

## Long-Term Oral Polyamine Intake Increases Blood Polyamine Concentrations

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**Summary** Although the intracellular de novo synthesis of the polyamines decreases with age, there is no similar trend in blood polyamine levels, but rather there is wide individual variability. We hypothesized that dietary polyamines attenuate a decrease in blood polyamine levels with age and augment the previously observed individual variability. The effect of a polyamine rich diet, in both mice and humans, on blood polyamine concentrations was examined in this study. Jc1:ICR male mice were fed test diets containing 3 different polyamine concentrations. Healthy human male volunteers added 50 to 100 g of the polyamine-rich fermented soybean product, natto, to their daily intake. After 26 wk, the mean blood spermine concentration in mice receiving the test diet with high polyamine concentrations was  $10.1 \pm 2.4 \mu\text{mol/L}$ , while the mean concentrations found in mice fed with a diet with normal or low polyamine concentrations were  $5.2 \pm 0.9$  and  $4.7 \pm 0.5 \mu\text{mol/L}$ , respectively ( $p < 0.05$ ). A mean daily intake of  $66.4 \pm 3.7$  g (range = 46.4–89.3 g) of natto for 2 mo by human volunteers increased the mean blood spermine concentration by a factor of 1.39 ( $n = 10$ ) ( $p < 0.01$ ), while in control volunteers ( $n = 7$ ), asked to exclude polyamine-rich foods from their diet, blood spermine concentration remained unchanged. The individual variability of blood polyamine levels was enhanced after polyamine intake in mice and, to a lesser extent, in humans. The long-term oral intake of enhanced polyamine diets increases blood polyamine levels in both mice and humans.

**Key Words** food, human, mice, spermine, polyamine.

The polyamines, spermine and spermidine, are synthesized in cells and indispensable for cell growth and differentiation (1–3). In addition to intracellular de novo synthesis, cells take up polyamines from the extracellular environment. We have shown that the changes in polyamine concentrations, especially spermine, directly affect the function of immune cells (4, 5).

The increase in blood polyamine concentrations in patients with malignancies and the subsequent decrease after tumor resection suggests that polyamine production and/or concentration in a local part of the body may affect blood polyamine concentrations. Food is considered one of the important sources of polyamines, because spermine and spermidine in the intestinal lumen are absorbed quickly without degradation and distributed throughout the body (6–9). Animal models have shown that polyamine deficient chow, along with concomitant administration of inhibitors of polyamine synthesis and/or antibiotics that suppress intestinal flora, lowers overall blood polyamine concentrations (6). In patients with malignancies, whose blood polyamine levels are often increased, reduced polyamine intake decreases blood polyamine concentration (10).

These findings indicate that the oral intake of polyamine can affect the concentration in the tissue and organs of the body. Therefore, it is plausible that increased oral polyamine intake can increase the total polyamine levels in the body, thereby causing elevated blood polyamine concentrations. However, there is little data to demonstrate that the long-term intake of enhanced oral polyamine diets increases blood polyamine levels, while short-term enhanced oral polyamine intake fails to increase blood polyamine concentration (11).

In a previous study of blood polyamine levels among healthy volunteers, while intracellular polyamine synthesis decreases with age, there is no age-dependent decrease in blood polyamine levels (4). Rather, we observed a very wide individual variability (4). We hypothesized that the polyamine supply from foods abrogates the age-dependent decrease in polyamine concentrations, and, because polyamine concentrations vary widely among foods, the individual variability of the amount of polyamines absorbed from the diet may contribute to the observed variability of blood polyamine concentrations.

In this study, we examined whether the long-term intake of polyamine-rich food can increase the whole

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blood polyamine levels in both mice and humans. Synthetic polyamines mixed in chows were used for the animal experiments, and natto, a traditional Japanese food that contains a high level of the polyamines (spermine and spermidine), was used for the human experiments.

### MATERIALS AND METHODS

**Animals.** This study was approved by the institutional animal welfare committee of Jichi Medical University, and applicable guidelines were followed. "Principles of Laboratory Animal Care" was followed regarding the use and care of the animals. Mice used in the study were euthanized via the intra-peritoneal injection of high dose pentobarbital. Male Jc1:ICR mice (Saitama Doubutsu, Saitama, Japan), were obtained at 8 wk of age. Five to six mice per cage were fed ad libitum with Rodent Laboratory Chow (Nippon Bio-Supply, Tokyo, Japan), and housed in a temperature-controlled (22°C) isolator with a high efficacy particulate-arresting (HEPA; 0.3 µm) filtered air supply, and maintained under a 12 h light/dark diurnal cycle. They were fed with Rodent Laboratory Chow until 24 wk of age and then randomly divided into 3 groups (high polyamine, normal polyamine, and low polyamine chow) and fed ad libitum with experimental chow containing the three different polyamine concentrations. Each group consisted of 30 mice.

