Food & Nutrition: Current Research
FNCR, 1(1): 9-18
wwww.scitcentral.com

Original Research: Open Access

Gestational Low-Salt Intake Decreases Offspring Survival Rates in Rats through Maternal and Neonatal Mechanisms

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Received February2, 2016; Accepted March 12, 2016; Published February 21, 2018

ABSTRACT

We investigated the influence of maternal salt-restriction during gestation on offspring growth in Dahl salt-sensitive (Dahl S) rats. Dahl S rats were fed a low-salt (0.3% NaCl, w/w) or high-salt (4%NaCl, w/w) diet during gestation. After giving birth, the mothers and their pups were arranged in the following manner, i.e., 1) high-salt fed mothers were grouped with pups from high-salt fed mothers (HM-HB), 2) high-salt fed mothers with pups from low-salt fed mothers (HM-LB), 3) low-salt fed mothers with pups from high-salt fed mothers (LH-HB) and 4) low-salt fed mothers with pups from low-salt fed mothers (LM-LB). The mothers and pups were maintained on a regular-salt chow (0.75%NaCl, w/w) chow until weaning with *ad libitum* access to water. The survival rate was 95% for HM-HB and the pups of LM-LB all died during the lactation period. The difference in survival was significant in the on Kaplan-Meier analysis (Chi-squared 19.88, $p<0.0001$). The survival rate of HM-LB pups was 20%, which was significantly lower than that of HM-HB pups (Chi-squared 26.3, $p<0.0001$). Furthermore, the survival rate of LM-HB pups was lower than that of HM-HB pups (Chi-squared 15.6, $p<0.0001$). The decrease in the survival of pups born to low-salt fed mothers was also observed in normal Wistar rats, and there was a decrease in the survival rate with the increase of generation. Thus, gestational low-salt intake is associated with low survival rate of offspring, and both neonatal and maternal mechanisms are involved.

Keywords: Low-salt intake, Neonate, Pregnancy, Growth, Dahl rat, Wistar rat

Abbreviations: Dahl S Rats: Dahl Salt-Sensitive Rats; RAS: Renin Angiotensin System; SD: Standard Deviation; ANOVA: One-Way Factorial Analysis Of Variance; HM: Mothers Fed A High-Salt Diet During Pregnancy; LM: Mothers Fed A Low-Salt Diet During Pregnancy; HB: Pups Born From Mothers Fed A High-Salt Diet During Pregnancy; LB: Pups Born From Mothers Fed a Low-Salt Diet During Pregnancy

INTRODUCTION

Since the 1980s investigators have reported serious health problems associated with excessive salt intake in humans. Salt restriction, as low as 6 g per day, has been the main strategy to prevent the occurrence hypertension and related organ damage, e.g., stroke, heart attack or renal failure. However, it is still unclear to what level can salt intake be lowered safely. Evidence for benefits and risks of salt restriction is lacking in neonates and elderly individuals.

On the other hand, numerous animal and human studies have recently reported that salt restriction during pregnancy may pose a health risk to neonates [1]. Gestational low-salt intake is often associated with intrauterine growth retardation, and the premature infants are likely to develop hypertension and insulin resistance in adulthood. In this regard, we have recently reported that there was an increase in salt craving for the neonates due to the gestational low-salt intake in salt-sensitive Dahl rats (Dahl S rats) [2].

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Citation: Sakuyama H, Kato M, Wakabayashi H, Shimizu N & Uehara Y (2018) Gestational Low-Salt Intake Decreases Offspring Survival Rates in Rats through Maternal and Neonatal Mechanisms. Food Nutr Current Res, 1(1): 9-18.

More interestingly, gestational low-salt intake has been associated with a decrease in birth rate and the survival rate of pups [3]. The pups of low-salt fed mothers are likely to die immediately after birth or during lactation. The survival rate of pups of low-salt fed mothers is as low as 65% while 95% of the pups of high-salt fed mothers survive (Chi-squared 19.8, $p<0.0001$). The reason for the high risk to pups is unclear; however, recent studies have reported modifications of maternal or fetal genes due to salt restriction. Epigenetics, the variations in DNA methylation patterns and chromatin remodeling, provide an intriguing explanation for how environmental factors or intrauterine nutrition influences the risk to developing neonates or metabolic diseases in adulthood [4-7]. However, the data for the effect of early nutrition on epigenetics modifications is limited.

It is of our interest to know the influence of gestational low-salt intake on behavior of mothers and their neonates during lactation. Accordingly, we proposed herein the hypothesis that gestational salt restriction affects both maternal behavior for pups and growth of the pups, thereby decreasing the survival rate. To test this hypothesis, we examined the survival of Dahl S pups born to low-salt and high-salt fed mothers, with exchange of mothers after birth. In addition, we examined whether the association of gestational low-salt intake to the decreased survival of pups is transmissible between generations using normal Wistar rat.

