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Sex Differences in *o*-Phenylphenol and Sodium *o*-Phenylphenate Rat Urinary Bladder Carcinogenesis: Urinary Metabolites and Electrolytes under Conditions of Aciduria and Alkalinuria

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F344 male and female rats were administered 1.25% *o*-phenylphenol (OPP) or 2% sodium *o*-phenylphenate (Na-OPP) in combination with 3% $NaHCO_3$ or 1% NH_4Cl for 8 weeks and changes in the urinary bladder histopathology and the urinary components were examined. Administration of OPP with $NaHCO_3$ resulted in marked urothelial hyperplasia in the urinary bladder of male rats, the response being less pronounced in females. OPP alone exerted no proliferative effect and $NaHCO_3$ induced only slight hyperplasia in males. Na-OPP alone induced mild hyperplastic lesions only in males, this being completely prevented by concomitant administration of NH_4Cl. The findings thus demonstrated a clear correlation between hyperplastic response and reported carcinogenic potential of these treatments. Of the urinary factors examined, increases in levels of pH and sodium ion concentration were positively associated with proliferative lesions especially in males, although the findings failed to explain the sex difference. Urinary concentrations of non-conjugated forms of OPP metabolites were also not directly correlated with the development of hyperplasias. Thus, changes in individual urinary factors presumably affect urothelial proliferation in combination rather than separately. The presence of OPP metabolites, including 2-phenyl-1,4-benzoquinone, in the urine may be unimportant in the OPP urinary carcinogenesis even under conditions of alkalinuria and high sodium ion concentration.

Key words: OPP— Na-OPP — Urinary bladder carcinogenesis —Sex difference — Rat

Sodium *o*-phenylphenate (Na-OPP) and *o*-phenylphenol (OPP) have been widely used as fungicides for citrus fruits. Neither is mutagenic in the Ames or other *in vitro* short-term tests,[1-3] although positive results were gained in CHO-K1 cells[4] and with RSa, a human cell line.[5] Since Hiraga and Fujii reported that dietary administration of both 2% Na-OPP[6] and 1.25% OPP[7] results in high incidences of urinary bladder tumors in male F344 rats, extensive studies have been performed on these non-mutagenic carcinogens. Resultant findings include: i) OPP is less active than Na-OPP with regard to induction of bladder tumors when administered in the diet[8,9]; ii) the rat is the most susceptible of the commonly used experimental animal species to Na-OPP tumorigenicity[10,11]; iii) male rats are more sensitive than females[6,12]; iv) OPP, phenylhydroquinone (PHQ), 2-phenyl-1,4-benzoquinone (PBQ), and conjugates of both OPP and PHQ with glucuronic acid are detectable in the urine of rats and other animal species as metabolites of OPP and Na-OPP[13-15]; v) PBQ is the most potent non-conjugated urinary metabolite regarding induction of DNA damage and hyperplastic changes in urinary epithelium of male and female rats when injected into the urinary bladder[16-18]; and vi) changes in urinary pH and electrolyte concentrations are involved in tumorigenesis caused by these chemicals.[19,20]

In the present study, urinary pH and levels of electrolytes and non-conjugated forms of the main OPP metabolites were analyzed in rats administered OPP or Na-OPP in the diet for 8 weeks in combination with an alkalizing ($NaHCO_3$) or acidifying (NH_4Cl) agent. We focused on differences in the response to these chemicals between male and female rats, with the aim of elucidating the basis for the observed sex dependence of OPP and Na-OPP carcinogenic potential.

MATERIALS AND METHODS

A total of 72 male and female F344 rats (Charles River Japan Inc., Atsugi), 6 weeks old at the commencement, were used. The rats were housed three to five per plastic cage on soft wood chip bedding in an air-conditioned room at a temperature of 22±2°C and a relative humid-

