The Effects of Maternal Mild Protein Restriction on Stroke Incidence and Blood Pressure in Stroke-Prone Spontaneously Hypertensive Rats (SHRSP)

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The effect of maternal protein restriction during pregnancy on the offspring’s blood pressure was assessed in stroke-prone spontaneously hypertensive rats (SHRSP) which are genetically predisposed to hypertension and stroke. After the confirmation of pregnancy, the control group was given a 20% casein diet, and the low-protein group was fed a 9% casein diet. After the confirmation of delivery, commercial feed was given to both of the groups. No differences were seen between the control and low-protein offspring in regard to body weight, blood pressure elevation, or life span. One percent saline solution was put in the control and low-protein groups after the age of 11 weeks. Blood pressure increased markedly in the low-protein group, on the blood pressure level in the low-protein group on week 2 after salt loading (242 ± 6 mmHg) was significantly higher than that in the control group (223 ± 9 mmHg; \( p < 0.05 \)). The survival duration was significantly shorter in the low-protein group (113 ± 4 days) than in the control group (135 ± 22 days; \( p < 0.05 \)). These results suggest that maternal protein malnutrition in SHRSP exerted a high salt sensitivity and a malignant influence on stroke incidence on offspring.

Key words: stroke-prone spontaneously hypertensive rats; pregnancy; protein restriction; offspring; salt-loading

Various studies have shown that blood pressure is affected by hereditary predisposition and environmental factors such as dietary habits.1–3) Studies in stroke-prone spontaneously hypertensive rats (SHRSP) have demonstrated that excess salt intake may affect blood pressure and cause cerebral stroke, leading to life-shortening,4) while the improvement of nutritional factors (including protein nutrition) are effective for the prevention of cerebral stroke and lengthen life span.5–8) These studies have revealed that individuals with genetic factors regarding hypertension or cerebral stroke can prevent the onset of stroke via the improvement of environmental factors after growth. On the other hand, although it is well known that maternal nutritional restriction during pregnancy delays fetal growth,9,10) its effect on the onset of diseases after birth is less understood. Recently, an epidemiological survey conducted in Britain, Sweden, Jamaica, India, and the U.S. has demonstrated that low birth weight can be a risk factor in hypertension, ischemic heart diseases, and type II diabetes mellitus after growth,11–16) attracting attention to the relationship between maternal nutritional state during pregnancy and the onset of diseases after growth. It has been hypothesized that maternal nutritional restriction may affect the development, metabolism, and physiology of a fetus in its adaptation to under-nutrition in utero, and that the effects can persist even after birth, and contribute to the onset of diseases.17) Langley-Evans et al. confirmed in an animal study using normotensive rats that maternal mild protein restriction during pregnancy elevated blood pressure levels of offspring, proving this hypotheses.18–20) In low-protein-exposed rats, the number of renal nephrons decreased,21) and the reactivity to glucocorticoids22) and angiotensin II increased,23) evidently showing the relationship between protein restriction in pregnancy and the onset of hypertension after growth. However, the effect of maternal nutritional restriction during pregnancy on the offspring of animals having genetic factors is unknown. In this study, we investigated the effect of protein restriction during pregnancy on the offspring’s blood pressure, using SHRSP that have a hereditary predisposition to hypertension and cerebral stroke. Furthermore, we examined the effect of post-growth environmental factors on blood pressure, the incidence of cerebral stroke, and life span, using salt loading in low-protein-exposed SHRSP.
Methods

Animals. This study was done according to the guidelines of the animal experimental care committee of the Kinki University Faculty of Agriculture, and the rats were managed in conformance with the guidelines for Care and Use of Laboratory Animals. Imbred SHRSP which had been raised in our laboratory were used for experiments. They were raised at a room temperature of 23 ± 1°C, humidity of 55 ± 5%, and under a 12-hour light-dark cycle (lighting from 7:00h to 19:00h).