MATERIALS AND METHODS

Design of Study 1

We obtained 4-week-old male and female Dahl salt-sensitive (Dahl S) rats from Sankyo Laboratories, Inc., Tokyo, Japan. The 12 paired rats were fed a regular-salt chow (0.75%NaCl, w/w CEL Rodent Diet CE-7; CLEA Japan Inc., Tokyo, Japan). Dahl S rats were divided into 2 groups **(Figure 1)**: (1) Dahl S rats that were fed a high-salt (4% NaCl, w/w) diet (F2Dahl-4%, Oriental Yeast Co., Ltd., Tokyo, Japan) during the gestational period (high-salt mothers), and (2) Dahl S rats that were fed a low-salt (0.3% NaCl, w/w) diet (F2Dahl-0.3%, Oriental Yeast) during the gestational period (low-salt mothers). After 1-week mating on the regular-salt chow, female rats were placed in separate cages and fed on each diet during the gestational period. Three weeks after mating, 5 high-salt mothers delivered 40 pups and 4 low-salt mothers gave birth to 24 pups.

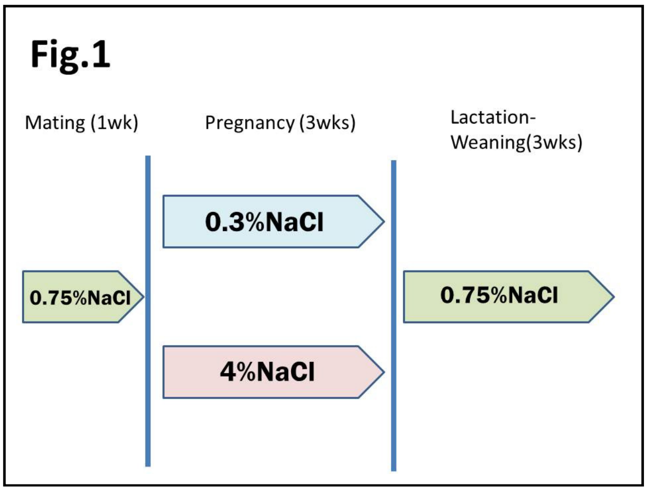

Figure 1. Salt group in mothers during mating, pregnancy and lactation

0.3%NaCl, low-salt chow containing 0.3NaCl (w/w); 0.75%NaCl, regular-salt chow containing 0.75%NaCl (w/w) and 4%NaCl, high-salt chow containing 4%NaCl (w/w). After delivery the chow for mothers and pups was changed to the regular-salt chow until weaning.

The pups born to the high-salt mothers were pooled and then, randomly assigned to; (1) the high-salt mothers (3 rats) (HM-HB) or (2) the low-salt mothers (2rats) (LM-HB). Those born to the low-salt mothers were assigned randomly to; (3) the high-salt mothers (2rats) (HM-LB) or (4) the low-salt mothers (2rats) (LM-LB) **(Figure 2)**. During the lactation period Dahl S rats were fed the regular-salt (0.75%NaCl, w/w) chow with free access to water. Number of survival pups was monitored until weaning.

Design of Study 2

1) 1st generation study

First, we examined whether gestational low-salt diet is associated with low survival rate of the pups in normal Wistar rat strain. Briefly, we obtained 4-week-old male and female rats from Sankyo Laboratories. The rats were fed the regular-salt (0.75%NaCl, w/w) chow until mating. At 10 weeks of age, they were divided into 2 groups **(Figure 3)**: (1) 6 pairs of male and female rats fed the high-salt (4% NaCl, w/w) diet during the gestational period, and (2) 3 pairs of male and female rats fed the low-salt (0.3% NaCl, w/w) diet during the gestational period. After mating for 1 week, the mated female rats were placed in separate cages for the gestational period and fed on each diet during the gestational period. Three weeks after the mating, the pregnant rats began delivering pups. After delivery, the rats were fed the regular-salt chow during the lactation period. Water was available *ad libitum*. The number and growth of pups were monitored until weaning.

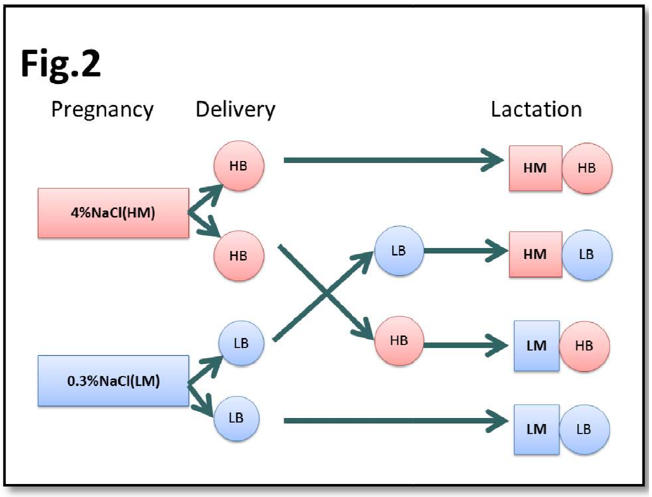

Figure 2. Design of exchange of mothers and pups relationship

Mothers and pups relationship was changed randomly in each salt group immediately after birth. Squares represent mothers and circles pups. HM, high-salt fed mother during gestation; HB, pups from high-salt fed mother; LM, low-salt fed mother during gestation; and LB, pups from low-salt fed mother.

