Effects of Liver-Supplemented Food on the Development of Embryos in Mice

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We examined whether dietary intake of cattle liver-supplemented food induces reproductive effects in dams and developmental effects in embryos in the mouse model. Seven groups of 19 to 35 female mice each were given either powdered food or the food supplemented with crude liver homogenate, its lipophilic component, the defatted liver homogenate or vitamin A (retinol palmitate) during a 25-d period spanning from a week prior to mating to gestation day 18 (GD18). Fetal mortality and incidence of external abnormalities of the fetuses whose dams were given the diet supplemented with the crude liver homogenate increased dose-dependently with an increase in the supplemented amount of the crude liver homogenate. On the other hand, the defatted liver homogenate did not induce any reproductive or teratological effect. The vitamin A (VA)-supplemented food (950 IU/5 g food as VA) induced approximately the same levels of the incidence of total external abnormalities appearing at the same affected regions or organs as the foods supplemented with the 700 mg crude liver homogenate (1029 IU/5 g food as VA) and its lipophilic component (950 IU/5 g food as VA). The content of VA (as 1029 IU/5 g food) in the crude liver homogenate was found to be approximately equal to that in the lipophilic component (950 IU/5 g food as VA). Therefore, it was concluded that VA plays an important role in induction of the lethal and teratogenic effects in the fetuses whose dams were given the powdered foods supplemented with the crude liver homogenate and its lipophilic component.

Key words fetal abnormality; liver; vitamin A; food supplement; retinol palmitate

It has been reported that about 50—75% of pregnant women suffer from anemia of the iron deficiency type[1—3] and that severe anemia during pregnancy causes serious consequence in the fetuses including prematurity, spontaneous abortions, low birth weight and fetus deaths.[3,4] Supplementary intake of iron has been used for improvement of the anemia. However, the iron supplement produced some deleterious effects including digestive disturbances. Alternatively, food containing liver has been generally recommended for the supply of iron, because the liver is rich in iron.[5,6] Although the liver is known as one of the best foods for a nutritional iron supplement, the supplementary intake of vitamin A (VA)-enriched liver might lead to excessive VA intake, resulting in some deleterious effects. Though VA is essentially important for growth, visual sensation, normal reproduction and so forth, the American College of Obstetricians and Gynecologists recommended in 1992 that due caution be taken concerning excessive VA intake for pregnant women,[7] because of an association between the use of high doses of vitamin A during pregnancy and birth defects. The excessive VA intake was reported to produce abnormalities of the skull neural crest in humans given 10000 IU/d or more during pregnancy,[8] and craniofacial dysmorphogenesis in mice given 10000 IU on GD8.7,[9] cleft palate and limb defects in mice given 100 mg/kg/d (333000 IU/kg/d) on GD11.[10] It was also reported that VA-supplemented food ($1.0—1.5\times10^6$ IU/kg) produced a high incidence of external and skeletal abnormalities in mouse fetuses.[11]

Since reproductive and teratological effects associated with the supplementary intake of foods containing liver have not been clarified by bioassay animal studies, the present study was undertaken to examine whether dietary administration of liver-supplemented food to female mice induces any hazardous effects on development of the fetuses, such as reproductive and teratological effects.

MATERIALS AND METHODS

Animals ICR mice of both sexes, 6 weeks of age, were purchased from Sankyo Labo Service (Tokyo, Japan). The animals were acclimatized in a laboratory room of the Showa Pharmaceutical University animal colony for at least one week prior to the start of the experiment. After the acclimatization period, seven groups of 19 to 35 female mice were selected for the study on the basis of body weight and lack of disease or injury. The animals were housed in polycarbonate cages with hardwood chip bedding in a room with temperature and relative humidity maintained at 23 ± 1 °C and 55 ± 5%, respectively. Fluorescent lighting was controlled automatically to provide a 12 h light (8:00—20:00)/dark (20:00—8:00) cycle. The animals were allowed free access to assigned powdered diets and sterilized water. All animals were treated in accordance with the Guidelines for Animal Care and Use published by the National Research Council.[12]

