

Studies on the Teratogenicity of Food Additives (3)

Teratogenic Effect of Dietary Salicylic Acid in Rats

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Salicylic acid was administered to pregnant rats at levels of 0.06, 0.1, 0.2 and 0.4% in diet from the 8th to 14th day of gestation, and its teratogenic effects were examined.

A temporal body weight loss with toxic symptoms such as salivation, piloerection following the administration was observed in 0.4% group, and also high mortality and growth retardation in fetuses were noticed. Not any toxic signs were observed in other three lower dose groups, but a significant growth retardation was noticed in fetuses of 0.2% group. Some kinds of anomalies with considerably high frequency were occurred in both groups of 0.4 and 0.2%, but not in the low dose groups below 0.1%.

In the postnatal observation, in 0.4% group only 6 newborn were obtained alive from one dam and all died within 1 day after birth. Among low dose groups below 0.2%, no marked difference in litter size and in weaning rate at 8 weeks after birth and no evidence of growth retardation were obtained. In the postmortem examination, neither external nor internal organ anomalies were found in all groups, but minor changes in cervical bone occurred in some pups of 0.2% group.

These findings support a view that salicylic acid acts as embryotoxic and teratogenic in rat, even after administration by dietary route as well as forced oral administration.

A number of investigations¹¹⁻¹⁴⁾ have been carried out on the teratogenicity of the compounds related to salicylic acid, since Warkany and Takacs¹⁵⁾ reported the teratogenic properties of methyl and sodium salicylates in rats.

Kimmel *et al.*¹⁶⁾ showed that salicylic acid reached the embryo as major metabolite after oral administration of carboxyl-¹⁴C-acetylsalicylic acid to pregnant rats, and they assumed that salicylic acid might be a causative agent in aspirin teratogenesis. In fact, Koshakji and Schulert¹⁴⁾ observed that when salicylic acid was given subcutaneously at a dose of 380 mg/kg to rats on the 9th day of gestation, fetal resorption and anomalies were produced. However, few data in connection with the teratogenicity of salicylic acid by oral administration is available, except experiments done by forced oral administration of salicylic acid and the teratogenic effect of the agent was

compared with those of acetylsalicylic acid¹⁷⁾.

The present work was then performed to examine whether the oral teratogenic effects of salicylic acid can also be produced in rats after ingestion of the agent with food.

Materials and Methods

The animals employed were a closed colony of Wistar strain rat supplied by Nihon Rat Co. Ltd., Tokyo. Animals were housed individually in an animal room kept under constant temperature of $25 \pm 2^\circ\text{C}$ and humidity of 50~60% and fed on a basal diet (NMF, manufactured by Oriental Yeast Co., Tokyo) and given tap water *ad libitum*. Nulliparous female rats of more than 12 weeks of age weighing about 200 g were mated with male overnight, and in the next morning those with vaginal plug or sperm in the vagina were taken to be day zero of pregnancy.

Salicylic acid diet was prepared to contain 0, 0.06, 0.1, 0.2 and 0.4% of the agent in the basal diet and 20 pregnant rats in each group fed the diets *ad libitum* for 1 week from the 8th to 14th day of gestation. Net amounts of salicylic acid ingested daily were calculated on their daily food consumptions.

On the 20th day of gestation, 15 of 20 animals in each group were sacrificed for fetuses examination. The fetuses were removed from dam under ether anesthesia. After gross observation, fetuses were divided into two groups. One was fixed in a 70% alcohol solution for clearing bones and then stained with alizarine red S⁽¹⁸⁾ for examination of skeletal bone anomalies. The other was fixed in a 10% neutral formalin solution for 2 weeks and internal organ anomalies were examined by the method of Wilson⁽¹⁹⁾.

Residual 5 dams in each group were allowed to give birth. The offspring were weaned on the 21st day after birth and were autopsied

on the 56th day for examinations of visceral and skeletal anomalies. During this period general appearance, behavior and survival were examined daily and the body weights were recorded every 3 days.