Mating. The animals were bred with sibling SHRSP. The SHRSP used in this study were of a strain in which blood pressure elevation is relatively mild and whose life span is long. Virgin female SHRSP weighing 180 g were mated to the same strain male (weighing 210 g or more, blood pressure 190 to 230 mmHg). Animals were paired with one male to one female and were placed together for one night. The date of pregnancy was determined with the confirmation of a vaginal plug and was set as pregnant day 0. Animals were provided pair feeding and given a 20% and a 9% casein diet in the control and low-protein groups, respectively, from pregnant day 0 to the confirmation of delivery (Table 1). During this period, the body weights and feed consumption of mother rats were measured every day. The feed was substituted by a commercial diet (Funabashi SP, Funabashi Farm Co., Ltd., Chiba, Japan) after the confirmation of delivery.

Observation of dams and fetus on pregnant day 21. Pregnant SHRSP (n = 5 per group) were killed by decapitation on pregnant day 21. After abdominal section, ovaries, and uteruses were isolated in order to confirm the establishment or non-establishment of pregnancy, the numbers of corpus lutea and implantation sites, and resorption on pregnant day 21 were measured. The brain, heart, lungs, and kidneys were weighed on postnatal day 4.

Breeding conditions for offspring rats. The day of delivery confirmation was set at postnatal day 0. Birth weight was measured on postnatal day 1. Offspring rats were culled on postnatal day 4, resulting in a uniform litter size of 6. Offspring rats were breast weaned at the age of 4 weeks. All offspring were provided commercial diet and tap water ad libitum. Offspring were weighed once weekly. Blood pressure was measured once weekly from 5 weeks of age. The brain, heart, lungs, liver, and kidneys were weighed on postnatal day 4.

Salt loading. The SHRSP in the control and low-protein groups were further divided into a tap water group or a 1% saline solution group, at 11 weeks of age. All rats were given a commercial diet and drinking water ad libitum until their natural deaths. The number of rats per cage was set to four or less. Dead rats were autopsied. The removed brains were fixed with 10% formalin and then divided into five parts in order to confirm the presence or absence of stroke lesions (cerebral hemorrhage, cerebral softening, etc.).

Blood pressure measurement. After preliminary heating at 37°C, blood pressure was measured using the tail-cuff method (BP-98A; Softron Inc, Tokyo, Japan).

Statistical analysis. Experimental data were expressed as mean ± standard deviation. Data were tested using the one-way layout analysis of variance (ANOVA). When a difference was seen, a test of homogeneity of variance was done for the values, and Student’s t-test or Welch’s t-test (for values showing no homogeneity of variance) was done in order to determine a significant difference.

Results

Observations of pregnant rats and fetuses

Figure 1 shows the body weight gain of dams during pregnancy. In the low-protein group, although weight gain was suppressed during early gestation, weight increased smoothly on and after pregnant day 4. The weight gain of this group, however, did not reach the level of the control group. The numbers of corpus lutea, implantation sites, and resorption on pregnant day 21 were similar in the control group to those in the low-protein group (Data was not shown). No differences were found in fetal weight on pregnant day 21 between the control and low-protein groups. Placental weight and fetus/placenta weight ratio were unaffected by the maternal diet (Table 2).

Table 2 shows fetal organ weight. Data show male and female fetus combined, because there was no effect of sex on organ weight at pregnant day 21 fetus. The control group consisted of 21 animals, and the male/female ratio was 12:9. The low-protein group consisted of 20 animals, and the male/female ratio was 12:8. The ratios of brain and lung weight to body weight were slightly greater, and the ratios of heart weight and kidney weight to body weight were lower in the low-protein group than in the control group, but did not reach significance.