Food Preparation Control animals were fed powdered food (F-2, Funabashi Farm, Chiba, Japan). Each experimental group was given the same powdered food mixed with a different component of powdered liver homogenate or VA. The crude liver homogenate was prepared as follows: marketed cattle liver was cut into small pieces, added to an equal volume of purified water, and homogenized at 4 °C in an ice bath. The homogenate was freeze-dried, and mixed with the powdered food. The defatted liver homogenate was prepared as follows: the crude liver homogenate was added to an equal volume of acetone, vigorously agitated and centrifuged at $400\times\boldsymbol{g}$ for 5 min. This procedure was repeated 4 times. After

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evaporating the acetone from the sediment, the dried sediment (0.237 g/g liver) was mixed with the powdered food. A lipophilic component of the crude liver homogenate was prepared as follows: after evaporating the acetone from the supernatant solution of the crude liver homogenate, the extracted lipophilic component (0.475 g/g liver) was dissolved in ethanol, and poured on the powdered food. The ethanol in the powdered food was removed by evaporation. The powdered food supplemented only with VA was prepared by mixing 5 g of the powdered food with 0.508 mg of VA (retinol palmitate; Sigma Chemical Co.). The food preparation was done under light-shielded at 4 °C, in order to prevent lipid oxidation. The powdered liver homogenate, the powdered defatted liver homogenate and the lipophilic component were preserved, until they mixed with the food, at −80 °C under N_2. The mixed foods thus prepared were stored at 4 °C in a refrigerator under N_2 and returned to normal temperature immediately before use.

Analysis of VA in the Foods The amounts of VA in the powdered food used for control animals, as well as the experimental foods supplemented with the crude liver homogenate, its lipophilic component and the defatted liver homogenate were determined by HPLC.[13] It was found in the present analysis that the powdered food used for control animals contained 137 IU of VA in 5 g of food, while the powdered foods supplemented with the crude liver homogenate (700 mg liver), its lipophilic component (700 mg liver) and the defatted liver homogenate (700 mg liver) contained 1029, 950 and 137 IU in 5 g of food including 137 IU as a base of the control powdered food, respectively.

Experimental Design The experiment was conducted on seven different groups of 19 to 35 female mice each. The feeding in all the groups was restricted to 5 g/d/mouse, which was an amount that was possible for them to eat in a day, from a week prior to the overnight mating until GD18. Animals of the control group (Group 1) were given the powdered food. Groups 2, 3 and 4 were fed the powdered food supplemented with the crude liver homogenate equivalent to 66, 200 and 700 mg of wet liver, respectively, in their daily 5 g of food. Group 5 was given the food supplemented with defatted liver homogenate equivalent to 700 mg of wet liver/5 g food. Group 6 was given the food supplemented with the lipophilic component equivalent to the crude liver homogenate of 700 mg of wet liver/5 g food. Group 7 was given the food supplemented only with VA at 813 IU/5 g food, an quantity of VA approximately equal to that contained in the crude liver homogenate of 700 mg/5 g food and its lipophilic component. The supplemented dose of crude liver homogenate equivalent to 66 mg wet liver/5 g food was considered to correspond to the recommended daily intake of liver-containing food as an iron supplement in humans. Higher doses of the crude liver homogenate were used for assessing the dose–response relationship for the reproductive and teratological effects. Female mice weighing 32—34 g were paired with males overnight and examined for a vaginal plug the morning after, and that day was counted as day 0 of pregnancy.

Teratological Examination The pregnant mice were killed by cervical dislocation on GD18 for caesarean section, and the corpora lutea, implantation sites, and the number of dead or live fetuses were counted. The live fetuses were individually weighed, and examined for their sex and external abnormalities under a stereomicroscope.

Statistical Analysis The copulation and fertility rates of the dams and the frequency of external abnormalities of live fetuses were analyzed using a Chi-square test. Other reproductive and teratological parameters were analyzed by the same statistical procedure using Bartlett's test, a one-way ANOVA and Dunnett's multiple comparison test as described by Kasai *et al.*[14]

Linear regression analysis of data for correlation between the total amount of VA and total external abnormalities was performed by the least-squares method. Statistical significance was considered at p values <0.05.