For the determination of salicylic acid concentration in fetuses and maternal organs, 5 pregnant rats were fed the diet containing 0.2% salicylic acid for one week from the 8th to 14th day of gestation. The animals were sacrificed on the last day of feeding and salicylic acid was determined fluorometrically by the method of Veresh *et al.*⁽²⁰⁾. One ml of serum or 2 ml of homogenized organ and fetus samples were acidified by 2 ml of a 2N hydrochloric acid solution and then 7 ml of chloroform was added and extracted. After centrifugation for 10 minutes at 3000 rpm, 3 ml of a 0.1 N sodium hydroxide solution was added to an aliquot of 5 ml of chloroform layer and shaken and centrifuged. An aliquot of 2 ml of aqueous layer was diluted with 4 ml of a 0.2 M sodium

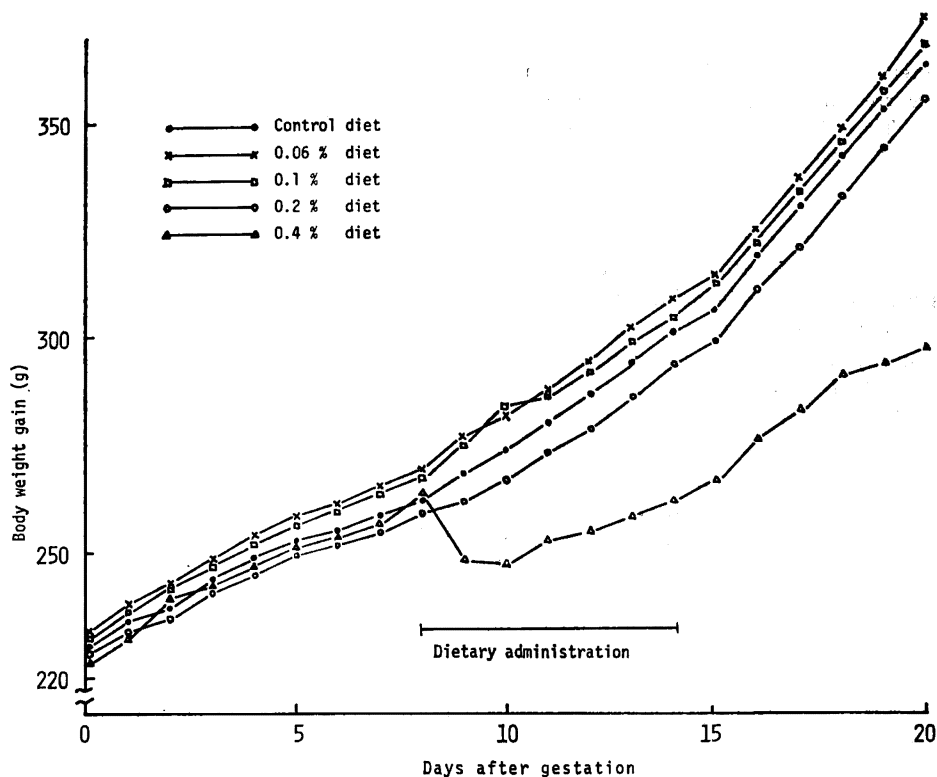


Fig. 1. Effect of dietary salicylic acid on body weight gain in pregnant rats

phosphate buffer solution (pH 11.0) and shaken and centrifuged. The fluorescence strength of an aliquot of 4 ml of the solution was determined by a spectrophotofluorometer (Hitachi, Model MPE-3) at the activation and emission wavelengths of 300 and 405 m μ , respectively.

Results

1. Prenatal observation

1) Effect of dietary salicylic acid on pregnant rats

A marked body weight loss was observed in 0.4% group at the beginning of salicylic acid administration, but the gradual increase was observed after the 11th day (Fig. 1). This change was assumed to be due to a decrease in food intake (Fig. 2), but no death was observed. No marked changes were noticed in other groups.

Table 1 shows the postmortem findings obtained on the 20th day of gestation. Very low uterine weight with fetuses and significantly low placental weight were obtained in 0.4% group, but there were no marked differences

in number of corpora lutea and in rate of nidation in all groups.

2) Effect of dietary salicylic acid on embryonic development in rats

In 0.4% group, no alive fetuses were obtained in 9 of 15 dams and the average litter size was very low (Table 2). However, the litter size in other low dose groups was almost similar to that of the control and remarkable difference among these groups in mortality was not found, but in higher two dose groups a tendency of retardation in the body weight and body and tail lengths was observed.