**Chows.** Test diets for the animal experiments were prepared by eliminating polyamine-rich materials from standard rodent chow (CE-2, CLEA Japan, Inc., Tokyo, Japan). Since soybean and soybean products contain highly concentrated polyamines (12–14), soybean cake, a major ingredient of commercially prepared

chow, was replaced with casein as the protein source, and soybean oil was replaced with lard as the source of fat. The complete ingredients and nutrition content of the test diets are shown in Table 1.

For the test diet with a high polyamine concentration, synthetic spermine (Wako Pure Chemical Industries, Ltd., Osaka, Japan), spermidine (Sigma Chem. Co., St. Louis, USA), and putrescine (Wako Pure Chemical Industries, Ltd.) were mixed in doses of 0.015, 0.060, and 0.015% (w/w), respectively, as compared to the test diet with a low polyamine concentration. For the test diet with normal polyamine concentration, spermine, spermidine, and putrescine were mixed in doses of 0.002, 0.008, and 0.002% (w/w), respectively, as compared to the test diet with low polyamine concentration. Polyamines were prepared as aqueous solutions and mixed with the raw materials of the chow at 60°C to avoid evaporation of the added polyamines. The test diets were prepared as pellets.

**Data collection.** The survival of each animal was confirmed every 2 d. Blood sampling was performed under anesthesia with pentobarbital (60 µg/g body weight) at the 8th, 16th, and 26th week after feeding with experimental chow. Anesthetized mice underwent a bilateral thoracotomy and blood was collected from the right atrium with a 1 mL syringe and a 26G needle. Whole blood samples were kept in EDTA-coated tubes at –80°C until the assay was performed.

**Polyamine-rich food and human volunteers.** In this study, we used the traditional Japanese food, natto, a fermented soybean product, as a rich source of polyamines. In order to exclude factors influenced by gender, 19 healthy male volunteers were asked to join the study. The study protocol was approved by the Institutional Review Committee of Saitama Medical Center and all volunteers who took part in the study provided informed consent after receiving a thorough explanation. The experimental group ( $n=10$ ) was asked to eat 50–100 g of natto every day in addition to their daily diet for 2 mo, and the control group ( $n=9$ ) was asked to exclude soybean products and fermented foods from their diet during the same period. During the study, two volunteers in the control group were removed from the study, because of a failure of blood collection in one and dropout due to the inability to eliminate soybean products and fermented foods from his diet in the other. This resulted in experimental and control groups consisting of 10 and 7 volunteers, respectively. The mean age of volunteers in the polyamine-rich diet group was  $49.8 \pm 2.9$  y ( $n=10$ , range=35–69 y) and in the control group was  $51.3 \pm 3.7$  y ( $n=7$ , range=40–68 y).

During the study period, volunteers were asked to submit detailed meal records during two separate weeks. According to their records, the daily intake of increased polyamines from natto was quantitated. Morning fasting blood samples were collected before and after the study. For the measurement of polyamine concentrations and analysis of polyamine fractions, 2 mL whole blood samples were stored at –80°C until the assay was performed.

Table 1. Ingredients and nutrition content of test diets used.<sup>1</sup>

	Low polyamine	Normal polyamine	High polyamine
Moisture (w/w %)	7.6	7.6	7.6
Protein (w/w %)	26.4	26.4	26.4
Fat (w/w %)	10.2	10.2	10.2
Fiber (w/w %)	2.5	2.5	2.5
Ash (w/w %)	6.0	6.0	6.0
Nitrogen free extract (w/w %)	47.3	47.3	47.3
Calorie (kcal/100 g)	397	397	397
Putrescine (w/w %)	0	0.002	0.015
Spermidine (w/w %)	0	0.008	0.060
Spermine (w/w %)	0	0.002	0.015

<sup>1</sup>The test diets include: milk casein, white-fish meal, yeast, wheat germ, lard, wheat bran, defatted rice bran, alfalfa meal, wheat meal, maize, milo, vitamin mixture (retinol 0.81 mg, vitamin B<sub>1</sub> 1.71 mg, vitamin B<sub>2</sub> 1.30 mg, vitamin B<sub>6</sub> 1.35 mg, vitamin B<sub>12</sub> 7.65 µg, total vitamin C 17 mg, vitamin E 6.15 mg, pantothenic acid 2.70 mg, niacin 17.95 mg, folic acid 0.26 mg, choline 0.17 g, biotin 45.4 µg, inositol 547 mg in 100 g of chow), and mineral mixture (Ca 1.05 g, P 1.06 g, Mg 0.35 g, K 1.28 g, Mn 10.15 mg, Fe 30.08 mg, Cu 0.75 mg, Zn 5.18 mg, Na 0.45 g in 100 g of chow).

