9 hr after the second administration. However, these were not observed in rats administered only 0.5% sodium carboxymethyl cellulose solution, which is a vehicle of TPA. In this study, in cases where the substances induce crystals in urine in a short period, it was suggested that the urinary crystals and the alterations on bladder epithelium could be detected using SEM in acute toxicity study.

Key words: Scanning electron microscope, TPA, Bladder, Acute toxicity, Crystal, Terephthalic acid

INTRODUCTION

Terephthalic acid (TPA) is a chemical substance used as a raw material for synthetic fibers and synthetic resins. However, hyperplasia and tumors in urinary bladders were observed in a carcinogenicity study (OECD, 2001a), and hyperplasia and calculi were frequently observed in male rats in a subacute toxicity study (i.e. during a 90-day feeding toxicity study) (Dai *et al*., 2005a, 2005b; Heck and Tyl, 1985). TPA had negative results from several genotoxicity tests and was known as a typical non-genotoxic bladder carcinogen whose Mode of Action was physical cytotoxicity due to calculi in the urine (Lee and Lee, 2007; Zeiger *et al*., 1982). If tumors due to calculi, precipitate or crystals were frequently observed in the bladder of male rats that were administered substances in high dose, the bladder carcinogenicity of the substances would not be extrapolated for humans (Cohen and Lawson, 1995; Cohen, 1998). Regarding typical non-genotoxic bladder carcinogens (e.g. sodium saccharin and uracil) which induce urinary calculi and precipitate, including TPA, there were some reports of scanning electron microscope (SEM) analyses about urinary precipitate or bladders (Cui *et al*., 2006; Cohen *et al*., 1995; Shirai *et al*., 1986), in addition, the alterations of bladder surface due to calculi were found on days 7 in uracil continuous feeding administration (Shirai *et al*., 1989). And, it has been reported that tetraethylorthosilicate caused the necrosis and exfoliation of the bladder surface which were considered due to urine crystals by SEM analysis in the acute

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toxicity study (Okamura *et al*., 1992). However, there were no reports to have been observed in crystals and corresponding alterations on the bladder epithelium at one time using SEM in the acute toxicity study of TPA. Conducting the SEM analysis of bladder surfaces in acute toxicity study has great significance in the viewpoint of short-term evaluation of extrapolation to humans in bladder carcinogenicity. Therefore, it was confirmed using SEM as to whether crystals and alteration of bladder epithelium occurred in the bladder of male rats in the acute phase after administration of TPA.

MATERIALS AND METHODS

Materials

Terephtharic acid (CAS No. 100-21-0) was purchased from Nacalai Tesque, Inc. (Kyoto, Japan). Sodium carboxymethyl cellulose (CMC) was purchased from Nacalai Tesque, Inc., and a 0.5% (w/v) aqueous solution (0.5% CMC) was prepared as the vehicle. Bouin's Fluid was purchased from Wako Pure Chemical Industries, Ltd. (Tokyo, Japan).

Animals

Male Sprague-Dawley rats (Crl:CD (SD)) were purchased from Charles River Laboratories Japan, Inc. (Kanagawa, Japan). Animals were subjected to the test after acclimation for more than a week and were seven weeks old at the start of the experiment. Rats were maintained on a 12-hr light/dark cycle at approximately 20°C and 50% relative humidity, and were fed a standard diet (MF Powder, Oriental Yeast Co., Ltd., Tokyo, Japan) and tap water *ad libitum*. Prior to the test, the animals were checked for any diseases or injuries, and those without weight or feeding abnormalities were used. All experimental procedures were performed in accordance with the animal study protocol approved by the Institutional Animal Care and Use Committee of the testing facility.

Animal treatment

Sufficiently crushed TPA was suspended in 0.5% CMC at a final concentration of 200 mg/mL. For TPA, the median lethal dose (LD_{50}) in acute oral toxicity is likely to be higher than 2,000 mg/kg (OECD, 2001a); therefore, the dose was set at 2,000 mg/kg. Furthermore, this dose is usually set as the highest dosage if there are no toxicological abnormalities in the acute toxicity study (OECD, 2001b).

The test substance was administered twice by oral gavage without fasting at a dose of 10 mL/kg at 21-hr intervals. All rats were weighed prior to administration of the test substance by using a PG2002-S balance (Mettler Toledo International Inc., Tokyo).

Necropsy

Necropsy was performed on all rats, excluding those which were deceased, at 3, 6, 9 hr after the second administration to observe bladder conditions, which was due to a report stating that over 80% of administered TPA was excreted within 8 hr (Hoshi and Kuretani, 1967). Two animals per group were subjected to necropsy at each time interval, excluding the control animals (which underwent necropsy at 6 hr only).