3) Incidence of external anomalies in fetuses of rats given dietary salicylic acid

Table 3 shows the occurrence of anomalies manifested in alive fetuses. No anomalies were found in 0.06 and 0.1% groups as in the control. Some kinds of anomalies were observed in 0.2% group and a high incidence of changes and severe anomalies were observed in 0.4% group. In this group the complicated anomalies were developed. Among them, cranioschisis,

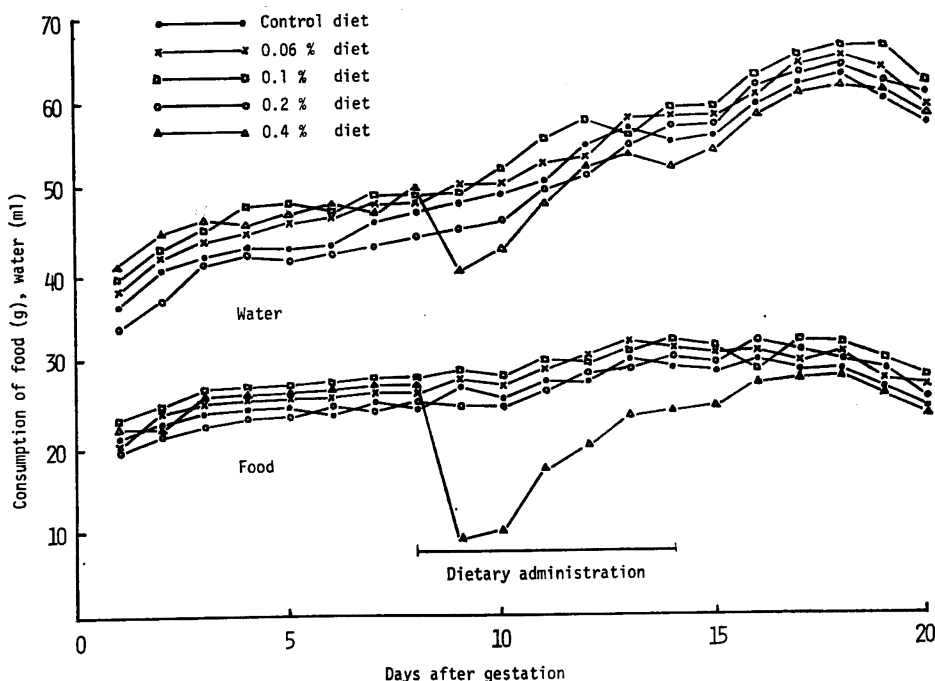


Fig. 2. Effect of dietary salicylic acid on food and water consumptions in pregnant rats

Table 1. Effect of Dietary Salicylic Acid on Pregnant Rats

Concn. of salicylic acid in diet (%)	0	0.06	0.1	0.2	0.4
Daily salicylic acid intake (mg/kg) [†]	0	50.7±0.6	77.4±1.0	165.4±2.1	205.9±18.9
No. of dams	15	15	15	15	15
Uterus weight (g) [†]	62.6±4.9	64.4±2.4	67.0±5.0	54.3±3.2	18.0±5.9**
Placental weight (g) [†]	0.44±0.01	0.42±0.01	0.41±0.01*	0.43±0.01	0.36±0.01**
No. of corpora lutea [†]	14.3±0.7	13.9±0.4	14.8±0.7	13.9±0.6	14.2±0.5
No. of implantation [†]	12.3±0.7	12.3±0.4	12.8±1.0	11.2±0.7	12.5±0.9
Rate of nidation (%)	86.1	88.1	86.5	80.4	88.0

[†]: Mean±S.E. *,** Significant from the control at 5% and 1% level

Table 2. Effect of Dietary Salicylic Acid on Embryonic Development in Rats

Concn. of salicylic acid in diet (%)	0	0.06	0.1	0.2	0.4
No. of dams	15	15	15	15	15
Alive fetuses					
No. of animals	172	178	190	160	54
Litter size [†]	11.47±0.96	11.87±0.49	12.71±0.92	10.67±0.65	3.61±1.39**
Body weight (g) [†]	3.46±0.03	3.47±0.02	3.47±0.02	3.11±0.04**	2.24±0.04**
Body length (cm) [†]	3.86±0.01	3.86±0.01	3.87±0.01	3.75±0.02*	3.21±0.03**
Tail length (cm) [†]	1.31±0.01	1.32±0.01	1.26±0.01*	1.29±0.01*	1.14±0.01**
Dead fetuses					
No. of animals	13 (7.0)	6 (3.2)	2 (1.0)	8 (4.8)	134 (71.2)
Resorption	2 (1.1)	1 (0.5)	1 (0.5)	1 (0.6)	45 (23.9)
Placental remnants	11 (5.9)	5 (2.8)	1 (0.5)	7 (4.2)	59 (31.4)
Implantation sites	0	0	0	0	30 (15.9)

[†]: Mean±S.E. *,** Significant from the control at 5% and 1% level. Numerals in parentheses indicate the ratio (%) to no. of implantations.