Table 1. Composition of Synthetic Diet

<table>
<thead>
<tr>
<th>Component (%)</th>
<th>20% Casein</th>
<th>9% Casein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Casein</td>
<td>20.00</td>
<td>9.00</td>
</tr>
<tr>
<td>Corn starch</td>
<td>66.56</td>
<td>77.56</td>
</tr>
<tr>
<td>dl-Methionine</td>
<td>0.20</td>
<td>0.20</td>
</tr>
<tr>
<td>Soy bean oil</td>
<td>5.00</td>
<td>5.00</td>
</tr>
<tr>
<td>Vitamin mixture</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>Mineral mixture</td>
<td>4.00</td>
<td>4.00</td>
</tr>
<tr>
<td>Cellulose powder</td>
<td>3.00</td>
<td>3.00</td>
</tr>
<tr>
<td>Choline chloride</td>
<td>0.24</td>
<td>0.24</td>
</tr>
</tbody>
</table>

1Crude protein 85.0%.
2AIN-76 prescription (Nippon Clea).
3Avicel (Nippon Clea).
Fig. 1. Effects of Low Protein Diet during Pregnancy on Maternal Weight Gain.

The rats were time-mated and then given either a 20% casein (C): n = 9) or 9% casein ( (): n = 8) diet throughout pregnancy. The dams were provided pair feeding. The body weight gain was measured daily. Data are mean±S.D. *Significant difference from the control (p < 0.05).

Table 2. Effects of Low Protein Diet during Pregnancy on Fetal Weight, Placental Weight, and Fetal Organ Size at Day 21 Gestation

<table>
<thead>
<tr>
<th></th>
<th>20% Casein</th>
<th>9% Casein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fetuses/litters</td>
<td>(21/5)</td>
<td>(20/5)</td>
</tr>
<tr>
<td>Fetus weight (g)</td>
<td>4.15 ± 0.19</td>
<td>3.94 ± 0.37</td>
</tr>
<tr>
<td>Placenta weight (g)</td>
<td>0.31 ± 0.02</td>
<td>0.32 ± 0.02</td>
</tr>
<tr>
<td>Fetus/placenta ratio</td>
<td>13.47 ± 0.94</td>
<td>12.86 ± 1.69</td>
</tr>
<tr>
<td>Brain wt./body wt. (%)</td>
<td>3.94 ± 0.15</td>
<td>4.07 ± 0.59</td>
</tr>
<tr>
<td>Heart wt./body wt. (%)</td>
<td>0.63 ± 0.06</td>
<td>0.59 ± 0.07</td>
</tr>
<tr>
<td>Lung wt./body wt. (%)</td>
<td>2.28 ± 0.47</td>
<td>2.81 ± 0.24</td>
</tr>
<tr>
<td>Kidney wt./body wt. (%)</td>
<td>1.08 ± 0.06</td>
<td>1.04 ± 0.07</td>
</tr>
</tbody>
</table>

Dams fed a 20% or 9% casein diets were killed at day 21 and fetuses and placenta were obtained. The male and female fetuses were obtained from 5 dams per group. Because of no effect of sex on organ weight at this point, data show male and female fetuses combined. Number of examined were 4–5 fetuses per dam. Male/female ratio was 12.9 in 20% casein diet, 12.8 in 9% diet. The values are expressed as mean±S.D.