RESULTS AND DISCUSSION

Table 1 shows reproductive and developmental effects of the dams given the powdered food as a control as well as the experimental foods supplemented with the crude liver homogenate, its lipophilic component, the defatted liver homogenate and VA. There was no difference in the copulation rate among the seven groups. The fertility rates of Groups 2 and 3 were significantly decreased as compared to that of the control group, but those of the other groups were not significantly different from the rate of the control group. The effects of liver homogenate and VA on fertility have not been reported until now. Spontaneous reproductive disorders of male mice, such as azoospermia may have been the reason for the low fertility rates. Fetal mortality increased dose-dependently with an increase in the supplemented amount of the crude liver homogenate. A statistically significant, 4-fold increase in the fetal mortality was observed in Group 4 as compared with the control group. Significantly increased fetal mortality was also observed in Groups 6 and 7. It is noteworthy that the fetal mortality in the group given the food supplemented with the defatted liver homogenate (Group 5) did not increase. The mean fetal body weight per litter significantly increased in the males of Group 6 and in both males and females of Group 7 over that of the control group. There was no statistical difference in the number of corpora lutea, the number of implantations, implantation index, the number of live fetuses or the sex ratio between the control and any treated group.

Table 2 shows incidences and types of external abnormalities of the fetuses whose dams were given the powdered foods supplemented with the crude liver homogenate, its lipophilic component, the defatted liver homogenate and VA. The incidence of total external abnormalities significantly increased dose-dependently in Groups 2, 3 and 4 with an increase in the supplemented amount of the crude liver homogenate. An 8 to 9-fold increase in the incidence of total external abnormalities was observed in Groups 4, 6 and 7 as compared with the control group, whereas the incidence of total external abnormalities did not increase in Group 5 given the defatted liver homogenate. There was a significant positive correlation ($r^2=0.9903$, $p<0.01$) between the total amount of VA and the total external abnormalities (Fig. 1). Various types of external abnormalities were observed in the body, head, ear, eye, nose, mouth, jaw, limb, paw/digit, tail and trunk. The main types observed in the present study were as follows: anasarca and hematoma in the body, domed head and exencephaly (Fig. 2A), malpositioned pinna and anotia

Table 1. Reproductive and Developmental Effects of the Dams Given the Powdered Foods Supplemented with the Crude Liver Homogenate (CLH), Its Lipophilic Component (LC) and the Defatted Liver Homogenate (DLH)

Experimental group	1 Control	2 CLH 66 mg	3 CLH 200 mg	4 CLH 700 mg	5 DLH 700 mg	6 LC 700 mg	7 VA 813 IU
Total amount of VA per 5 g of food	137 IU	221 IU	392 IU	1029 IU	137 IU	950 IU	950 IU as retinol
No. of female animals purhased	30	30	35	30	30	30	30
No. of mated pairs	27	24	35	25	19	23	30
No. of copulated pairs	27	24	35	25	19	23	30
Copulation rate (%)[b]	100	100	100	100	100	100	100
No. of pregnant animals	25	16	25	24	16	19	24
Fertility rate (%)[c]	92.6	66.6*	71.4*	96.0	84.2	82.6	80.0
No. of corpora lutea	13.5±2.5[a]	12.6±1.5	14.1±2.3	14.1±2.2	12.6±2.8	14.5±1.5	13.3±2.7
Implantation sites	13.2±3.1	12.3±1.8	13.4±3.1	12.7±4.1	11.8±4.5	13.5±3.0	12.4±3.8
Implantation index (%)[d]	96.4±8.6	96.7±5.8	93.8±11.7	87.6±23.8	89.2±24.5	92.7±16.8	91.5±15.9
No. of fetal deaths	0.4±0.8	1.1±1.4	1.3±1.6	1.7±2.5*	0.4±0.8	1.5±1.0**	1.6±1.6**
Fetal mortality (%)[e]	3.7±7.2	9.2±12.6	11.1±15.3	15.1±21.9*	4.7±8.2	10.9±7.0**	16.1±20.6*
No. of live fetuses	12.7±3.3	11.2±2.4	12.1±3.5	11±4.6	11.3±4.5	12±2.8	10.8±4.3
Sex ratio[f]	1.04±0.19	1.00±0.30	0.93±0.30	0.90±0.40	1.00±0.20	0.90±0.20	0.88±0.31
Fetal body weight (g) male	1.17±0.13	1.19±0.07	1.16±0.24	1.11±0.35	1.20±0.14	1.23±0.06**	1.27±0.64**
female	1.10±0.09	1.11±0.04	1.12±0.06	1.22±0.43	1.10±0.10	1.13±0.07	1.45±0.77**