Table 3. Incidence of External Anomalies in Fetuses of Rats Given Dietary Salicylic Acid

Concn. of salicylic acid in diet (%)	0	0.06	0.1	0.2	0.4
No. of fetuses examined	172	178	190	160	54
No. of fetuses with anomalies	0	0	0	6	16
Occurrence ratio (%)	0	0	0	3.8	29.6
Cranioschisis					4
Pseudomacroglossia					2
Myeloschisis				2	6
Subcutaneous hematocoele				1	
Cracked skin				2	1
Pes varus				1	8
Oligodactyly					6
Syndactyly					3
Flexure of the tail					2
Total	0	0	0	6	32

myeloschisis, pseudomacroglossia, oligodactyly of left fore limb and of both hind limbs, and pes varus of both fore limbs were combined in several fetuses. Cracked-skin of about 10 mm in length was found in flank or dorsal region of some fetuses.

4) Incidence of internal organ anomalies in fetuses of rats given dietary salicylic acid

Anomalies were found only in 0.4% group (Table 4). The fetuses with anomalies were obtained from one dam, and its anomalies were

Table 4. Incidence of Internal Organ Anomalies in Fetuses of Rats Given Dietary Salicylic Acid

Concn. of salicylic acid in diet (%)	0	0.06	0.1	0.2	0.4
No. of fetuses examined	85	85	97	78	22
No. of fetuses with anomalies	0	0	0	0	3*
Occurrence ratio (%)	0	0	0	0	13.6

* Anomalies indicate deformed and dislocated kidney and all cases were obtained from one dam in the group.

Table 5. Incidence of Skeletal Bone Anomalies in Fetuses of Rats Given Dietary Salicylic Acid

Concn. of salicylic acid in diet (%)	0	0.06	0.1	0.2	0.4
No. of fetuses examined	87	93	98	82	32
No. of fetuses with anomalies	0	0	0	12	15
Occurrence ratio (%)	0	0	0	14.6	46.8
Skull					2
Cervical vertebrae				8	12
Thoracic vertebrae				2	4
Rib				2	5
Lumber vertebrae				2	3
Sacral vertebrae					3
Caudal vertebrae				2	1
Radius					5
Total	0	0	0	16	35
No. of fetuses with 14 ribs	6	5	9	54	26
No. of fetuses with 15 ribs	0	0	0	0	3

Table 6. Effect of Dietary Salicylic Acid During Pregnancy on Offspring of Rats

Concn. of salicylic acid in diet (%)	0	0.06	0.1	0.2	0.4
No. of dams	5	5	4	5	5
Litter size†					
at birth	11.0±0.7	11.6±0.5	13.4±1.0	10.2±0.6	6*
3 weeks after birth	10.6±1.5	11.6±0.8	13.8±1.0	9.6±1.8	0
8 weeks after birth	8.4±1.9	8.4±1.4	10.3±2.2	8.2±1.8	0
Weaning rate (%)					
at 3 weeks	96.4	100	100	94.1	—
at 8 weeks	76.4	72.4	78.2	80.4	—

†: Mean±S.E. *: Youngs were obtained only from 1 dam, but all died within 1 day after birth.

manifested only in kidney. In the two cases with deformed kidney, unilateral enlargement of right kidney was observed, and both were located near to the urinary bladder. The size was about 1.5 times that of the opposite ones

and they had a transversal groove around the middle part of the organ. In the remained one case site of dislocation (left) was similar to those cited above, but was normal in size and shape.