Changes in body weight and blood pressure during the growth of offspring rats

The birth weight of male pups was significantly lower in the low-protein group than in the control group (p < 0.05) (Table 3). In female pups, however, no significant differences were observed between these groups. Table 4 shows organ weights on postnatal day 4. Since body and organ weights varied between males and females but the ratios of organ to body weight presented no differences between the sexes, the data of males and females were dealt with together. The control group consisted of 13 animals, and the male/female ratio was 2:11. The low-protein group consisted of 17 animals, and the male/female ratio was 5:12. The ratios of kidney weight to body weight were significantly lower in the low-protein group than in the control group (p < 0.05), whereas other organ weight as a proportion of body weight was unaffected by the maternal diet. Body weight was similar until weaning but was subsequently slightly higher in the low-protein group than in the control group after weaning. Body weight at the age of 10 weeks was almost the same in the control group (male, n = 10; 201 ± 19 g, female, n = 6; 168 ± 8 g) as in the low-protein group (male, n = 8; 200 ± 7 g, female, n = 6; 166 ± 6 g). Blood pressure reached similar levels in the control group (male, n = 10; 189 ± 5 mmHg, female, n = 6; 172 ± 6 mmHg) and in the low-protein group (male, n = 8; 190 ± 7 mmHg, female, n = 6; 168 ± 9 mmHg). No differences in blood pressure were seen in the control and low-protein-exposed SHRSP ever since. Life span and stroke incidence were observed on male offspring rats. The average life span (control group; 270 ± 89 days, low-protein group; 293 ± 104 days) and incidence of stroke (control and low-protein group; 100%) were similar in both groups.

Table 3. Birth Weight of SHRSP Exposed to Low Protein in Utero

<table>
<thead>
<tr>
<th></th>
<th>Average per litter (g)</th>
<th>Mean (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male</td>
<td>5.3 ± 0.3 (n = 6)</td>
<td>5.4 ± 0.4 (19/4)</td>
</tr>
<tr>
<td>20% Casein</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>4.9 ± 0.3 (n = 6)</td>
<td>5.2 ± 0.2 (18/4)</td>
</tr>
<tr>
<td>9% Casein</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>4.5 ± 0.8 (n = 6)</td>
<td>4.7 ± 0.2* (18/3)</td>
</tr>
<tr>
<td>Female</td>
<td>4.5 ± 0.6 (n = 6)</td>
<td>4.7 ± 0.4 (18/3)</td>
</tr>
</tbody>
</table>

Table 4. Organ Size of 4 Days after Birth SHRSP Exposed to Low Protein Diet

<table>
<thead>
<tr>
<th></th>
<th>20% Casein</th>
<th>9% Casein</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>13</td>
<td>17</td>
</tr>
<tr>
<td>Brain wt./body wt. (%)</td>
<td>5.15 ± 0.42</td>
<td>5.25 ± 0.64</td>
</tr>
<tr>
<td>Heart wt./body wt. (%)</td>
<td>0.77 ± 0.08</td>
<td>0.80 ± 0.12</td>
</tr>
<tr>
<td>Lung wt./body wt. (%)</td>
<td>2.24 ± 0.47</td>
<td>2.11 ± 0.24</td>
</tr>
<tr>
<td>Liver wt./body wt. (%)</td>
<td>3.73 ± 0.58</td>
<td>3.49 ± 0.98</td>
</tr>
<tr>
<td>Kidney wt./body wt. (%)</td>
<td>0.75 ± 0.03</td>
<td>0.71 ± 0.03*</td>
</tr>
</tbody>
</table>

*Significant difference from the 20% casein diet (p < 0.05).
Changes in blood pressure, life span, and the incidence of stroke by salt loading on low-protein-exposed SHRSP

Salt was administered to male offspring rats at the age of 11 weeks. At the start of the experiment, blood pressure levels of low-protein-exposed SHRSP were 206 \pm 7 \text{mmHg} and those of the control rats were 206 \pm 8 \text{mmHg}. After 2 weeks of salt loading, blood pressure levels were 223 \pm 9 \text{mmHg} in the control group and 242 \pm 6 \text{mmHg} in the low-protein group, showing a marked elevation in the low-protein group (Fig. 2B). Subsequently in the low-protein group, some rats showed weakness as well as symptoms commonly observed with the onset of stroke, such as convulsions and paralysis of the lower limbs. Feed consumption decreased (data not shown), and body weight decreased markedly (Fig. 2A). Blood pressure measurement was impossible in some rats 4 weeks after salt loading. Figure 3 shows the survival curves of these rats. Death began to occur at 105 days of age. The average life span was significantly lower in the low-protein group (113 \pm 4 days) than in the control group (135 \pm 22 days; \( p < 0.05 \)) (Table 5). The incidence of stroke was 80% in the control group and 100% in the low-protein group. The brain/body weight ratio was slightly lower in the low-protein group than in the control group, but did not show significance.