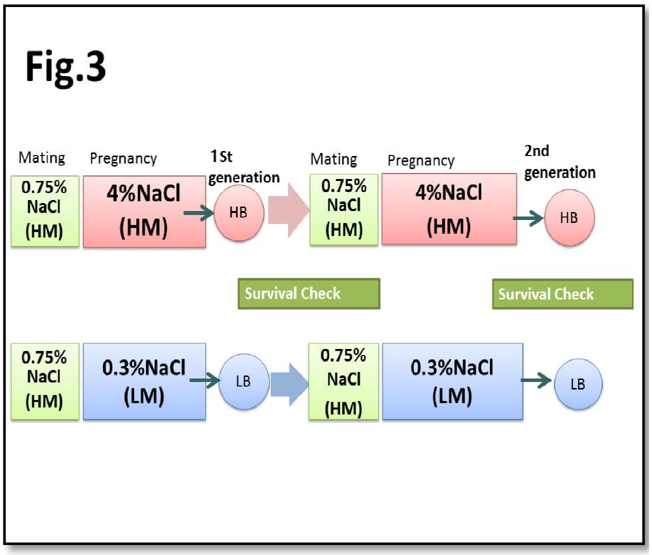

Figure 3. Protocol of the study 2 on a difference in surviving between generations

Study on survival rate of Wistar pups from mothers fed the low- and high-salt diets during gestation and on the effects of generation on the association.

2) 2nd generation study

Male and female Wistar rats born to low-salt and high-salt fed mothers in the 1st generation study were used in this test. At 10 weeks of age, the male and female rats of low-salt mothers (1st generation) were mated, and those of high-salt mothers (1st generation) were mated. After mating, the mated female rats were placed in separate cages for the gestational period, and each animal was fed a chow with a different salt content. After delivery, the rats were fed the regular-salt chow during the lactation period with free access to water (2nd generation). The number of survival pups was monitored until weaning.

Statistical analysis

Values were expressed as means ± SD. Differences were assessed by the χ^2 test, one-way or multifactorial analysis of variance (ANOVA/MANOVA) followed by post-hoc least significant difference (LSD) test or Kaplan-Meier survival curve analysis. *P*-values less than 0.05 were considered statistically significant. All statistical analyses were performed using STATISTICA software (StatSoft, Tulsa, OK, USA) and MedCalc Statistical Software version 14.12.0 (MedCalc Software bvba, Ostend, Belgium; http://www.medcalc.org; 2014) for Kaplan-Meier survival curve analysis.

Guidelines for handling rats

We followed the guidelines for handling of experimental animals, and our study was approved by the Animal Care Committee of the Kyoritsu Women's University. The experiment was conducted in accordance with the National Institutes of Health (NIH) guidelines.

Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

RESULTS

Study 1

We investigated the relationship between gestational low-salt intake and survival of pups after birth in Dahl S rats according to our previous studies [1-3]. Using the data from the pups of mothers fed the low- and high-salt diets during gestation, we analyzed the Kaplan-Meier survival curve for the offspring in order to increase our understanding of the time course before death **(Figure 4)**. Only one of 31 pups born to high-salt fed mothers and nursed by high-salt fed mothers (HM-HB) died during the lactation period. In contrast, the pups born to low-salt fed mothers and nursed by low-salt fed mothers (LM-LB) began to die immediately after birth and all were dead by 15 days after birth. The difference in pup survival was highly significant (vs HM-HB, Chi-squared 55.53, p<0.0001).