Table 7. Effect of Dietary Salicylic Acid on Organ Weights of Male Offspring of Rats

Concn. of salicylic acid in diet (%)	0	0.06	0.1	0.2
No. of offspring examined	22	10	12	16
Body weight (g)	174.1±7.8	171.9±10.1	222.3±7.4**	187.9±6.8
Body length (cm)	15.2±0.2	15.1±0.3	17.0±0.2**	15.2±0.2
Tail length (cm)	14.4±0.2	13.6±0.3	15.7±0.2**	15.0±0.2
Carcass (g)	122.0±5.5	118.8±6.8	159.5±5.4**	132.0±4.3
Brain (g)	1.64±0.04	1.68±0.02	1.63±0.08	1.65±0.04
Pituitary (mg)	5.96±0.72	4.74±0.96	6.70±0.82	5.78±0.82
Thyroid (mg)	18.91±1.07	16.97±1.33	19.66±1.43	18.72±1.16
Thymus (g)	0.57±0.12	0.54±0.02	0.68±0.07	0.57±0.04
Heart (g)	0.88±0.03	0.89±0.04	1.05±0.04*	0.96±0.04
Lung (g)	2.01±0.14	1.61±1.01	2.22±0.19	1.56±0.08*
Liver (g)	11.43±0.60	11.55±0.76	14.23±0.61	12.01±0.51
Spleen (g)	0.57±0.03	0.72±0.05*	0.63±0.04	0.79±0.03**
Kidney (g)	2.08±0.08	0.14±0.14	2.52±0.09**	2.42±0.11*
Adrenal (mg)	42.46±1.19	42.91±2.01	49.19±2.21*	44.92±1.31
Testis (g)	1.65±0.08	1.58±0.11	1.89±0.05*	1.76±0.07

Figures in the Table represent mean±S. E. *,** Significant from the control at 5% and 1% level.

Table 8. Effect of Dietary Salicylic Acid on Organ Weights of Female Offspring of Rats

Concn. of salicylic acid in diet (%)	0	0.06	0.1	0.2
No. of offspring examined	19	21	29	25
Body weight (g)	152.4±4.5	156.5±3.7	175.6±2.4**	151.0±5.4
Body length (cm)	14.5±0.2	14.8±0.2	15.7±0.1**	14.5±0.2
Tail length (cm)	13.0±0.2	13.3±0.1	15.1±0.1**	13.7±0.3
Carcass (g)	102.8±3.3	111.7±2.5*	126.0±1.8**	104.0±3.7
Brain (g)	1.61±0.03	1.59±0.04	1.62±0.01	1.53±0.03
Pituitary (mg)	6.60±1.19	5.68±1.12	6.36±0.69	6.04±0.30
Thyroid (mg)	18.41±1.33	19.21±0.77	19.39±0.57	18.74±0.70
Thymus (g)	0.57±0.04	0.57±0.04	0.53±0.02	0.58±0.06
Heart (g)	0.74±0.01	0.78±0.03	0.82±0.03*	0.73±0.02
Lung (g)	1.49±0.11	1.63±0.08	1.55±0.09	1.48±0.06
Liver (g)	9.95±0.52	10.19±0.37	12.57±0.30**	10.51±0.52
Spleen (g)	0.51±0.02	0.55±0.02	0.55±0.02	0.58±0.03
Kidney (g)	1.76±0.06	1.77±0.05	1.81±0.02	1.77±0.06
Adrenal (mg)	46.01±1.55	49.81±1.78	59.25±1.33**	49.37±2.50
Ovary (mg)	71.02±5.01	92.72±4.57*	100.50±3.19**	79.02±4.17
Uterus (g)	0.23±0.02	0.24±0.02	0.27±0.01	0.21±0.01

Figures in the Table represent mean±S. E. *,** Significant from the control at 5% and 1% level.

Table 9. Summarized Data on Macroscopic Findings in Offspring of Rats Given Dietary Salicylic Acid

Concn. of salicylic acid in diet (%)	0	0.06	0.1	0.2	0.4
External examination					
No. of offspring examined	55	58	44	51	0
No. of offspring with anomalies	0	0	0	0	—
Occurrence ratio (%)	0	0	0	0	—
Internal organ examination					
No. of offspring examined	42	42	41	41	0
No. of offspring with anomalies	0	0	0	0	—
Occurrence ratio (%)	0	0	0	0	—
Skeletal examination					
No. of offspring examined	28	29	28	29	0
No. of offspring with anomalies	0	0	0	4*	—
Occurrence ratio (%)	0	0	0	13.8	—
No. of offspring with 14 ribs	0	0	0	5	—

* Anomalies in cervical vertebrae.