Discussion

Low birth weight due to maternal undernutrition has been considered a risk factor in hypertension and heart diseases, and studies have been done on the relationship between maternal nutrition and the onset of diseases in offspring. \(^{11-16}\) It was reported that maternal undernutrition and protein restriction during pregnancy might cause hypertension in adult rat offspring. \(^{18-20}\) This phenomenon, however, has not been examined in animals with hereditary factors regarding hypertension. We investigated the effects of maternal protein restric-

![Fig. 2. Effects of Salt Loading from 11 Weeks of Age on Body Weight (A) and Blood Pressure (B) of SHRSP Exposed to Low Protein Diet in Utero.](image)

Eleven week-old offspring born to dams fed a 20% casein (\( \circ \): \( n = 10 \)) or 9% casein (\( \bullet \): \( n = 9 \)) were given 1% saline from eleven weeks old. Offspring were obtained from 4 dams fed a 20% casein diet and from 3 dams fed a 9% casein diet. Offspring were given commercial diets (Funahashi SP diet, Funahashi Farm Co., Ltd., Chiba, Japan). Body weight was measured once a week for 4 weeks, with a final measurement at 15 weeks. To avoid possible gender differences, only male pups were studied. Systolic blood pressure was measured using the tail-cuff method once weekly. (\( \circ \捆 \)): (No. of blood pressure measurable rats/No. of examined rats). Data are mean\( \pm \)S.D. *Significant difference from the control (\( p < 0.001 \)).

![Fig. 3. Effects of Salt-loading from 11 Weeks of Age on Survival Curve of SHRSP Exposed to Low Protein Diet in Utero.](image)

Eleven week-old male offspring born to dams fed a 20% casein (\( \circ \): \( n = 10 \)) or 9% casein (\( \bullet \): \( n = 8 \)) were given 1% saline. Offspring were obtained from 4 dams fed a 20% casein diet and from 3 dams fed a 9% casein diet. Offspring were given commercial diets (Funahashi SP diet, Funahashi Farm Co., Ltd., Chiba, Japan). To avoid possible gender differences, only male pups were studied. Data represents percentage survival.
tion during pregnancy on offspring’s blood pressure in SHRSP, which are the animal models for human essential hypertension and stroke.

The minimum protein requirement is 9% for adult rats and 12% for pregnant rats. Langley-Evans et al. advocated that the use of a 9% casein diet constituted mild protein restriction, because the breeding of normotensive rats on a 9% casein diet suppressed body weight gain in mothers but did not change the litter size and offspring’s body weight after birth. We adopted a 9% protein level for the low-protein diet in this study. It has been reported that a 9% casein diet during pregnancy in normotensive rats did not affect reproductive outcome but did increase placental weight, leading to a decrease in fetal weight. In SHRSP, it was shown that maternal weight gain and litter size were smaller, the placental weight was higher, and offspring’s birth weight was lower than in the original strain, normotensive Wistar Kyoto rats (WKY). Since SHRSP are programmed to develop hypertension due to genetic factors, we presumed that a 9% casein diet during pregnancy might significantly affect reproductive outcome and the offspring’s growth. In SHRSP, however, protein restriction during pregnancy resulted in similar changes in mothers and neonatal rats to those reported by Langley-Evans et al.

