INTRODUCTION

It has been reported that nutritional changes such as restricted feeding or vitamin imbalance induced abortions and/or premature birth in pregnant rabbits (Clark et al., 1986; DiGiacomo et al., 1992; Petere et al., 1993; Matsuoka et al., 2006). In addition, it is well known that progesterone is important to maintain pregnancy and an inhibition of progesterone secretion during the perinatal period induces abortion in pregnant rabbits (Jost, 1986; González-Mariscal et al., 1994).

We have previously reported that pregnant rabbits subjected to restricted feeding of 20 g/day showed significant changes in the blood coagulation system suggesting a tendency to bleed and a clear decline in serum progesterone concentrations on gestation day 22 (GD 22), and that half of them showed blood progesterone concentrations less than 4.0 ng/ml which resulted in abortions on and after GD 23 (Matsuoka et al., 2009).

In humans and domestic animals, it is well known that malnutrition and changes in metabolic functions in dams induce intrauterine growth retardation such as inhibition of embryo-fetal and placental growth, bringing about adverse effects on the maintenance of pregnancy (Redmer et al., 2004; Wu et al., 2006; Murphy et al., 2006). Rabbits have been used in various studies as a model animal of naturally occurring intrauterine growth retardation (Buchmiller-Crair et al., 2001, Skarsgard et al., 2001; Cellini et al., 2006). However, there are a somewhat small number of reports of a relationship between
intrauterine growth retardation and the occurrence of abortion and/or structural changes in the placenta.

In this study, the relationship between maintenance of pregnancy, placental development and embryo-fetal development was examined in pregnant rabbits subjected to the restricted feeding as described in our previous report (Matsuoka et al., 2009).

MATERIALS AND METHODS

Animals
Twenty-one pregnant rabbits (New Zealand White strain, Kitayama Labs, Co., Ltd., Nagano, Japan) at 5 to 7 months of age were used. The animals were housed individually in aluminum cages (W 360 × D 550 × H 350 mm; Riko Denki Co., Ltd., Tokyo, Japan) in an air-conditioned animal room (temperature: 22.5 ± 3.5°C; relative humidity: 50 ± 20%; air ventilation: 10 to 16 times per hour; lighting: 12 hr/12 hr light/dark cycle). The animals were allowed free access to pelleted diet (RC4; Oriental Yeast Co., Ltd., Tokyo, Japan) and to water provided by an automatic water supply system (Fujimi Water Union, Shizuoka, Japan). Female rabbits exhibiting swollen and dark-purple vulva were judged to be in estrus, and were housed together with male rabbits from the same colony in circles (650 mm in diameter and 500 mm in height) for mating on a 1:1 basis. Females which copulated twice based on the cage-side observation and were positive for semen in the vagina and around the vaginal region were regarded to have successfully copulated, and the day of mating was designated as GD 0. The animal care and experimental procedures were done in accordance with the animal welfare guidelines of Bozo Research Center Inc. and carried out in accordance with the standards of the Institute of Laboratory Animal Resources Guide.

Group composition
The animals were divided into 2 groups (Fig. 1). One group, composed of 6 animals, was allowed free access to food (NT group, measured food consumption from GD 6 to GD 22) and the other group, composed of 15 animals, was subjected to restricted feeding of 20 g/day from GD 6 to GD 22 (RF group) as described in our previous report (Matsuoka et al., 2009).

Clinical signs and body weight gains
All animals were observed for clinical signs daily, and body weights were measured on GD 0, 6, 8, 10, 12, 14, 16, 18, 19 and 22.

Examination of blood coagulation-related parameters
Blood samples were obtained from each animal on GD 6, 13 and 22. Approximately 4 ml of blood was collected from the auricular artery, and 1 ml of blood was collected into tubes containing EDTA-2K (SB-41: Sysmex Corporation, Kobe, Japan, Lot No. G7012) and subjected to examination for the parameters listed in Table 1. In addition, blood samples of 2 ml collected into tubes containing 3.8w/v% sodium citrate (1 volume to 9 volumes of blood, Terumo Corporation Co., Ltd., Tokyo, Japan, Lot No. 071108B) were centrifuged (approximately 1,600 × g for 10 min) and the resulting plasma samples were examined for the parameters listed in Table 1.