a) Values are expressed as mean per litter ±S.D. *b*) Copulation rate (%)=(No. of copulated pairs/No. of mated pairs)×100. *c*) Fertility rate (%)=(No. of pregnant animals/No. of copulated pairs)×100. *d*) Implantation index (%)=(Implantation sites/No. of corpola lutea)×100. *e*) Fetal mortality (%)=(No. of fetal death/Implantation sites)×100. *f*) Sex ratio=No. of males/No. of females. Significant difference at $p \leqq 0.05$ (*) and $p \leqq 0.01$ (**) by Chi-square and Dunnet tests.

Table 2. External Abnormalities of Fetuses Whose Dams Were Given the Powdered Food Supplemented with the Crude Liver Homogenate (CLH), Its Lipophilic Component (LC), the Defatted Liver Homogenate (DLH) and Vitamin A (VA)

Experimental group	1 Control	2 CLH 66 mg	3 CLH 200 mg	4 CLH 700 mg	5 DLH 700 mg	6 LC 700 mg	7 VA 813 IU
Total amount of VA per 5 g of food	137 IU	221 IU	392 IU	1029 IU	137 IU	950 IU	950 IU as retinol
No. of fetuses examined	318	179	302	263	181	228	260
No. of fetuses with external abnormalities	7 (2.5±5.8)	10 (5.6±7.2)*	26 (8.2±7.5)**	41 (22.9±26.8)**	7 (3.2±4.4)	36 (19.8±24.3)**	32 (22.6±32.4)**
Regions or organs in which external abnormalities were observed[a]							
General	1 (0.3)		8 (2.5)	7 (7.4)	1 (0.5)	6 (6.3)	12 (14.9)
Cranium	2 (0.9)	3 (2.4)	6 (1.8)	5 (1.6)	2 (0.9)	12 (9.5)	5 (3.0)
Ear		1 (0.5)			2 (1.0)		
Eye		1 (0.5)	1 (0.3)		2 (1.0)	2 (1.1)	
Nose				2 (0.6)			
Mouth/Jaw		1 (0.4)	1 (0.3)	5 (1.6)		2 (1.1)	
Limb			1 (0.3)	1 (0.3)			1 (0.8)
Paw/Digit	3 (1.0)	5 (3.0)	7 (2.5)	10 (3.3)	1 (0.4)	4 (2.3)	8 (2.3)
Tail	1 (0.3)	2 (1.1)	2 (0.8)	9 (3.0)	3 (1.2)	9 (4.2)	7 (3.9)
Trunk		1 (0.5)	1 (0.3)	11 (3.8)	1 (0.4)	10 (5.3)	6 (3.7)

a) Main external abnormalities ; General (anasarca, hematoma), Cranium (domed head, exencephaly), Ear (malpositioned pinna, anotia), Eye (absent eye bulge, exophthalmos), Nose (naris atresia), Mouth/Jaw (mandibular micrognathia, cleft palate), Limb (limb hyperextension), Paw/Digit (acheiria, malpositioned digit), Tail (bent tail, kinked tail), Trunk (spina bifida, short trunk). ( ): Incidence, Mean or Mean per litter±S.D. Significant difference at $p \leqq 0.05$ (*) and $p \leqq 0.01$ (**) by Chi-square test.