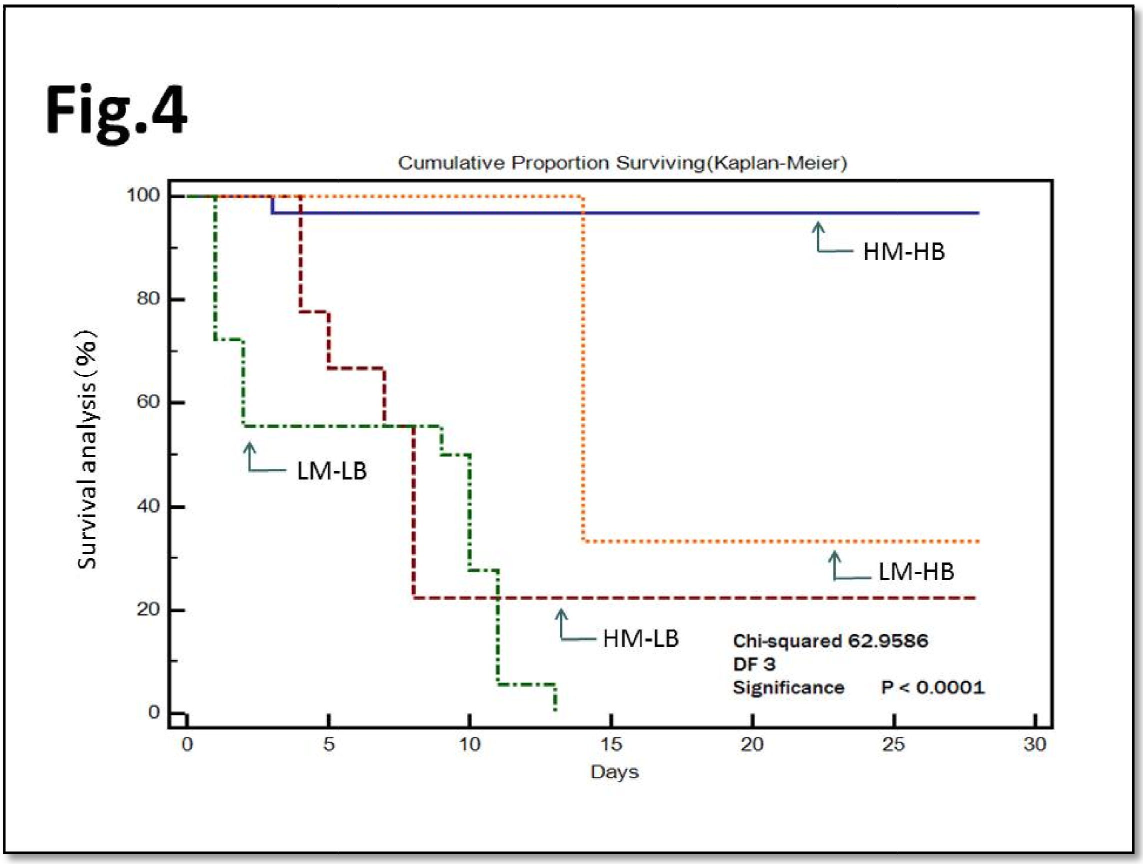

Figure 4. Kaplan Meier surviving analysis of Study 1 on Dahl S rats

The number of pups used for Kaplan Meier surviving analysis was 31 for HM-HB, 18 for LM-LB, 6 for LM-HB and 9 for LM-LB. HM, high-salt fed mothers during gestation; HB, pups from high-salt fed mothers; LM, low-salt fed mothers during gestation; and LB, pups from low-salt fed mothers. The difference in survival rate among the 4 groups was statistically significant as indicated in the graph. The decline of survival in LM-LB, HM-LB and LM-HB was significant compared with HM-HB (Chi-squared=55.53, p<0.0001; Chi-squared=26.39, p<0.0001; Chi-squared=15.62, p<0.0001). There was no difference between LM-LB and HM-LB. MedCalc Statistical Software version 14.12.0 (MedCalc Software bvba, Ostend, Belgium; http://www.medcalc.org; 2014) were used for Kaplan-Meier survival curve analysis.

The pups born to low-salt fed mothers and nursed by high-salt fed mothers (HM-LB) died immediately after birth. The difference in pup survival was highly significant (vs HM-HB, Chi-squared 26.39, p<0.0001). However, there was no difference in the Kaplan-Meier survival between LM-LB and HM-LB. On the other hand, the pups born to high-salt fed mothers and nursed by low-salt fed mothers (LM-HB) survived the early period of lactation, but died around 15 days after birth. The difference in the Kaplan-Meier survival between HM-HB and LM-HB was statistically significant (Chi-squared 15.62, p<0.0001).

HM-HB pups gained body weight in a time-dependent manner **(Figure 5)**. However, LM-LB pups gained less body weight than HM-HB pups. The body weight of HM-LB pups was low during the early lactation period; however, the weight caught up until weaning and at weaning, the body weight was equal to that of HM-HB. LM-HB pups underdeveloped during the 1st week; thereafter, the deviation of body weight became greater because of growth retardation in some of the pups. After they were dead, the rest of pups grew as HM-HB until weaning. Overall group difference from day-1 through day-21 was p<0.0001 for LM-LB vs HMHB, p<0.0001 for HMLB vs HMHB and p<0.01 for LMHB vs HMHB.

Study 2

In this experiment, we examined whether the association between gestational low-salt intake and low pup survival rate can be observed and it is transmitted to the 2nd generation **(Figure 6)**. Fifteen pups were born to high-salt fed mothers (3 rats) and 33 to low-salt mothers (3 rats) in the

1st generation **(Figure 6a)**. The survival of pups was monitored until weaning. In the first generation, the survival rate was 93% for HM-HB pups and 91% for LM-LB (Chi-squared 0.06, p=0.806). The offspring of high-salt fed mothers was mated and maintained on the high-salt diet during gestation, and the offspring of low-salt fed mothers was mated and maintained on the low-salt diet during gestation. After birth, the survival rate of pups in the 2nd generation was monitored during lactation period **(Figure 6b)**. The survival rate in the 2nd generation tended to be lower in the pups born to low-salt fed mothers (4 rats) than those that in those born to high-salt fed mothers (3 rats) (60% vs 79%; Chi-squared 2.56, p<0.10). Particularly, the survival rate of LM-LB pups declined significantly with each new generation (91% vs 60%; Chi-squared 8.58, p<0.005).