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ity of 55±5% with a 12-h light-dark daily cycle. The rats were maintained on commercial pelleted basal diet (Oriental M, Oriental Yeast Co., Tokyo). OPP (Dowcide 1 flake, Lot. MM850425, active ingredient: OPP 99.45%, inert ingredients 0.55%) and Na-OPP (Dowcide A, Lot 850425, active ingredients: Na-OPP 72.02%, H_2O 26.78, NaOH 1.25%) were purchased from Dow Chemical Co., Midland, MI. Sodium bicarbonate ($NaHCO_3$) and ammonium chloride (NH_4Cl) were obtained from Wako Pure Chemical Co., Osaka. All chemicals were incorporated into powdered diet at the following dose levels: 1.25% for OPP, 2% for Na-OPP, 3% for $NaHCO_3$ and 1% for NH_4Cl. The concentration of 1.25% OPP is equivalent to 2% Na-OPP by weight on a molar basis.[19]

Groups of 5 or 6 rats of each sex were treated with OPP plus $NaHCO_3$, OPP alone, $NaHCO_3$ alone, Na-OPP plus NH_4Cl, Na-OPP alone, NH_4Cl alone, or were maintained without any chemical supplementation (no treatment) for 8 weeks. Food and water were available *ad libitum* throughout the experimental period. Body weights and food and water consumption levels were determined weekly.

Fresh urine specimens were obtained from all rats by forced micturition at 09.00–10.00 h at week 8 and examined for pH using a pH meter with a flat electrode (Model F-8DP, Hitachi-Horiba, Tokyo). For urinalysis, urine samples were collected from all rats during 07.00–13.00 h in the last week of the experiment using metal metabolic cages without supply of diet or water. Each animal was sampled over 3 consecutive days to obtain enough pooled urine. Collected urine was stored in a freezer at −70°C. The urine volume was measured and its osmolality was determined by measuring freezing-point depression using an Osmett A, Precision Systems Inc., MA. Concentrations of sodium and potassium were measured by flame photometry, calcium by the cresolphthalein complexone method and chloride with a chloride meter. For urinary metabolite analysis, 3 ml of each sample was mixed with the same volume of diethyl ether and stirred, and the upper layer was pipetted into a tube. After one more ether extraction, the extracts were combined and the ether evaporated off at room temperature. The residue was dissolved in 0.5 ml of diisopropyl ether and examined for OPP metabolites. Levels of OPP, PHQ and PBQ in the urine were determined from calibration curves by gas-chromatographic analysis as described earlier[17] using a Shimadzu GC-7APF gas chromatograph (Kyoto) equipped with a hydrogen flame ionization detector under the following conditions: 1 m×3 mm glass tubing column packed with 1% OV-17; flow rates of 60 ml/min for N_2, 50 ml/min for H_2, and 500 ml/min for air; initial temperature of 150°C, programmed rate at 10.0°C, final temperature of 220°C, injection temperature of 230°C, detection temperature of 220°C. The retention times for OPP, PBQ and PHQ were 2.8, 4.2 and 4.5 min, respectively.

All rats were killed at the end of week 8. For histological examination, urinary bladders were gently inflated with 10% phosphate-buffered formalin and cut into 8 longitudinal strips after fixation. Tissue sections were cut, after routine processing, from paraffin blocks and stained with hematoxylin and eosin.

Statistical analyses were performed by using Student's t and Welch's t tests in combination with the F test for variability between means, and by the cummulative chi-square method[21] in 2×5 tables for analysis of proliferative lesions in the urinary bladder.

RESULTS

Body weight and general condition No animals died before termination of the experiment. There were no significant differences in food intake between groups for either sex. Water intake was slightly higher in $NaHCO_3$-treated groups and was apparently unchanged in NH_4Cl-treated groups. Body weights at week 8 were significantly lower in all male treated groups (263–281 g against 313 g for the control) and both OPP- and Na-OPP-treated groups in females (165–174 g against 185 g for the control). The effects of the combined treatment on body weight gain were apparent only in female rats treated with OPP plus $NaHCO_3$.

Urine volume and urinary pH Total urine volume over 3 days (18 h) was 4.0 to 7.4 ml for males and 3.6 to 6.2 ml for females as shown in Table I. The volume was increased in groups given OPP plus $NaHCO_3$, Na-OPP plus NH_4Cl and NH_4Cl in males, and OPP plus $NaHCO_3$ and Na-OPP in females. The combined treatment with OPP and $NaHCO_3$ increased urine volume compared to each single chemical treatment. Osmolality of the urine was generally high in groups for which urine volume were low (Table I). However, the $NaHCO_3$ group did not show increased urine volume, as previously observed.[22]

Values for pH at week 8 were 6.39 and 6.69 for OPP and 7.06 and 6.81 for Na-OPP in male and female rats, respectively. The pH levels were significantly increased by $NaHCO_3$ in OPP-treated animals and decreased by NH_4Cl in the Na-OPP groups to the values in $NaHCO_3$- or NH_4Cl-alone group in both sexes. There was no significant difference in pH level for each treatment between the sexes (Table I).

