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 and that severe anemia during pregnancy causes serious consequence in the fetuses including prematurity, spontaneous abortions, low birth weight and fetus deaths. Supplementary intake of iron has been generally recommended for improving the anemia of the iron deficiency type. However, the iron supplement produced some deleterious effects in the fetuses whose dams were given the powdered foods 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

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.

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×g for 5 min. This procedure was repeated 4 times. After...
eliminating the acetone from the sediment, the dried sedi-
ment (0.237 g/g liver) was mixed with the powdered food. A
lipophilic component of the crude liver homogenate was pre-
pared as follows: after evaporating the acetone from the su-
pernatant solution of the crude liver homogenate, the ex-
tracted 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 pow-
dered food supplemented only with VA was prepared by mix-
ing 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 de-
fatted liver homogenate and the lipophilic component were
preserved, until they mixed with the food, at −80 °C under
N₂. The mixed foods thus prepared were stored at 4 °C in a
refrigerator under N₂, and returned to normal temperature im-
mediately before use.

Analysis of VA in the Foods  The amounts of VA in the
powdered food used for control animals, as well as the experi-
mental foods supplemented with the crude liver ho-
rogenate, its lipophilic component and the defatted liver ho-
logenate were determined by HPLC.13) It was found in the
present analysis that the powdered food used for control ani-
mals contained 137 IU of VA in 5 g of food, while the pow-
dered 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. Ani-
mals of the control group (Group 1) were given the powdered
food. Groups 2, 3 and 4 were fed the powdered food supple-
mented with the crude liver homogenate equivalent to 700,
200 and 700 mg of wet liver, respectively, in their daily 5 g of
food. Group 5 was given the food supplemented with defat-
ted 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 ho-
rogenate 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
amount 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 cor-
respond 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–re-
sponse relationship for the reproductive and teratological ef-
facts. Female mice weighing 32—34 g were paired with
males overnight and examined for a vaginal plug the morn-
ing 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 indi-
vidually 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 re-
productive 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 signifi-
cance 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 ho-
logenate, its lipophilic component, the defatted liver ho-
logenate 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 signifi-
cantly different from the rate of the control group. The ef-
ects 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-de-
pendently 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 abnormali-
ties 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 in-
creased dose-dependently in Groups 2, 3 and 4 with an in-
crease in the supplemented amount of the crude liver ho-
logenate. 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 posi-
tive 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

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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)