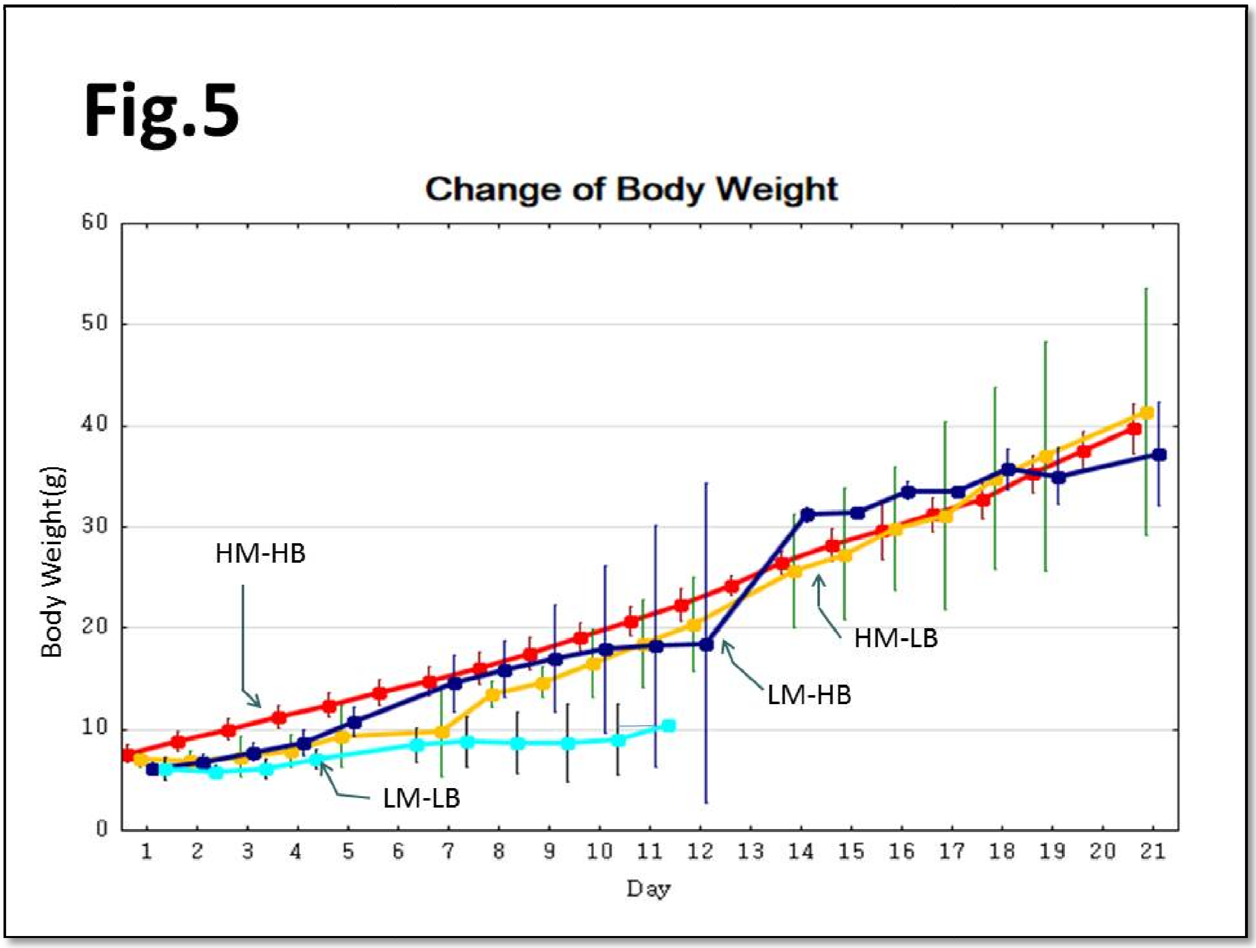

Figure 5. Body weight changes of Dahl S rats

HM, high-salt fed mother during gestation; HB, pups from high-salt fed mother; LM, low-salt fed mother during gestation; and LB, pups from low-salt fed mother. The group different was significant between HM-HB and LM-LB. The difference in day 1 to day 10 was significant between HM-HB and HM-LB, and in day 2 to day day 5 was significant between HM-HB and LM-HB. Thereafter, the deviation of body weight became greater because of growth retardation in some of the pups. After they were dead, the rest of pups grew as HM-HB until weaning. Overall group difference, assessed by MANOVA, from day-1 through day-21 was p<0.0001 for LM-LB vs HMHB, p<0.0001 for HMLB vs HMHB and p<0.01 for LMHB vs HMHB.

HM-HB pups in the 1st generation gained body weight in a time-dependent manner **(Figure 7a)**. However, LM-LB pups gained less body weight than HM-HB pups. The overall group difference day 1 through day 22 was statistically different (p<0.0001). Similarily, HM-HB pups in the 2st generation gained body weight in a time-dependent manner and LM-LB pups gained less body weight than HM-HB pups **(Figure 7b)**. The overall group difference day 1 through day 22 was statistically different (p<0.0001).