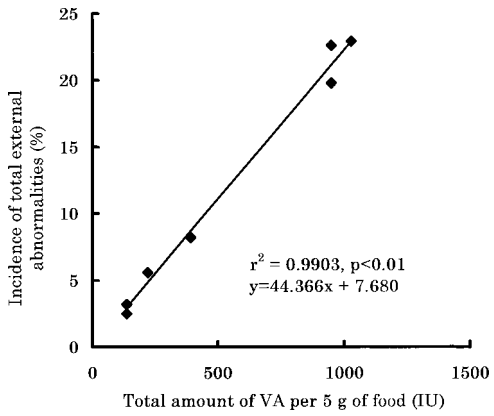

Fig. 1. Correlations between Total Amount of Vitamin A and Total External Abnormalities in Mice

The line is the least-squares regression line.

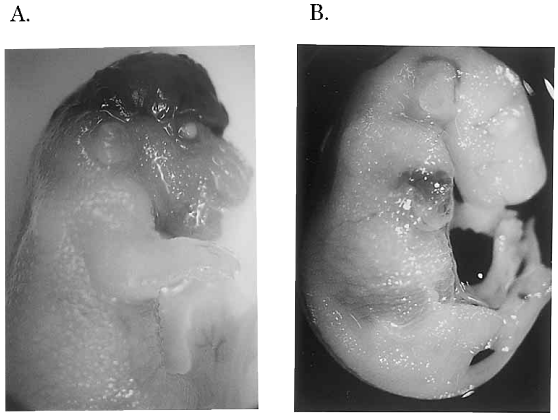

Fig. 2. Typical Major External Abnormalities of Fetuses Observed Following the Ingestion of Crude Liver Homogenate and VA

(A) Exencephaly. (B) Acheiria in the paw.

in the ears, absent eye bulge and exophthalmos in the eyes, naris atresia in the nose, mandibular micrognathiain and cleft palate in the mouth/jaw, hyperextension in the limbs, acheiria in the paw (Fig. 2B) and malpositioned digits, bent and kinked tail, spina bifida in the trunk and short trunk. It was noteworthy that the incidences of the total external abnormalities and the regions or organs in which the abnormalities were observed were similar among Groups 4, 6 and 7. In the present study, it was found that the fetal mortality and the incidence of total external abnormalities increased dose-dependently with an increase in the supplemented amount of the crude liver homogenate, and that the lipophilic component and VA alone also increased the fetal mortality and the incidence of total external abnormalities, while the defatted liver homogenate did not. These findings implicate that the lethal and teratological effects on the fetuses were induced by maternal administration of the supernatant-layered fraction of the crude liver homogenate, because the defatted liver homogenate as the sediment did not produce any positive effects. It was found in the vitamin analysis that the content of VA in the crude liver homogenate [0.268 mg (892 IU) retinol/700 mg liver] was approximately equal to that in its lipophilic component [0.244 mg (813 IU) retinal/700 mg liver]. The incidences of total external abnormalities and the affected regions or organs of the fetuses whose dams were given the crude liver homogenate were in essential agreement with those of the fetuses whose dams were given the lipophilic component. Besides, the dietary intake of the powdered food supplemented only with the same level of VA as in both the crude liver homogenate and its lipophilic component was found to bring about the increased incidence of total external abnormalities with the same affected regions or organs as observed in both the crude liver homogenate and its lipophilic component. Oral VA administration to female mice at a dose of 400 IU/d was reported to induce teratogenic effects in the fetuses.[15] However, the types of abnormalities observed were not similar to those seen in the present study. Mulder *et al.* reported that VA elicited external abnormalities in fetuses whose dams were given VA at the dose of 10 mg/kg (29000 IU/kg),[16] and the results were similar to those of the present study. Rothman *et al.* reported that ingestion of 10000 IU/d or more of VA by pregnant woman was associated with an incresed rate of fetal malformations.[8] In the present study, the total incidence of external abnormalities in mice was increased following the daily ingestion of 66 mg/d of liver or more, (equivalent to 84 IU/d of VA or more), and this liver intake could be converted into an equivalent of approximately 100 g/d (VA 127273 IU) in humans (50 kg body weight). No relation between the ingestion of liver and teratogenicity was reported in humans, although pregnant women should be advised to exercise caution while eating liver with a high VA content.

It was concluded from the present study that VA plays an important role in induction of lethal and teratological effects in the mouse fetuses whose dams received the dietary administration of liver-supplemented food.

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