<table>
<thead>
<tr>
<th>Experimental group</th>
<th>1 Control</th>
<th>2 CLH 66 mg</th>
<th>3 CLH 200 mg</th>
<th>4 CLH 700 mg</th>
<th>5 DLH 700 mg</th>
<th>6 LC 700 mg</th>
<th>7 VA 813 IU</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total amount of VA per 5 g of food</td>
<td>137 IU</td>
<td>221 IU</td>
<td>392 IU</td>
<td>1029 IU</td>
<td>137 IU</td>
<td>950 IU</td>
<td>950 IU</td>
</tr>
<tr>
<td>No. of female animals purchased</td>
<td>30</td>
<td>30</td>
<td>35</td>
<td>30</td>
<td>30</td>
<td>30</td>
<td>30</td>
</tr>
<tr>
<td>No. of mated pairs</td>
<td>27</td>
<td>24</td>
<td>35</td>
<td>25</td>
<td>19</td>
<td>23</td>
<td>30</td>
</tr>
<tr>
<td>No. of copulated pairs</td>
<td>27</td>
<td>24</td>
<td>35</td>
<td>25</td>
<td>19</td>
<td>23</td>
<td>30</td>
</tr>
<tr>
<td>Copulation rate (%)(^a)</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>No. of pregnant animals</td>
<td>25</td>
<td>16</td>
<td>25</td>
<td>24</td>
<td>16</td>
<td>19</td>
<td>24</td>
</tr>
<tr>
<td>Fertility rate (%)(^b)</td>
<td>92.6</td>
<td>66.6*</td>
<td>71.4*</td>
<td>96.0</td>
<td>84.2</td>
<td>82.6</td>
<td>80.0</td>
</tr>
<tr>
<td>No. of corpora lutea</td>
<td>13.2 ± 2.5</td>
<td>12.6 ± 1.5</td>
<td>14.1 ± 2.3</td>
<td>14.1 ± 2.2</td>
<td>12.6 ± 2.8</td>
<td>14.5 ± 1.5</td>
<td>13.3 ± 2.7</td>
</tr>
<tr>
<td>Implantation sites</td>
<td>13.2 ± 3.1</td>
<td>12.3 ± 1.8</td>
<td>13.4 ± 3.1</td>
<td>12.7 ± 4.1</td>
<td>11.8 ± 2.4</td>
<td>15.3 ± 3.0</td>
<td>12.4 ± 3.8</td>
</tr>
<tr>
<td>Implantation index (%)^c</td>
<td>96.4 ± 8.6</td>
<td>96.7 ± 5.8</td>
<td>93.8 ± 11.7</td>
<td>87.6 ± 23.8</td>
<td>89.2 ± 24.5</td>
<td>92.7 ± 16.8</td>
<td>91.5 ± 15.9</td>
</tr>
<tr>
<td>No. of fetal deaths</td>
<td>0.4 ± 0.8</td>
<td>1.1 ± 1.4</td>
<td>1.3 ± 1.6</td>
<td>1.7 ± 2.5^*</td>
<td>0.4 ± 0.2</td>
<td>1.5 ± 1.0**</td>
<td>1.6 ± 1.6**</td>
</tr>
<tr>
<td>Fetal mortality (%)^d</td>
<td>3.7 ± 7.2</td>
<td>9.2 ± 12.6</td>
<td>11.1 ± 15.3</td>
<td>15.1 ± 21.9*</td>
<td>4.7 ± 8.2</td>
<td>10.9 ± 7.0**</td>
<td>16.1 ± 20.6*</td>
</tr>
<tr>
<td>No. of live fetuses</td>
<td>12.7 ± 3.3</td>
<td>11.2 ± 2.4</td>
<td>12.1 ± 3.5</td>
<td>11.4 ± 6.1</td>
<td>11.3 ± 4.5</td>
<td>12.2 ± 2.8</td>
<td>10.8 ± 4.3</td>
</tr>
<tr>
<td>Sex ratio(^e)</td>
<td>1.04 ± 0.09</td>
<td>1.00 ± 0.30</td>
<td>0.93 ± 0.30</td>
<td>0.90 ± 0.40</td>
<td>1.00 ± 0.20</td>
<td>0.90 ± 0.20</td>
<td>0.88 ± 0.31</td>
</tr>
<tr>
<td>Fetal body weight (g) male</td>
<td>1.17 ± 0.13</td>
<td>1.19 ± 0.07</td>
<td>1.16 ± 0.24</td>
<td>1.11 ± 0.35</td>
<td>1.20 ± 0.14</td>
<td>1.23 ± 0.06**</td>
<td>1.27 ± 0.64**</td>
</tr>
<tr>
<td>Fetal body weight (g) female</td>
<td>1.10 ± 0.09</td>
<td>1.11 ± 0.04</td>
<td>1.12 ± 0.06</td>
<td>1.22 ± 0.43</td>
<td>1.10 ± 0.10</td>
<td>1.13 ± 0.07</td>
<td>1.45 ± 0.77**</td>
</tr>
<tr>
<td>Fertility rate (%)</td>
<td>96.4</td>
<td>96.0</td>
<td>96.0</td>
<td>96.0</td>
<td>96.0</td>
<td>96.0</td>
<td>96.0</td>
</tr>
<tr>
<td>Fetal mortality (%)</td>
<td>3.6</td>
<td>4.0</td>
<td>4.0</td>
<td>4.0</td>
<td>4.0</td>
<td>4.0</td>
<td>4.0</td>
</tr>
<tr>
<td>Sex ratio</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>Fetal body weight (g)</td>
<td>1.17</td>
<td>1.17</td>
<td>1.17</td>
<td>1.17</td>
<td>1.17</td>
<td>1.17</td>
<td>1.17</td>
</tr>
</tbody>
</table>