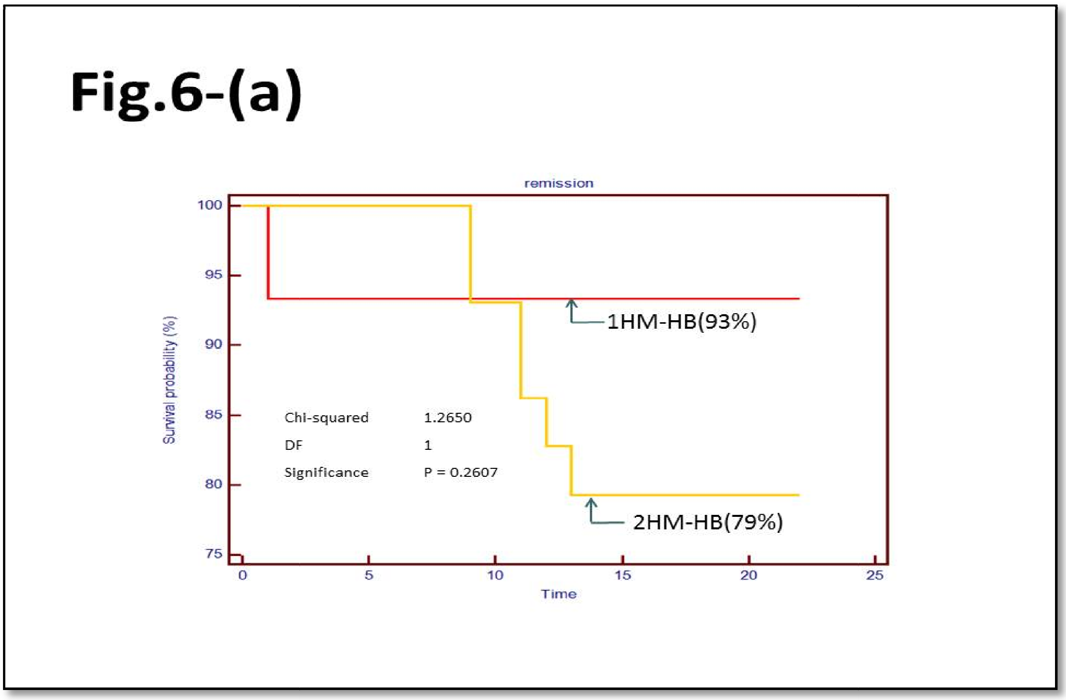

Figure 6a. Effects gestational high-salt intake on survival of pups in the 1st generation

The number of pups used for analysis was 15 for HM-HB in the 1st generation and 29 for HM-HB in the 2nd generation. HM, high-salt fed mother during gestation; HB, pups from high-salt fed mother; LM, low-salt fed mother during gestation; and LB, pups from low-salt fed mother. There was no difference in survival of HM-HB pups between the 1st and 2nd generations.

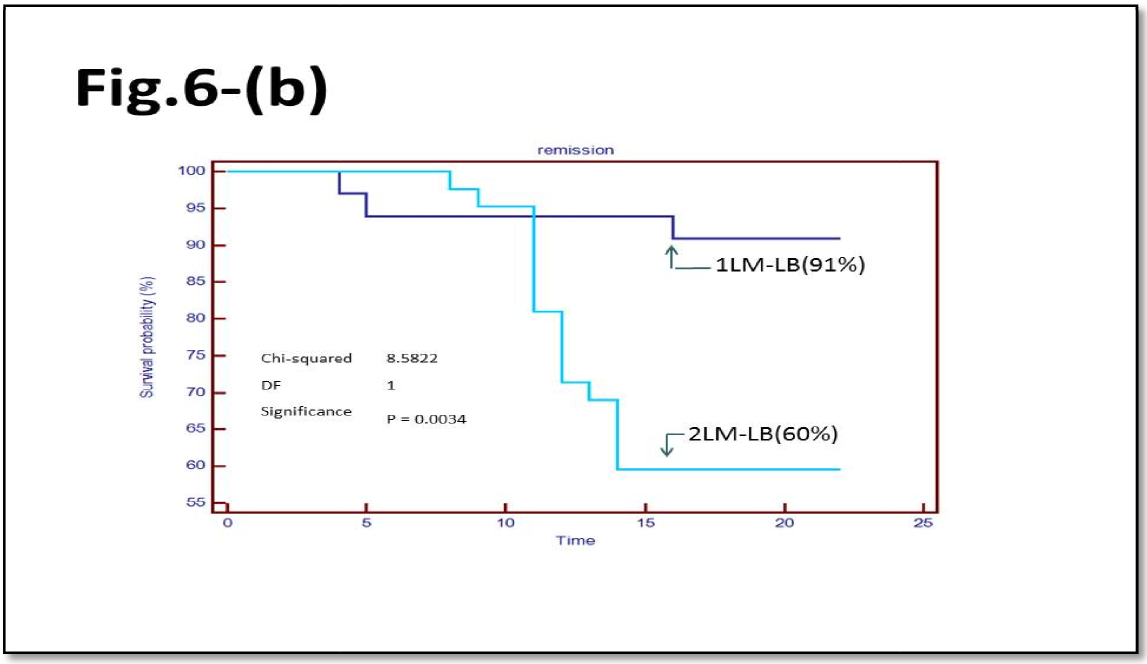

Figure 6b. Effects gestational high-salt intake on survival of pups in the 2nd generation

The number of pups used for analysis was 33 for LM-LB in the 1st generation and 42 for the 2nd generation. HM, high-salt fed mother during gestation; HB, pups from high-salt fed mother; LM, low-salt fed mother during gestation; and LB, pups from low-salt fed mother. Particularly, the survival rate of LM-LB pups declined significantly with each new generation (91% vs 60%; Chi-squared 8.58, p<0.005). MedCalc Statistical Software version 14.12.0 (MedCalc Software bvba, Ostend, Belgium; http://www.medcalc.org; 2014) were used for Kaplan-Meier survival curve analysis.

DISCUSSION

In the present study, we demonstrated the association between gestational low-salt intake and low survival rate of Dahl S pups. This finding corresponded to our previous reports that pups from low-salt fed mothers died during early and mid-lactation periods [3]. It is noted that the pups from low-salt fed mothers died even if the pups were nursed by high-salt fed mothers. This finding suggested that deaths that occurred immediately after birth were due to the neonatal factors, but not to maternal factors. The pathophysiological changes in the pups during intrauterine development are not clear; however, gestational low-salt intake is often associated with intrauterine growth retardation, premature birth and metabolic disease in adulthood.