Determination of serum progesterone concentrations
Serum progesterone concentrations were measured using the remaining blood sample (1 ml) by Chemiluminescent Enzyme Immunoassay using IMMULYZE (Diagnostic Products Corporation, Los Angeles, CA, USA).

Cesarean section and histological examination of placentae
On GD 23, all animals were euthanized by exsanguination from the abdominal aorta under pentobarbital

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The table and figure are shown below:

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of animals</th>
<th>Food consumption</th>
<th>CS</th>
</tr>
</thead>
<tbody>
<tr>
<td>NT</td>
<td>6</td>
<td>116~144 g</td>
<td></td>
</tr>
<tr>
<td>RF</td>
<td>15</td>
<td>20 g</td>
<td></td>
</tr>
</tbody>
</table>

**Fig. 1.** Experimental schedule. GD, gestational day; Group Non-treated was allowed free access to food throughout the experimental period of GD 0 to GD 22. Group Restricted feeding was subjected to restricted 20 g/day from GD 6 to GD 22. CS; cesarean section on GD 23.
anesthesia, and gestation status was determined by cesarean section. The ovary and uterus were excised from each animal. Then, the number of corpora lutea was counted in the ovaries, and the uterine wall was cut and the numbers of live fetuses and embryo-fetal death as well as their stage (resorbed embryo, placental remnant, macerated fetus and dead fetus) were counted and recorded. The sum of the numbers of live fetuses and embryo-fetal death was taken as the number of implantations. For live fetuses, body weight and placental volume (longer axis × shorter axis × height) were measured.

For histological examination, the placenta was fixed in 10% phosphate-buffered formalin without exfoliating from the uterus. They were embedded in paraffin, cut into 4 μm sections and then stained with hematoxylin and eosin (HE) for histological examination. Some sections were also stained by periodic acid-Schiff (PAS) staining. The thicknesses of the labyrinth zone and basal zone of the placenta and the decidual basalis and the myo-metrium were measured on HE-stained sections under the light microscope.

### Statistical analysis

The means ± standard deviations (SDs) were calculated for the parameters examined in each group, and they were first analyzed for homogeneity of variance by F test between the NT and RF groups. Then, the homogeneous data were analyzed by Student’s $t$-test and the heterogeneous data by Aspin-Welch’s $t$-test, respectively.

### RESULTS

#### Clinical signs and body weight gains

No abnormal clinical signs were observed in any group. The body weight gain during the gestation period from GD 0 to GD 22 was about 0.3 kg in the NT group while that in the RF group was about -0.2 kg (Fig. 2).

#### Changes in blood coagulation-related parameters and serum progesterone concentrations

On GD 22, the values of PLT, Fib, APTT and ATIII...
II were significantly lower while that of PT was significantly longer in the RF group than in the NT group (Table 2). In addition, the serum progesterone concentration was significantly lower in the RF group than in the NT group (Fig. 3 and Table 3).

Findings at cesarean section

The embryo-fetal death index showed a tendency to increase in the RF group, and the values of body weights and placental volumes for live fetuses were significantly lower in the RF group than in the NT group (Table 4). In a classification of embryo-fetal death, resorbed embryo, macerated fetus and dead fetus were mostly observed in the RF group (Table 5). There were no significant differences in the numbers of corpora lutea, implantations and live fetuses, and in the implantation index between the NT group and the RF group.

Animals in the RF group could be divided into 2 subgroups according to the minimum serum progesterone value of the NT group (Min = 4.0 ng/ml) on GD 22 (Table 3). In the subgroup showing a value less than 4.0 ng/ml, the embryo-fetal death index showed a tendency to increase compared with the other subgroup showing a value higher than 4.0 ng/ml and with the NT group, suggesting a relation between a decrease in progesterone concentration and an increase in embryo-fetal death index. There was no clear relation detected between the live fetus numbers, body weights or placental volumes for live fetuses and the progesterone concentrations.