In low-protein-exposed Wistar rats, blood pressure levels have been 20 to 30 mmHg higher even at 4 weeks of age than in control rats, and the levels remain high throughout their lifetimes. In low-protein-exposed SHRSP, blood pressure levels were slightly higher than in the control group from 7 to 9 weeks of age but showed no differences between the two groups after 10 weeks of age. Likewise, the effect of maternal protein malnutrition does not alter the offspring’s life span and incidence of stroke. Changes in blood pressure during the growth process vary fundamentally between SHRSP and normotensive rats. In WKY rats, blood pressure levels are 110 to 120 mmHg at 4 weeks of age and remain unchanged at 130 mmHg throughout the adult lifetime. In SHRSP, the levels are 120 to 130 mmHg at 5 weeks of age, then elevate by approximately 10 mmHg weekly up to 12 weeks of age, and increase to 230 mmHg or higher after growth. The possible mechanisms of blood pressure elevation in SHRSP are considered to be hyperactivity of the renin-angiotensin system (RAS), the sympathetic nervous system, or the endocrine system, or increased reactivity of peripheral resistance arteries, decreased number of renal nephrons, etc. Blood pressure elevation resulting from a low protein diets in normotensive Wistar rats, on the other hand, has been suggested to be related to a decreased number of renal nephrons and RAS hyperactivity. The mechanisms of the development of high blood pressure in low-protein Wistar rats and that in SHRSP share certain common characteristics. In SHRSP, the effects of intrauterine low-protein exposure were considered to have been concealed by the genetic mechanism of pressure elevation and to have not been revealed as an increase in blood pressure.

In this context, we loaded low-protein-exposed SHRSP with salt from 11 weeks of age in order to investigate the effects of low-protein exposure in utero. The changes of blood pressure elevation and stroke incidence were not observed in low-protein-exposed SHRSP in water drinking. However, the low-protein-exposed SHRSP had a marked elevation in blood pressure as compared with the control group after salt loading, suggesting that low-protein exposure in the fetal period could continue to affect blood pressure even after growth. These results indicated that maternal mild protein restriction induces the incidence of stroke in salt-loading SHRSP. Moreover, the survival duration after salt loading was shorter in the low-protein-exposed SHRSP. In a report observing the morphologic changes of the brain in offspring rats exposed to low protein during gestation, anastomotic abnormality in the cerebral artery and decreased vascular density were demonstrated. In the low-protein-exposed SHRSP group, it is unclear whether the shortened survival duration after salt loading might result from vascular disorders advanced by blood pressure elevation or might be associated with a morphologic developmental anom-
aly of the cerebral vessels. The incidence of stroke was almost identical between the control and low-protein groups, whereas the brain/body weight ratio was lower in the low-protein group than in the control group. This indicates that the period from the onset of stroke to death was short in the low-protein group. An epidemiological survey demonstrated that low kidney weights and a small number of renal nephrons in babies with low birth weight contributed to the onset of hypertension after growth.39,40 Speculating from the fact that the ratio of kidney weight of body weight were smaller than those of the control rats at postnatal day 4, we consider that the decreased number of renal nephrons is likely to be involved in increased sensitivity to salt. However, maternal mild protein restriction may not determine later blood pressure in SHRSP. Generally, blood pressure elevation after salt loading is often caused by decreased Na excretion. Recently, it has been suspected that salt sensitivity may be related to an enzyme, 11beta-hydroxysteroid dehydrogenase type 2 (11β-HSD2),41,42 which regulates the binding of aldosterone to mineralocorticoid receptors. Maternal malnutrition and glucocorticoid loading to mother rats during pregnancy have been reported to reduce renal 11β-HSD2 activity in offspring rats.43,44 It is known that salt sensitivity is higher in SHRSP than in WKY rats. Salt loading in low-protein-exposed rats after growth did not elevate blood pressure in normotensive rats45 but increased blood pressure in SHRSP. In SHRSP with low Na excretion, low-protein exposure might decrease renal 11β-HSD2 activity, resulting in further reduced Na excretion. We consider it necessary to investigate the mechanism governing the increase in salt sensitivity in low-protein-exposed SHRSP in terms of the Na excretion in offspring rats.

References