Urinary electrolytes Results regarding urinary electrolytes are also summarized in Table I. Sodium ion levels were higher in males than in females in all groups except for the NH_4Cl and no treatment groups. Sodium ion values were highest in the groups treated with $NaHCO_3$

Table I. Urinalysis Data for Rats Fed OPP or Na-OPP in Combination with $NaHCO_3$ or NH_4Cl

Treatment	No. of rats	pH	Volume of urine (ml/18 h)	Osmolality (mOsm/kg H_2O)	Electrolytes (mEq/liter)			
					Na^+	K^+	Ca^{2+}	Cl^-
Male								
OPP + $NaHCO_3$	5	8.39±0.07[a]	7.4±1.6[b]	1271±213	404±59[a]	88±13[a]	3.3±0.8	132±18[c]
OPP	5	6.39±0.05[b,d]	4.3±1.0[e]	1497±224	164±12[a,d]	184±12[d]	3.5±0.4	156±64
$NaHCO_3$	5	8.55±0.10[a,f]	5.2±1.2[f]	1430±178	354±81[b]	130±25[b,f]	6.0±1.9[f]	136±46
Na-OPP + NH_4Cl	5	6.05±0.11[a]	6.4±1.0[b]	1205±165	123±79	113±15[a]	5.0±0.9	247±28
Na-OPP	5	7.06±0.15[d]	5.2±0.6	1035±167[c]	170±42	110±30[b]	4.6±0.8	125±45[c,d]
NH_4Cl	6	5.91±0.22[a]	5.7±0.8[c]	879±290[d]	38±23[a]	117±41[b]	6.5±0.8[c,f]	206±66
No treatment	5	7.00±0.28	4.0±1.2	1536±308	125±9	197±33	4.8±1.3	201±48
Female								
OPP + $NaHCO_3$	6	7.94±0.14[a]	5.6±1.0[c]	1165±240	290±50[a]	82±26[c]	6.0±1.3	122±20[b]
OPP	5	6.69±0.22[d]	5.0±1.1	967±107	127±16[d]	113±15[f]	8.4±2.8	133±14[c]
$NaHCO_3$	5	8.52±0.08[a,d]	3.6±1.0[f]	1471±380	313±42[a]	99±10	9.7±0.6[d]	139±35
Na-OPP + NH_4Cl	5	5.93±0.05[b]	4.8±1.1	1074±205	108±26[c]	107±21	8.4±1.9	206±52
Na-OPP	5	6.81±0.17[d]	6.2±1.1[e]	953±216	143±55	97±25	7.0±1.6	118±34[c,f]
NH_4Cl	5	5.89±0.13[a]	4.5±0.5	1187±366	82±30[b]	128±40	10.3±4.3	216±58
No treatment	5	6.81±0.32	4.1±1.0	1326±352	155±35	143±41	8.7±2.9	167±26

Data are mean±SD values.
Significantly different from no treatment group at: *a*, $P<0.001$; *b*, $P<0.01$; *c*, $P<0.05$.
Significantly different from combined treatment group at: *d*, $P<0.001$; *e*, $P<0.01$; *f*, $P<0.05$.

Table II. Urinary Metabolites in Rats Fed OPP or Na-OPP in Combination with $NaHCO_3$ or NH_4Cl

Treatment	No. of rats examined	Urinary metabolites (nmol/ml)		
		OPP	PBQ	PHQ
Male				
OPP + $NaHCO_3$	5	13.1±5.5	0.0±0.0	32.6±20.6
OPP	5	103.1±92.2	10.2±2.2[a]	296.4±214.3
Na-OPP + NH_4Cl	3	59.5±79.7	2.2±3.8	85.7±64.2
Na-OPP	4	74.4±116.6	6.6±7.5	224.9±320.1
Female				
OPP + $NaHCO_3$	5	8.3±4.2	0.0±0.0	28.2±14.8
OPP	5	10.3±5.5	8.2±2.5[a]	40.1±20.9
Na-OPP + NH_4Cl	4	32.9±26.4	0.0±0.0	73.3±24.0
Na-OPP	4	13.3±6.8	2.8±5.5	36.7±26.4