Table 10. Organ Distribution of Salicylic Acid in Pregnant Rats

Organ	Concentration, $\mu\text{g/ml}$ or g.
Serum	115.96 \pm 9.02
Brain	4.14 \pm 0.46
Liver	21.68 \pm 1.37
Kidney	60.89 \pm 4.87
Adrenal	28.06 \pm 3.12
Ovary	35.23 \pm 1.05
Uterus	28.10 \pm 1.54
Placenta	27.37 \pm 1.80
Fetus	13.86 \pm 1.20
Amniotic fluid	12.35 \pm 0.72

Five pregnant rats fed *ad libitum* a diet containing 0.2% of salicylic acid for one week from the 8th to 14th day of gestation. The animals were sacrificed on the last day of feeding, and salicylic acid in organs was determined fluorometrically. Figures in the Table represent mean \pm S.E.

5) Incidence of skeletal bone anomalies in fetuses of rats given dietary salicylic acid

As shown in Table 5, no anomalies were found in 0.06 and 0.1% groups as in the control but the fetuses with anomalies were observed in 0.2 and 0.4% groups. The anomalies were occurred most frequently in cervical bone as absence or fragmentation or conjugation. Similar anomalies were observed in costal, lumbar, sacral and caudal bones, but their

incidences were low compared with those of the cervical bone.

The fetuses with 14 ribs increased with a dose-dependent manner, and also some fetuses with 15 ribs were observed in the highest dose group.

2. Postnatal observation

1) Effect of dietary salicylic acid on postnatal development in rat's offspring

In 0.4% group only 6 newborn were obtained alive from one dam, but all died within 1 day after birth. The other 4 dams delivered no pups alive (Table 6). Among low dose groups below 0.2%, no marked difference in litter size at birth and in weaning rate at 3 weeks after birth was observed. At 8 weeks after birth, the weaning rate was above 70% in all groups and there were no evidences of growth retardation in body weight, body length and tail length.

Their organ weights are shown in Tables 7 and 8. Some changes in several organs were obtained but these changes were not dose dependent.

2) Incidence of anomalies in offspring of rats given dietary salicylic acid

Table 9 shows the summary of the results obtained in the terminal stage of experiment at 8 weeks after birth. Neither external nor internal organ anomalies were found in all groups examined, but in skeletal bone exami-

nation youngs with minor change (shortening in pterygoid process) in cervical bone were found in 0.2% group, and also youngs with 14 ribs were observed in the same dose group but none with 15 ribs as observed in the fetal examination.

3. Organ distribution of salicylic acid in pregnant rats

As shown in Table 10, the highest concentration of salicylic acid in dams was found in serum and the lowest in brain. Kidney showed a little high level and the levels in other organs were almost same including placenta. The levels in fetuses and amnionic fluid were lower than those obtained in maternal organs and placenta.

Discussion

It is clear from the results obtained in the present and previous experiments¹⁷⁾ that salicylic acid through oral route has a teratogenic effect on rat as salicylic acid derivatives^{11)~15)}.

When a dose of salicylic acid was administered orally through a stomach tube or diet during the critical period of fetal organ development of rat, various anomalies were produced in fetuses. With a higher dose, embryonic death in early stage of development was observed frequently. However, the frequency of anomalies manifested by dietary administration was less than those by forced oral administration, despite dietary daily dose was almost equivalent to force-fed dose.

No information is available with regard to the teratogenic effect of oral administration of salicylic acid, though there are some reports on other salicylates^{11), 16), 19)}. Obbink and Dalderup¹¹⁾ reported that a high death rate was induced in fetuses of mice or rats received a dietary aspirin at a dose of 1.2 or 0.6 g/kg/day from the 6th to last day of pregnancy. Baba *et al.*¹⁶⁾ observed various types of anomalies in rats after oral administration by a stomach tube with 100~660 mg/kg doses of acetyl or phenyl salicylate on different days of gestation. The present results are well in accord with these findings. Moreover, general hydrops have been observed in fetuses of rat received orally 330~660 mg/kg of aspirin on the later stage of pregnancy from the 14th to 17th day of

gestation¹⁹⁾. Similar findings were also obtained in our laboratory with rats given oral salicylic acid (unpublished data). It seems to provide further evidences that salicylic acid has an untoward effect on rat's fetuses with the sublethal doses to dams.

Koshakji and Schulert¹⁴⁾ reported that none of the metabolites of salicylic acid produce congenital anomalies. The teratogenic effects of salicylic acid may be attributable to a direct action of the compound on fetal tissues as relatively well distribution was found in fetus and amnionic fluid in the present experiment. Though the levels of the compound were sustained about as one half as found in the placenta, this result will indicate that salicylic acid penetrates into the fetus considerably well across the placental barrier.

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