Scanning electron microscope (SEM) analysis

The method of sample preparation was made with reference to the previous paper (Cohen *et al*., 2007). Laparotomy was performed on the animals under isoflurane anesthesia, and 0.1 M phosphate buffer was injected into the bladder via an indwelling needle placed into the lower bladder for irrigation (once). The 0.1 M phosphate buffer was then removed from the bladder, the syringe was exchanged, and approximately 300 μL of Bouin's fluid was injected. The puncture opening was then tied with a suture to prevent fluid leakage, and the bladder was isolated and immersed in Bouin's fluid. After one to four hr of fixation, the bladder was cut into two pieces and rinsed with 70% ethanol to remove Bouin's fluid. Next, the bladder samples were immersed in 80%, 90% and 95% ethanol for three min each and finally in 100% ethanol for five min, twice, for full dehydration. The samples were completely air-dried, fixed via a carbon tape to an aluminum SEM stub (Nisshin EM Co., Ltd., Tokyo, Japan) and coated with evaporated gold for SEM observation. The aluminum stub was fixed to the special sample stage, which in turn was fixed to the sample stage holder of the S-3400N (Hitachi High-Technologies Corporation, Tokyo, Japan). The sample stage was then transported to the observation area and observations were conducted under a vacuum at an accelerating voltage of 15 kV.

SEM evaluation method

If substances (e.g. crystals) were observed inside the bladder, they were evaluated using the criteria as below (severe +++: present at high density throughout the bladder; moderate ++: scattered throughout the bladder or concentrated in limited areas; slight +: present throughout the bladder, but sparsely or in limited areas when observed under low power. Densities lower than ++; minimal ±: present in very limited areas). Abnormality changes observed in the bladder epithelium (e.g. raised ridge, swollen cell, crater and exfoliation) were also recorded

and qualitatively evaluated using the same criteria.

RESULTS

General observation

During the test period, no deaths or toxicity symptoms suspected of having been caused by the test substance were observed. A decrease in body weight after the first administration was observed in three of the six rats in the TPA group (Table 1).

Necropsy

The results of the necropsy are shown in Table 2. In the TPA group, crystal-like substances were observed in the bladder of some of the animals (Nos. 1103, 1104, 1105 and 1106), when the 0.1 M phosphate buffer was injected into the bladder. In No. 1103, in particular, the Bouin's fluid injected into the bladder became whitish (photo not shown).

SEM analysis

The results of the SEM analysis are shown in Table 2 and representative photos are shown in Figs. 1-6. In the TPA group, micro crystals were observed in all animals at 6 hr and later time points after administration (Figs. 1, 2, 3 and 4); the degree of crystal formation was moderate (++) in No. 1103 (Fig.1) , minimal (±) in No. 1104 (Fig. 2), severe (+++) in No. 1105 (Fig. 3), and slight (+) in No. 1106 (Fig. 4). In the 0.5% CMC group, however, this crystal formation was not observed (Fig. 5). In the TPA group, very little raised ridges were observed on the bladder surface of one rat at 9 hr after the second administration (Fig. 4). This change was observed over a large region, relatively, but evaluated as minimal (±) due to only experiencing a slight change. Although this change was very minor compared with the 0.5% CMC group, it was possible to distinguish between the TPA group and the 0.5% CMC group as the bladder urothelial surface in the 0.5% CMC group was extensively smooth (Figs. 4 and 5). A pleated bladder epithelium was observed in some animals, although this was suspected to be an artifact due to insufficient fixation (Fig. 6). The injection of more Bouin's fluid was considered to be necessary in order to spread the bladder surface.

DISCUSSION

To begin with, TPA was determined to be administered with sufficient doses from results of the body weight

Table 1. Body weight (BW) and body weight gain (BWG) - Individual values (g), mean and standard deviation (S.D.).

Chemical Dose	Animal No.	BW		BWG
		1st adm.	2nd adm.	2nd -1st
0.5% CMC	1001	259.7	265.5	5.8
	1002	250.8	264.7	13.9
Mean		255.3	265.1	9.8
TPA 2,000 mg/kg	1101	253.7	255.5	1.8
	1102	261.0	259.7	-1.3
	1103	256.0	261.3	5.3
	1104	250.0	247.3	-2.7
	1105	251.9	246.2	-5.7
	1106	246.7	248.1	1.4
Mean		253.2	253.0	-0.2
S.D.		5.0	6.7	3.9

Table 2. Necropsy and SEM analysis data.