Histological examination of the uterus and placenta

For the placenta, the labyrinth zone was significantly thinner and the basal zone was significantly thicker in the RF group than in the NT group (Table 6). There were no significant differences observed in the thicknesses of the decidual membrane and the myo-metrium in the uterus between the NT group and the RF group. In addition, in the RF group, many PAS-positive glycogen-containing cells still remained in the basal zone in the placenta (Fig. 4). Histological findings of the decidua membrane and myo-metrium in the uterus were similar between the NT and RF groups.

DISCUSSION

As mentioned in the Introduction, in our previous study (Matsuoka et al., 2009), restricted feeding of 20 g/day from GD 6 brought about significant changes in blood coagulation-related parameters indicating an inhibition of blood coagulation and prominent decreases in serum concentrations of progesterone, an important factor to maintain pregnancy, in pregnant rabbits on GD 22. A half of them showed serum progesterone concentrations lower than 4.0 ng/ml which resulted in abortions on and after GD 23.

In the present study, based on the above-mentioned results, the effects of restricted feeding (20 g/day from GD 6 to GD 22) on the development of on embryo / fetus and placenta on GD 23 as well as on blood coagulation-related parameters and serum progesterone concentrations on GD 22 were examined.

On GD 22, the RF group showed changes in blood coagulation-related parameters and a decrease in serum progesterone concentrations as previously reported (Matsuoka et al., 2009). The changes in blood coagulation-related parameters indicate an inhibition of blood coagulation, i.e. a tendency of bleeding, in mother and neonates (Patnaik et al., 2007), and the decrease in serum progesterone concentrations during pregnancy will be a trigger of abortion in pregnant rabbits (Fail and Reynolds, 1987).

Following cesarean section on GD 23, weights of the body and placenta were significantly lower in the RF group than in the NT group, and the embryo-fetal death index was higher in the RF group than in the NT group,

Table 2. Hematological examination, coagulation-related parameters

<table>
<thead>
<tr>
<th>GD</th>
<th>n</th>
<th>PLT (x10^4/μl)</th>
<th>PT (s)</th>
<th>APTT (s)</th>
<th>ATIII (%)</th>
<th>Fib (mg/dl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NT</td>
<td>13</td>
<td>6</td>
<td>49.4 ± 19.5</td>
<td>6.5 ± 1</td>
<td>23.6 ± 2.8</td>
<td>96 ± 8</td>
</tr>
<tr>
<td>22</td>
<td>6</td>
<td>45.4 ± 12.6</td>
<td>5.5 ± 0.7</td>
<td>31.7 ± 5.7</td>
<td>125 ± 12</td>
<td>265 ± 26</td>
</tr>
<tr>
<td>RF</td>
<td>13</td>
<td>15</td>
<td>38.3 ± 10.4</td>
<td>6.5 ± 2</td>
<td>23.6 ± 2.9</td>
<td>99 ± 14</td>
</tr>
<tr>
<td>22</td>
<td>15</td>
<td>20.1 ± 6.5++</td>
<td>5.5 ± 1.2</td>
<td>20.9 ± 2.7</td>
<td>96 ± 8</td>
<td>291 ± 72</td>
</tr>
</tbody>
</table>

GD, Gestational day; NT, Non-treated group; RF, Restricted feeding group.
++: p < 0.01, significantly different from NT group (Aspin-Welch t-test).
**: p < 0.01, significantly different from NT group (Student's t-test).
suggesting an adverse effect of restricted feeding. In the histological examination of the placenta, the labyrinth zone was significantly thinner and the basal zone was significantly thicker in the RF group than in the NT group. An abnormally thin labyrinth zone is a common finding of intrauterine growth retardation in rats (Yokoi et al., 2008) and rabbits (Zhang et al., 1995), and a decreased placental surface area with decreased numbers of end villi is said to be related to decreased transport of nutrition to the fetus, resulting in fetal growth inhibition (Constância et al., 2002, Zygmunt et al., 2003; Olausson et al., 2003). It is known that in the placenta of rabbits, PAS-positive glycogen-containing cells in the basal zone almost disappear during the late gestational period. However, in the present study, many PAS-positive glycogen-containing cells still remained in the thick basal zone in the RF group. Together with the above-mentioned thin labyrinth zone, this suggests placental growth retardation with functional decline.