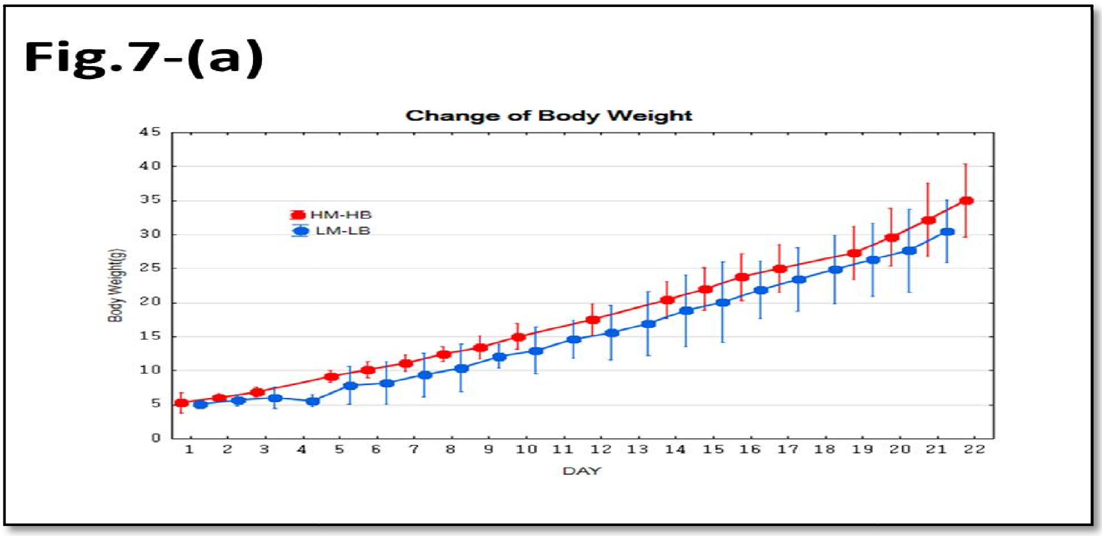

Figure 7a. Body weight changes of Wistar rats

Body weight changes of Wistar rats in the 1st generation were demonstrated in graph (a) and those in the 2nd generation in graph

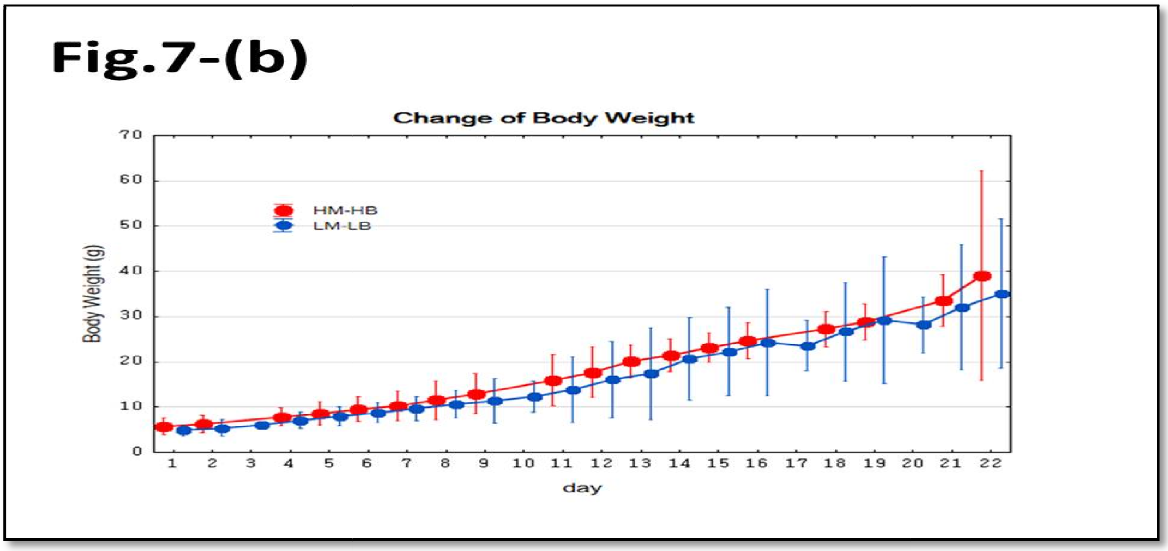

Figure 7b. Body weight changes of Wistar rats

Body weight changes of Wistar rats in the 2nd generation in graph (b). HM, high-salt fed mother during gestation; HB, pups from high-salt fed mother; LM, low-salt fed mother during gestation; and LB, pups from low-salt fed mother. The group difference was assessed by MANOVA. HM-HB pups in the 1st generation gained body weight in a time-dependent manner (graph a). However, LM-LB pups gained less body weight than HM-HB pups. The overall group difference in body weight day 1 through day 22 was statistically different (p<0.0001). Similarly, HM-HB pups in the 2nd generation gained body weight in a time-dependent manner and LM-LB pups gained less body weight than HM-HB pups (graph b). The overall group difference in body weight day 1 through day 22 was statistically different (p<0.0001).