\(^a\) Values are expressed as mean per litter ± S.D. \(^b\) Copulation rate (%) = (No. of copulated pairs/No. of mated pairs)×100. \(^c\) Implantation index (%) = (Implantation sites/No. of corpora lutea)×100. \(^d\) Fetal mortality (%) = (No. of fetal death/Implantation sites)×100. \(^e\) Sex ratio = No. of males/No. of females. Significant difference at p≤0.05 (+) and p≤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)

<table>
<thead>
<tr>
<th>Experimental group</th>
<th>1 Control</th>
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<td>392 IU</td>
<td>1029 IU</td>
<td>137 IU</td>
<td>950 IU</td>
<td>950 IU</td>
</tr>
<tr>
<td>No. of fetuses examined</td>
<td>318</td>
<td>179</td>
<td>302</td>
<td>263</td>
<td>181</td>
<td>228</td>
<td>260</td>
</tr>
<tr>
<td>No. of fetuses with external abnormalities</td>
<td>7 (2.5 ± 5.8)</td>
<td>10 (5.6 ± 7.2)*</td>
<td>26 (8.2 ± 7.5)**</td>
<td>41 (22.9 ± 26.8)**</td>
<td>7 (3.2 ± 4.4)</td>
<td>16.8 ± 24.5</td>
<td>36 (19.8 ± 24.3)**</td>
</tr>
<tr>
<td>Regions or organs in which external abnormalities were observed(^f)</td>
<td>General</td>
<td>1 (0.3)</td>
<td>8 (2.5)</td>
<td>7 (7.4)</td>
<td>1 (0.5)</td>
<td>6 (6.3)</td>
<td>12 (14.9)</td>
</tr>
<tr>
<td></td>
<td>Cranium</td>
<td>2 (0.9)</td>
<td>3 (2.4)</td>
<td>6 (1.8)</td>
<td>5 (1.6)</td>
<td>2 (0.9)</td>
<td>12 (9.5)</td>
</tr>
<tr>
<td></td>
<td>Ear</td>
<td>1 (0.5)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Eye</td>
<td>1 (0.5)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Nose</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Mouth/Jaw</td>
<td>1 (0.4)</td>
<td>1 (0.3)</td>
<td>5 (1.6)</td>
<td></td>
<td></td>
<td>1 (1.1)</td>
</tr>
<tr>
<td></td>
<td>Limb</td>
<td>1 (0.3)</td>
<td>1 (0.3)</td>
<td>1 (0.3)</td>
<td></td>
<td></td>
<td>1 (0.8)</td>
</tr>
<tr>
<td></td>
<td>Paw/Digit</td>
<td>3 (1.0)</td>
<td>5 (3.0)</td>
<td>7 (2.5)</td>
<td>10 (3.3)</td>
<td>1 (0.4)</td>
<td>4 (2.3)</td>
</tr>
<tr>
<td></td>
<td>Tail</td>
<td>1 (0.3)</td>
<td>2 (1.1)</td>
<td>2 (0.8)</td>
<td>9 (3.0)</td>
<td>3 (1.2)</td>
<td>9 (4.2)</td>
</tr>
<tr>
<td></td>
<td>Trunk</td>
<td>1 (0.5)</td>
<td>1 (0.3)</td>
<td>11 (3.8)</td>
<td>1 (0.4)</td>
<td>10 (5.3)</td>
<td>6 (3.7)</td>
</tr>
</tbody>
</table>

\(^a\) Main external abnormalities: General (anasarca, hematoma), Cranium (domeled head, encephaly), 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). \(^b\) Incidence, Mean or Mean per litter ± S.D. Significant difference at p≤0.05 (+) and p≤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 micrognathia 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. In the present study, 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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**REFERENCES**