Data are mean±SD values.
a) Significantly different from combined treatment group at $P<0.01$.

with or without OPP in both sexes. In the Na-OPP alone groups, sodium concentration was elevated slightly over control values in males but not in females. However, the levels were much lower than in the $NaHCO_3$-treated groups even for males. The depressive effect of NH_4Cl on sodium ion level was also less pronounced in females. Changes in potassium ion levels were generally opposite to those for sodium, decreases being observed in all treatment groups.

Calcium ion level did not appear to be clearly related to treatments, the highest levels being obtained in both sexes treated with NH_4Cl. $NaHCO_3$ treatment increased calcium ion levels in both sexes. Chloride ion levels were increased by NH_4Cl administration and decreased by the other treatments.

Urinary metabolites Urinary non-conjugated metabolites of OPP were examined only in the OPP- and Na-OPP-treated groups. Marked differences in concentra-

Table III. Hyperplastic Changes in the Urinary Bladders of Rats Fed OPP or Na-OPP in Combination with $NaHCO_3$ or NH_4Cl

Treatment	No. of rats	Normal	Simple hyperplasia		Papillary or nodular hyperplasia		Significant levels[a]			
			Mild	Severe	Mild	Severe	vs. no treatment[b]	vs. combined treatment[b]	vs. OPP[b]	vs. female
Male										
OPP+$NaHCO_3$	5	0	0	0	1	4	$P<0.01$	*b*)		—
OPP	5	5	0	0	0	0	—	$P<0.01$	*b*)	—
$NaHCO_3$	5	1	3	0	1	0	—	$P<0.01$		—
Na-OPP+NH_4Cl	5	5	0	0	0	0	—	*b*)		—
Na-OPP	5	0	2	2	1	0	$P<0.01$	$P<0.01$	$P<0.01$	$P<0.01$
NH_4Cl	6	6	0	0	0	0	—	—		—
No treatment	5	5	0	0	0	0	*b*)			—
Female										
OPP+$NaHCO_3$	6	0	0	2	3	1	$P<0.01$	*b*)		
OPP	5	5	0	0	0	0	—	$P<0.01$	*b*)	
$NaHCO_3$	5	5	0	0	0	0	—	$P<0.01$		
Na-OPP+NH_4Cl	5	5	0	0	0	0	—	*b*)		
Na-OPP	5	5	0	0	0	0	—	—	—	
NH_4Cl	5	5	0	0	0	0	—	—		
No treatment	5	5	0	0	0	0	*b*)			

Each animal is tabulated in the column of the most advanced lesion present in the urinary bladder as determined by histological examination.

a) Significancy by the cummulative chi-square method in 2×5 contingency tables.[21]

b) Group against which statistical significancy is examined. —; Not significant ($P \geqq 0.05$).

tions of the metabolites among individual animals were observed, and the standard deviations were very large as shown in Table II, which summarizes the quantitative data. Levels were generally much higher in males than in females for all three metabolites examined.

The OPP level in males was decreased from 103.1 to 13.1 nmol/ml by co-administration of $NaHCO_3$, the drop being significant even if the difference in urine volume was taken into consideration. In females, the highest level of OPP was obtained in the Na-OPP plus NH_4Cl group. PBQ was not detectable in any animals treated with OPP plus $NaHCO_3$ in both sexes. However, OPP treatment alone resulted in nmol/ml values of 10.2 in males and 8.2 in females, the respective levels for Na-OPP being 6.6 and 2.8. PBQ was also not detectable in the female Na-OPP plus NH_4Cl group. Thus, PBQ was excreted only in small amounts even in male rats, and was conspicuously absent in the male group treated with OPP plus $NaHCO_3$ which showed the highest potential for induction of urothelial hyperplasia. The PHQ metabolite showed the highest concentrations among the 3 metabolites tested in both sexes irrespective of the treatment, the decreasing or increasing tendencies mimicking those observed for OPP levels.