Necropsy time (After 2nd adm.)	Chemical Dose	Animal No.	Necropsy	SEM analysis		
				Crystal	Abnormality of bladder surface	Remarks
3 hr	TPA (2,000 mg/kg)	1101	None	-	None	Pleated surface
		1102	None	-	None	
6 hr	0.5% CMC	1001	None	-	None	Pleated surface
		1002	None	-	None	
	TPA (2,000 mg/kg)	1103	Crystals in bladder	++	None	Pleated surface
		1104	Crystals in bladder	±	None	Pleated surface
9 hr	TPA (2,000 mg/kg)	1105	Crystals in bladder	+++	None	
		1106	Crystals in bladder	+	Raised ridges (±)	Pleated surface

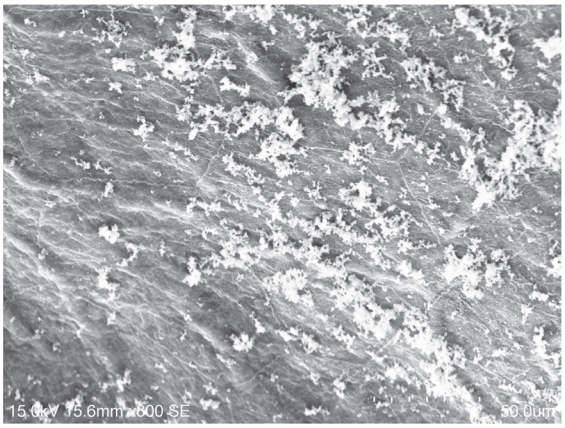

Fig. 1. Crystals on the bladder surface (++) of the rat (No. 1103) administered 2,000 mg/kg TPA.

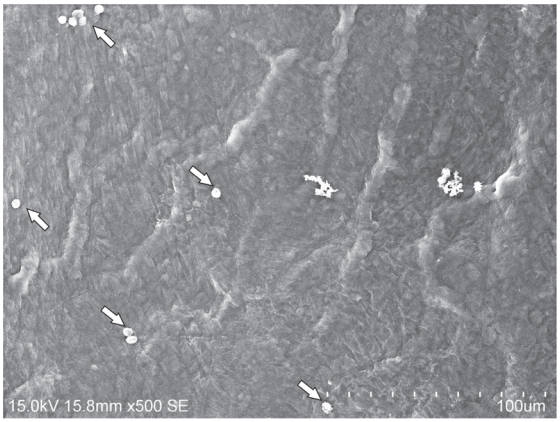

Fig. 2. Crystals on the bladder surface (±) of the rat (No. 1104) administered 2,000 mg/kg TPA. White round materials (arrows) are not crystals (they may possibly be red and white blood cells).

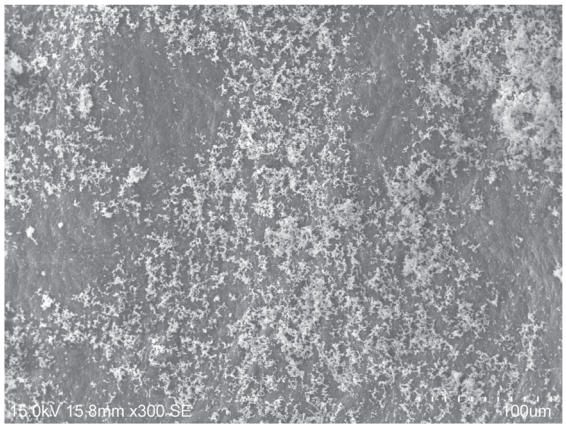

Fig. 3. Crystals on the bladder surface (+++) of the rat (No. 1105) administered 2,000 mg/kg TPA.

(Table 1). In the TPA group, micro crystals were observed on the bladder surface at 6 and 9 hr after the second administration. The majority of the administered TPA was excreted to urine smoothly within a 24-hr period (Hoshi and Kuretani, 1967). Thus, a large section of TPA had previously been excreted at 3 hr after the second administration (at 24 hr after the first administration), and, it was too early a time point to collect micro crystals after the second administration. However, the number of administrations and necropsy times in this study were suitable for the substances such as TPA, which is excreted into urine with ease. The result in this study is a rare case, because crystal and precipitate were mostly observed in the urine trapped on the filter in SEM analysis (Cohen *et al.*, 2000; Cohen *et al*., 2007). This is a valuable case example that