Recent studies suggest an epigenetic mechanism for the growth retardation and predisposition to hypertension and metabolic disease in adulthood [1,8-10]. Up-regulation of renin-angiotensin system by low-salt intake may play an important role in activating this epigenetic mechanism [11-14]. Notably, Dahl S rats are sensitive to angiotensin-mediated-events. Slight changes in angiotensin II levels are known to cause renal damage [15]. It is because the response of regulator of Gαq signaling-2 mRNA to angiotensin II is impaired, thereby predisposing the animals to renal injury [16]. Regulator of Gαq signaling-2 down-regulates G-protein-mediated intracellular signal transduction of angiotensin II. However, there are no reports on the role of the renin-angiotensin system in the low survival of pups from low-salt fed mothers.

Furthermore, we demonstrated that LM-HB pups died during the lactation period rather than immediately after birth. Relative to the body weight gain in HM-HB pups, LM-HB pups exhibited growth retardation during the early phase of lactation period. This caused high variation in the body weight, and the death of the pups with growth retardation was related to the increase in body weight at the late phase of lactation. These findings strongly suggested that gestational low-salt intake influenced mothers, thereby impairing growth of the pups. In the present study, we didn't measure body weight of mothers during gestation period. However, in general, body weight gain slightly decreased in Dahl S rats fed a high-salt (4% NaCl, w/w) chow compared with those fed a low-salt (0.3% NaCl, w/w) chow [17]. These suggested that the lowered survival rate of the pups from mothers fed a low-salt diet during gestation was not due to poor nutrition of mothers.

We have investigated over years the influence of gestational low-salt intake in birth and survival of pups in Dahl salt sensitive rats. Dahl salt sensitive rats are a genetic model of salt-induced hypertension in humans. This rat strain exhibits salt-dependent hypertension with hypertensive organ damage, e.g., cerebral stroke, heart failure, kidney impairment and decreased insulin sensitivity. In this sense it is a model for metabolic syndrome in humans. On the other hand, Wistar strain is a salt-independent, normotensive rat without any apparent metabolic abnormality, and widely used as a model for normal humans. In study 2, we attempted to test whether the findings on the relationship between gestational low-salt intake and poor survival of the pups would be observed in salt-resistant settings. The association between gestational low-salt intake and low survival rate of the pups was found not only in rats with salt-sensitive genetic background, but also in normal Wistar rats. In addition, decrease in pup survival was more obvious in the 2nd generation than in the 1st generation. These observations suggested that the effects of gestational low-salt intake on offspring survival persist over generations, and the association becomes stronger with each new generation although it is unclear whether genetic background influenced this association. Nevertheless, this is a very important point when considering the role of epigenetic mechanisms in the health risks attributable to low-salt intake.

The survival rate of LM-LB Wistar was 91% in the 1st generation and down to 60% in the 2nd generation. This suggested that gene drift may occur during gestational low-salt intake. The genetic background of salt-sensitivity may be involved in the possible gene drift through gestational low-salt intake.

Several studies have reported the health risk of gestational low-salt intake [1-3]. In animal studies, 0.3% NaCl (w/w) chow is used for a low-salt diet. Regular-salt chow for breeding contains 0.75%NaCl (w/w). Thus, a 50% reduction in the usual salt intake potentially poses a significant risk to neonates. Although care should be taken when extrapolating the data from animal studies to humans, the data are so clear that there is urgent need to investigate this association in humans.

For over 50 years, the role of salt in health has been an important topic in the field of medicine as well as in cardiovascular research. Increased salt intake has been implicated in hypertension and various other vascular diseases. A recent meta-analysis of 25 previous studies on salt intake and an average 3.7-y observational study of 101,945 people in 17 countries wherein salt intake was assessed by determining urinary sodium excretion demonstrated an association between low-salt intake and increase in cardiovascular events or death [18,19]. This is consistent with a J-shaped association between sodium intake and health outcomes [18]. The mechanism by which low-salt intake confers this risk is unclear. An interesting hypothesis that some forms of cardiovascular or metabolic diseases in adulthood are programmed during fetal growth in the uterus has been proposed.

In the present we did not check blood pressure of mothers during gestation. According to our previous study on female

Dahl S rats taking the low-salt (0.3%NaCl, w/w) diet, systolic blood pressure was around 140 mmHg after 2 weeks [20]. These suggested that the decreased survival rate of the pups was not due to collapse of cardiovascular system of mothers fed the low-salt diet during gestation. In addition, since it is technically difficult to measure blood pressure of pups until weaning using the tail-cuff method, it was unclear whether cardiovascular system of the pups was influenced by gestational low-salt intake. These points needs to be clarified in more precise experimental settings. Moreover, it also remains to be elucidated the cardiovascular changes in adulthoof the pups from low-salt mothers.

In conclusion, low-salt intake during pregnancy is associated with high death rates among offspring owing to both neonate and maternal factors. This sensitivity to salt restriction has been proved in this paper to be transmissible at least to the second generation. We urge additional investigations into the effects of para-gestational salt reduction in humans because the level of salt intake required for optimal health varies with subjects' age or stage of growth. Furthermore, the current finding provide evidence that support the recommendation that the amount of salt consumed during pregnancy should be considered carefully, which is based on the data regarding the relationship between mothers and their offspring.

ACKNOWLEDGEMENTS

The authors acknowledge Grant-in-Aids from the Center for Interdisciplinary Studies of Science and Culture, Kyoritsu Women's University & Junior College.

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