Table 3. Serum progesterone concentrations on GD 22

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Min</th>
<th>Max</th>
<th>Mean</th>
<th>S. D.</th>
</tr>
</thead>
<tbody>
<tr>
<td>NT</td>
<td>6</td>
<td>4.0</td>
<td>8.7</td>
<td>6.0</td>
<td>1.8</td>
</tr>
<tr>
<td>RF</td>
<td>15</td>
<td>1.0</td>
<td>7.1</td>
<td>3.8**</td>
<td>1.4</td>
</tr>
<tr>
<td>P &gt; 4.0**</td>
<td>6</td>
<td>4.1</td>
<td>7.1</td>
<td>5.1</td>
<td>1.1</td>
</tr>
<tr>
<td>P &lt; 4.0**</td>
<td>9</td>
<td>1.0</td>
<td>3.7</td>
<td>3.0</td>
<td>0.8</td>
</tr>
</tbody>
</table>

Unit, ng/ml; P, progesterone value; GD, Gestational day; NT, Non-treated group; RF, Restricted feeding group.

*: The minimum value in NT group.
**: p < 0.01, significantly different from NT group (Student’s t-test).

Table 4. Observation at cesarean section and intrauterine examination on GD 23

<table>
<thead>
<tr>
<th>Group</th>
<th>N</th>
<th>No. of Copora Lutea</th>
<th>No. of Implants</th>
<th>Implantation Index (%)</th>
<th>Embryo-fetal Death Index (%)</th>
<th>No. of Live Fetuses</th>
<th>Fetal Body Weight (g)</th>
<th>Placental Volume (mm³)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NT</td>
<td>6</td>
<td>Mean 9.2</td>
<td>8.5</td>
<td>93.9</td>
<td>5.2</td>
<td>8.0</td>
<td>10.28</td>
<td>479</td>
</tr>
<tr>
<td></td>
<td></td>
<td>S.D. 2.7</td>
<td>2.3</td>
<td>7.4</td>
<td>8.5</td>
<td>2.1</td>
<td>0.91</td>
<td>48</td>
</tr>
<tr>
<td>RF</td>
<td>15</td>
<td>Mean 10.4</td>
<td>8.9</td>
<td>86.1</td>
<td>11.1</td>
<td>7.9</td>
<td>7.84**</td>
<td>411*</td>
</tr>
<tr>
<td></td>
<td></td>
<td>S.D. 2.1</td>
<td>2.0</td>
<td>11.1</td>
<td>12.2</td>
<td>2.2</td>
<td>1.06</td>
<td>66</td>
</tr>
</tbody>
</table>

NT, Non-treated group; RF, Restricted feeding group.

*: p < 0.05, **: p < 0.01, significantly different from NT group (Student’s t-test).
Index was calculated mean value in each dam.
**Table 5.** A classification of embryo-fetal death

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Resorbed embryo (%)</th>
<th>Placental remnant (%)</th>
<th>Macerated fetus (%)</th>
<th>Dead fetus (%)</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>NT</td>
<td>6</td>
<td>0</td>
<td>2 (1)</td>
<td>0</td>
<td>1 (1)</td>
<td>3 (2)</td>
</tr>
<tr>
<td>RF</td>
<td>15</td>
<td>5 (3)</td>
<td>2 (2)</td>
<td>5 (4)</td>
<td>3 (1)</td>
<td>15 (9)</td>
</tr>
<tr>
<td>P &gt; 4.0</td>
<td>6</td>
<td>2 (2)</td>
<td>1 (1)</td>
<td>0</td>
<td>0</td>
<td>3 (3)</td>
</tr>
<tr>
<td>P &lt; 4.0</td>
<td>9</td>
<td>3 (1)</td>
<td>1 (1)</td>
<td>5 (4)</td>
<td>3 (1)</td>
<td>12 (6)</td>
</tr>
</tbody>
</table>

NT, Non-treated group; RF, Restricted feeding group.