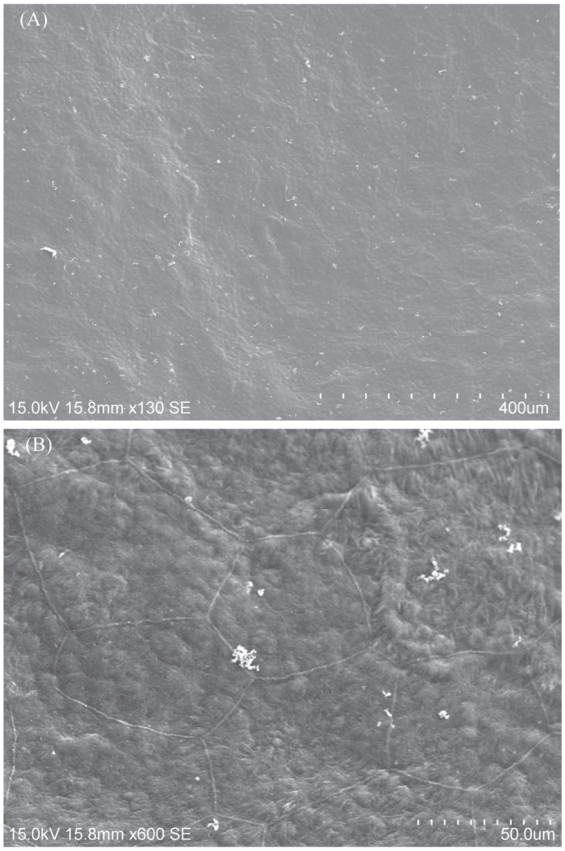

Fig. 4. Crystals on the bladder surface (+) and raised ridges on the urothelial surface of the bladder (±) of the rat (No. 1106) administered 2,000 mg/kg TPA. (A): Low power. (B): High power.

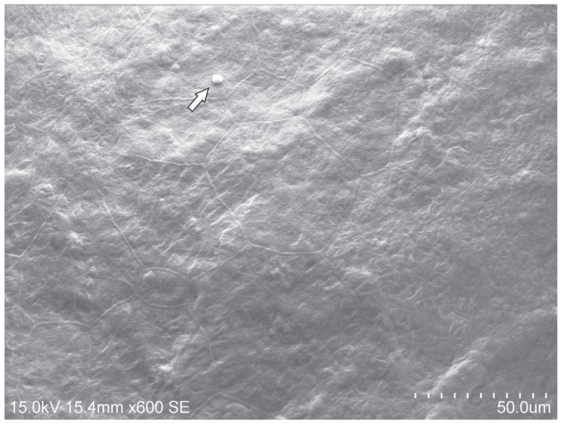

Fig. 5. The urothelial surface of the bladder of the rat administered 0.5% CMC (No. 1002). White round material (arrow) is not crystal (maybe red blood cell).

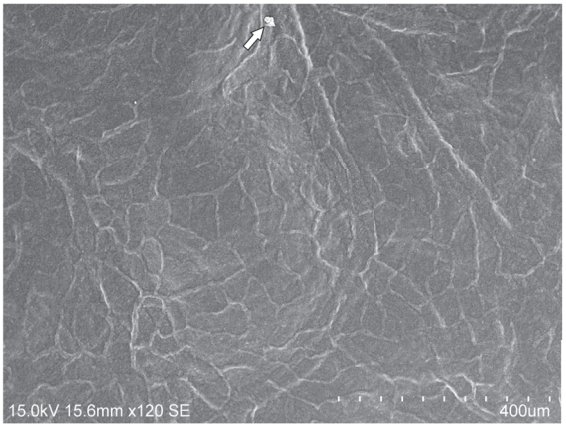

Fig. 6. Pleated surface of the bladder urothelium of the control rat (No. 1001). White materials (arrow) are not crystals (they may possibly be red and/or white blood cells).

micro crystals were detected in the bladder using SEM in the acute toxicity study.

The minor raised ridges on the bladder surface were observed in one out of six animals in the TPA group. Physical damage due to crystals in urine is one of the most common causes of cytotoxicity in bladder epithelium. The cytotoxicity on the bladder surface is often observed as swollen or elevated cells, craters and exfoliations (Cohen, 2002; Cohen *et al*., 2007; Fava *et al*., 2015; Fukushima and Cohen, 1980), and it seems that the raised ridges were one of the shapes of bladder epithelium damage because of the similarity of the shapes to swollen and elevated cells. It was believed that weak cytotoxicity occurred quickly after the administration of TPA.

Six animals were given a small sample size of TPA, nevertheless four out of them had micro crystals in the urinary bladder. One out of six also had slightly raised ridges on the bladder surface that were considered to be caused by the cytotoxicity. It was suggested that acute toxicity study with SEM analysis to observe crystal and epithelial cells in the bladder under this conditions was effective against substances like TPA which are immediately excreted after administration.

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Conflict of interest---- The authors declare that there is no conflict of interest.