Histopathological changes Hyperplastic and preneoplastic bladder epithelial lesions were histologically classified into simple hyperplasia and papillary and/or nodular (PN) hyperplasia as described previously.[23] Each lesion was further classified into mild and severe based on the height of urothelial thickening and the numbers in the PN hyperplasia case.

The histopathological findings are summarized in Table III. Each animal is tabulated in the column of the most advanced lesion present in the urinary bladder as determined by histopathological examination for the purpose of statistical analysis with the chi-square test in a 2×5 table. In males, the most advanced lesions in terms of hyperplasia development were observed in the OPP plus $NaHCO_3$-treated group: severe PN hyperplasia was induced in 4 of 5 rats and mild PN hyperplasia was found in one rat. In the other groups, some PN hyperplasia and simple hyperplasia were induced in the Na-OPP and $NaHCO_3$-treated groups. In females, hyperplastic lesions were only induced in the OPP plus $NaHCO_3$ group, where PN hyperplasia was induced in 4 rats, 1 with severe and 3 with mild PN hyperplasia. Numbers of PN hyperplasia per 10 cm^2 of the basement membrane (BM) are shown in Table IV. In both sexes, OPP plus $NaHCO_3$

Table IV. Number of PN Hyperplasia

Treatment	No. of rats	No./10 cm BM
Male		
OPP+$NaHCO_3$	5	12.0±6.8[a]
OPP	5	0
$NaHCO_3$	5	0.38±0.85
Na-OPP+NH_4Cl	5	0
Na-OPP	5	0.42±0.94
NH_4Cl	6	0
No treatment	5	0
Female		
OPP+$NaHCO_3$	6	4.50±3.67[b]
OPP	5	0
$NaHCO_3$	5	0
Na-OPP+NH_4Cl	5	0
Na-OPP	5	0
NH_4Cl	5	0
No treatment	5	0

a) Significantly different from all other groups at $P<0.05$.
b) Significantly different from no treatment and male at $P<0.05$.

showed the highest values, the levels being much greater in males (12.0 against 4.5 per 10 cm BM in females). Na-OPP induced only 0.42 PN hyperplasias per 10 cm BM even in males. OPP induced no proliferative lesions in either sex. Papillomas and carcinomas were not observed in the present study.

In the kidney, mild calcification was observed in control female rats, and this lesion was severe in other groups except for NH_4Cl-treated groups, which showed a similar degree to the control. Such renal calcification was not observed in males, and may have reflected the levels of urinary Ca^{2+} concentration.

DISCUSSION

In experimental urinary bladder carcinogenesis, male rats are usually more susceptible to carcinogenic and promoting agents,[24,25] and this is also applicable to Na-OPP and OPP.[6,7] Na-OPP is stronger in terms of carcinogenic or promoting activity than OPP.[8,9] An earlier dose-response study using 2.0, 1.0, 0.5 and 0% in males and 2.0 and 0% in females revealed similar carcinogenic activity for 2.0% Na-OPP in females to that achieved with 0.5% in males.[12]

In the present experiment, urothelial hyperplastic changes were not observed with OPP alone in either sex and equimolar Na-OPP induced mild PN hyperplasia or simple hyperplasia only in males. $NaHCO_3$ treatment, while itself inducing mild hyperplasia limited to males in the present study, when combined with OPP caused the development of advanced urothelial hyperplasia in both sexes and particularly in males. Proliferative effects of $NaHCO_3$ on the bladder urothelium have also been reported.[22,26] Thus, the induction of hyperplastic changes in the urothelium in the present 8-week study was essentially comparable to the results obtained in long-term experiments and earlier data for combined effects of $NaHCO_3$ or NH_4Cl with OPP-related chemicals.[19]