**: p < 0.01, significantly different from NT group (Student’s t-test).

**: p < 0.01, significantly different from NT group (Aspin-Welch t-test).

**Table 6.** Results of placental measurement.

<table>
<thead>
<tr>
<th>Group</th>
<th>N</th>
<th>Labyrinth zone (Unit: ×100mm)</th>
<th>Basal zone</th>
<th>Decidual membrane</th>
<th>Myometrium</th>
</tr>
</thead>
<tbody>
<tr>
<td>NT</td>
<td>6</td>
<td>Mean 20.7</td>
<td>11.6</td>
<td>51.1</td>
<td>16.1</td>
</tr>
<tr>
<td>RF</td>
<td>15</td>
<td>Mean 15.6*</td>
<td>14.4**</td>
<td>52.0</td>
<td>17.2</td>
</tr>
</tbody>
</table>

NT, Non-treated group; RF, Restricted feeding group.

*: p < 0.01, significantly different from NT group (Student’s t-test).

**: p < 0.01, significantly different from NT group (Aspin-Welch t-test).

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**Fig. 4.** Histopathological findings of placenta on GD 23. Tissue sections on Non-treated group (A and C) or Restricted feeding group (B and D) were stained with HE (A and B) or PAS (C and D). LZ, labyrinth zone. BZ, basal zone. The thickness of the labyrinth zone is thinner in Restricted feeding group than in Non-treated group, and the number of glycogen-containing cells (arrow) is greater in Restricted feeding group than in Non-treated group.

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T. Matsuoka et al.

Vol. 37 No. 1
Restricted feeding on fetal and placental development in pregnant rabbits

bringing about adverse effects on embryonic viability and growth.

Macerated or dead fetuses, which occur during the period from the late stage of the embryogenesis to the early stage of the fetal development, were observed mostly in dams showing a significant decrease in blood progesterone concentration on GD 22. As detected in our previous study, pregnant rabbits showing serum progesterone concentration less than 4.0 ng/ml resulted in abortion. In the present study, in the subgroup showing serum progesterone concentrations less than 4.0 ng/ml, the embryofetal death index showed a tendency to increase compared with the other subgroup showing serum progesterone concentrations higher than 4.0 ng/ml. In this connection, it is said that a higher serum progesterone concentration is necessary during the above-mentioned period for maintaining embryofetal development (Inskeep, 2004). On the other hand, an increase in resorbed embryos that died at the early stage after implantation was considered not to be related to the level of blood progesterone concentration as previously reported in pregnant rabbits subjected to restricted feeding from GD 6 to GD 18 (Clark et al., 1986; Matsuoka et al., 2006). It has been reported that the restriction of nutrition during the peri-implantation period has effects on the development of the blastocyst and its implantation and on the later placental growth (Cross and Mickelson, 2006). In the present study, restricted feeding may also cause adverse effects on embryos during the peri-implantation period and later placental growth in pregnant rabbits.

It is generally recognised that the placenta of normal pregnant rabbits is structurally established on GD 16 and GD 17 and then the developmental change in the labyrinth zone and the retrograde change in the decidual basalis occurs. The role of uteroplacental blood flow volume shifts from the uterus to the placenta on GD 22 to GD 24, and this stage is thought to have a great effect on fetal viability as well as on maintenance of pregnancy in dams (Hafez and Tsutsumi, 1966). In our previous study on the effects of restricted feeding on the pregnancy outcome under similar experimental conditions to those in the present study (Matsuoka et al., 2009), abortions occurred on and after GD 23. This suggests that abortions might occur due to placental growth retardation with functional decline under restricted feeding.

In conclusion, restricted feeding of 20 g/day from GD 6 to GD 22 brought about developmental impairment of the embryo-fetus and placenta in pregnant rabbits with inhibition of blood coagulation and decline in progesterone concentrations.

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