REFERENCES

Cohen, S.M., Cano, M., Garland, E.M., St John, M. and Arnold, L.L. (1995): Urinary and urothelial effects of sodium salts in male rats. Carcinogenesis, **16**, 343-348.

Cohen, S.M. and Lawson, T.A. (1995): Rodent bladder tumors do not always predict for humans. Cancer Lett., **93**, 9-16.

Cohen, S.M. (1998): Urinary bladder carcinogenesis. Toxicol. Pathol., **26**, 121-127.

Cohen, S.M., Arnold, L.L., Cano, M., Ito, M., Garland, E.M. and Shaw, R.A. (2000): Calcium phosphate-containing precipitate and the carcinogenicity of sodium salts in rats. Carcinogenesis, **21**, 783-792.

Cohen, S.M. (2002): Comparative pathology of proliferative lesions of the urinary bladder. Toxicol. Pathol., **30**, 663-671.

Cohen, S.M., Ohnishi, T., Clark, N.M., He, J. and Arnold, L.L. (2007): Investigations of rodent urinary bladder carcinogens: collection, processing, and evaluation of urine and bladders. Toxicol. Pathol., **35**, 337-347.

Cui, L., Shi, Y., Dai, G., Pan, H., Chen, J., Song, L., Wang, S., Chang, H.C., Sheng, H. and Wang, X. (2006): Modification of N-Methyl-N-Nitrosourea initiated bladder carcinogenesis in Wistar rats by terephthalic acid. Toxicol. Appl. Pharmacol., **210**, 24-31.

Dai, G., Cui, L., Song, L., Cheng, J., Zhong, Y., Zhao, R. and Wang, X. (2005a): Bladder epithelial cell proliferation of rats induced by terephthalic acid-calculi. Food Chem. Toxicol., **43**, 217-224.

Dai, G.D., Cui, L.B., Song, L., Zhao, R.Z., Cheng, J.F., Liu, M.X., Zhou, J.W., Xiao, H. and Wang, X.R. (2005b): Induction of bladder lesion by terephthalic acid and its mechanism. Biomed. Environ. Sci., **18**, 211-219.

Fava, R.M., Ferragut Cardoso, A.P., da Rocha, M.S., Nascimento, E., Pontes, M.G., de Camargo, J.L. and de Oliveira, M.L. (2015): Evaluation of early changes induced by diuron in the rat urinary

bladder using different processing methods for scanning electron microscopy. Toxicology, **333**, 100-106.

Fukushima, S. and Cohen, S.M. (1980): Saccharin-induced hyperplasia of the rat urinary bladder. Cancer Res., **40**, 734-736.

Heck, H.D. and Tyl, R.W. (1985): The induction of bladder stones by terephthalic acid, dimethyl terephthalate, and melamine (2,4,6-triamino-s-triazine) and its relevance to risk assessment. Regul. Toxicol. Pharmacol., **5**, 294-313.

Hoshi, A. and Kuretani, K. (1967): Metabolism of terephthalic acid. III. Absorption of terephthalic acid from gastrointestinal tract and detection of its metabolites. Chem. Pharm. Bull., **15**, 1979-1984.

Lee, K.H. and Lee, B.M. (2007): Study of mutagenicities of phthalic acid and terephthalic acid using *in vitro* and *in vivo* genotoxicity tests. J. Toxicol. Environ. Health A, **70**, 1329-1335.

OECD SIDS Initial Assessment Report for 12th SIAM (2001a): Terephthalic Acid (TPA).

OECD (2001b): OECD Guidelines for the Testing of Chemicals, Acute Oral Toxicity - Fixed Dose Procedure, Test No. 420.

Okamura, T., Garland, E.M., Johnson, L.S., Cano, M., Johansson, S.L. and Cohen, S.M (1992): Acute urinary tract toxicity of tetraethylorthosilicate in rats. Fundam. Appl. Toxicol., **18**, 425-441.

Shirai, T., Fukushima, S., Tagawa, Y., Okumura, M. and Ito, N. (1989): Cell proliferation induced by uracil-calculi and subsequent development of reversible papillomatosis in the rat urinary bladder. Cancer Res., **49**, 378-383.

Shirai, T., Ikawa, E., Fukushima, S., Masui, T. and Ito, N. (1986): Uracil-induced urolithiasis and the development of reversible papillomatosis in the urinary bladder of F344 rats. Cancer Res., **46**, 2062-2067.

Zeiger, E., Haworth, S., Speck, W. and Mortelmans, K. (1982): Phthalate ester testing in the National Toxicology Program's environmental mutagenesis test development program. Environ. Health Perspect., **45**, 99-101.