The mechanism underlying the urinary bladder carcinogenicity of these apparently non-genotoxic chemicals in animals may involve altered urine pH and electrolytes concentrations,[19,20] urinary stone and crystal formation[9] and urinary excretion of possible active metabolites.[13-15,17] Fujii *et al.*[19] reported enhancing effects of $NaHCO_3$ co-administration on OPP tumorigenicity and prevention of Na-OPP tumorigenicity by NH_4Cl co-administration in male rats, concluded that Na-OPP was more carcinogenic than OPP because of its higher alkalinity. The importance of elevated pH in association with increased urinary sodium ion concentration in promotion has been stressed by Fukushima and his colleagues, not only for OPP[20] but also for ascorbate and other chemicals.[22,26-28] Similar involvement in the hyperplastic response of the urinary bladder epithelium was also reported for the sodium salt of saccharin,[29] and in modification of N-butyl-N-(4-hydroxybutyl)nitrosamine urinary bladder carcinogenesis by $NaHCO_3$ or K_2CO_3.[30,31] These studies were commonly performed in male rats and it was usually noted that urine alkalinity and high sodium content, while themselves not absolute determining factors, could possibly be associated with altered general conditions,[19] urine volume,[22,30,32] stone or crystal formation[6,26,33] or altered charge in particular molecules.[34] In the present experiment, changes in pH and sodium ion concentration were positively correlated with proliferative findings in males, but not in females. Kidney lesions were not correlated with the urinary bladder proliferative changes. Thus, the sex difference in hyperplastic response could not be explained on the basis of urinary alkalinity and sodium ion level alone.

Urinary excretion levels of non-conjugated forms of OPP metabolites have been reported to be related to relative tumor-inducing potential.[12,17] The urinary levels of PBQ were about 1/10 to 1/80 of PHQ or OPP values after 5 months' feeding of Na-OPP in males: 12.7, 13.1 and 17.8 nmol PBQ/ml and 171.6, 574.5 and 1506.6 nmol PHQ/ml for 0.5, 1.0 and 2.0% Na-OPP, respectively. In females, PBQ concentration was 9.6 nmol/ml and PHQ was 62.3 nmol/ml after 5 months' administration of 2.0% Na-OPP.[17] The results in the present exper-

iment after 8 weeks were similar to those from the earlier feeding study. Although present at only low urinary concentration, PBQ has been suspected to be a metabolite playing a major role in urinary bladder carcinogenesis, since, when injected into the urinary bladder, it is more effective than Na-OPP or PHQ with regard to induction of epithelial injury, subsequent hyperplasia and induction of DNA single-strand breakage.[16-18] Furthermore, synergism with sodium saccharin promotion was exerted only by PBQ, resulting in development of bladder preneoplastic lesions with the two-stage protocol applied.[18] Although conjugated forms of OPP metabolites were not examined in any of the studies, they are commonly regarded as being biologically inactive.[13] The available data demonstrate a good correlation between bladder tumor incidence and urinary concentrations of OPP, PBQ and PHQ,[12, 17] strongly suggesting that these non-conjugated metabolites are mainly responsible for Na-OPP urinary bladder carcinogenesis. However, in the present study no correlation between their levels and induction of urothelial hyperplasia was apparent. Of particular interest, PBQ was undetectable in animals treated with OPP plus $NaHCO_3$, in which the most advanced proliferative lesions were observed. It is not yet clear if any of the urinary changes described above are critical to this increased cell proliferation.

As other possible mechanisms underlying the response differences between sexes to various chemical forms of OPP, the following deserve mention: excretion of other types of metabolite-like conjugated forms; altered function of ion-exchange pump systems in the membrane; generation of oxygen radicals; and formation of tiny crystals. The recent discovery of silicate crystals in the urine of rats fed sodium saccharin raises another provocative mechanistic hypothesis and may be relevant to other similar chemicals.[35] Acid pH tends to inhibit the formation of urinary silicate crystals in rats, though it increases with increasing protein and sodium concentration.[36, 37] Since it is well known that male rats have considerably more urinary protein than females and also most other species including man,[38] silicate crystals, which appear to increase the numbers of urothelial cells exfoliating into the urine with consequent mild, focal regenerative hyperplasia, may be involved in OPP carcinogenesis, especially in males.

It is widely recognized that cell proliferation plays important roles in the carcinogenic process with regard to both fixation of altered genetic information and as a general stimulus to tumor development.[39, 40] It has also been found to exert profound effects in the bladder,[41] a particularly strong promoting influence being reported for uracil-induced urolithiasis.[42] Although crystals or stones have not been observed in Na-OPP- or OPP-fed animals, possible formation of microscopic particles, such as silicate crystals, clearly deserves investigation.

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