Table 2. Polyamine concentrations in test diets.

	Spermine (nmol/g)	Spermidine (nmol/g)	Putrescine (nmol/g)
Low polyamine test diet	143	224	496
Normal polyamine test diet	160	434	625
High polyamine test diet	374	1,540	1,075

Polyamine concentrations were measured by HPLC.

*Determination of polyamine concentrations in chows and whole blood.* Chow was cut into small pieces and homogenized with 5% trichloroacetic acid. Blood samples were thawed, vortexed, and sonicated twice for 5 min. The samples of chow homogenates and blood were then centrifuged at 18,000  $\times g$  for 10 min, the supernatants transferred to a new microtube, and an equal volume of 20% trichloroacetic acid, containing 20  $\mu M$  *N*-(3-aminopropyl)cadaverine as an internal standard, was added. After centrifugation at 18,000  $\times g$  for 10 min, the supernatant was separated and stored at  $-20^{\circ}C$  until the time of the assay.

For measurement, supernatant was directly injected into a high performance liquid chromatography (HPLC) apparatus (Shimadzu, Kyoto, Japan) for analysis. The HPLC conditions were as follows: column, cation exchange resin, JEOL LC-R-2, 4.6 mm $\times$ 8 cm; elution buffer, a mixture of 12.5% (v/v) of methanol and 87.5% (v/v) of 0.28 M sodium citrate buffer, pH 5.5, containing 2.0 M sodium chloride, 0.5 mL/min; column temperature, 65 $^{\circ}C$ ; post-column reagent solution, 6 mM *o*-phthaldialdehyde in 0.4 M potassium borate buffer, pH 10.4, containing 0.2% mercaptoethanol, 0.1% Brij 35, 0.25 mL/min at 65 $^{\circ}C$ ; fluorescence detection, excitation 345 nm, emission 450 nm.

*Statistical analysis.* Data are expressed as mean  $\pm$  SE (standard error). The results for each experimental mouse group were compared by a post hoc test (Student-Newman-Keuls), and blood polyamine concentrations in human volunteers were compared by a paired *t* test. A *p* value  $<0.05$  was considered to be statistically significant. Relationships between the changes in blood polyamine concentration and the amount of daily natto intake or volunteer's age were tested by linear regression analysis using StatView 5.0 and *p* $<0.05$  was considered significant.

## RESULTS

### *Polyamine concentrations in test diets*

Polyamine concentrations as measured by HPLC of the three different test diets are shown in Table 2. Neither the process of mixing synthetic polyamines with the test diets nor the molding process degrades the polyamines or decreases the polyamine concentrations.

### *Appearance and mortality in mice*

The external appearance of mice fed test diets with a high polyamine concentration did not differ from that of mice fed with test diets with either normal or low polyamine concentrations. After 26 wk of receiving the test

Table 3. Whole blood polyamine concentrations in mice after 8 and 16 wk of feeding with experimental chow.

Test diets	Low polyamine	Normal polyamine	High polyamine
Spermine concentration			
8 wk	3.6 $\pm$ 0.5	4.3 $\pm$ 0.5	4.0 $\pm$ 0.8
16 wk	4.9 $\pm$ 0.7	4.3 $\pm$ 0.4	5.2 $\pm$ 0.7
Spermidine concentration			
8 wk	33.8 $\pm$ 2.8	33.3 $\pm$ 3.7	41.5 $\pm$ 4.1
16 wk	34.8 $\pm$ 2.6	34.6 $\pm$ 1.9	39.7 $\pm$ 2.1

Blood polyamine concentrations were measured by HPLC. Data are presented as mean  $\pm$  SE of 6 mice. There are no significant differences (*p* $>0.05$ ) among the three groups of mice tested.

diets, one mouse had died in each of the low and normal polyamine diet groups. At 50 wk of age, there was no difference in mortality among the three groups.

### *Polyamine concentrations in murine whole blood*

Eight weeks of feeding with test diets having 3 different polyamine concentrations failed to show significant differences in blood polyamine concentrations in mice (Table 3). After 16 wk of consuming the test diets, the mean blood polyamine concentrations in mice did not significantly differ between the three groups (Table 3). However, after 26 wk of consuming the test diets, the mean blood spermine concentration in mice fed the high polyamine diet was 10.1 $\pm$ 2.4  $\mu mol/L$  (range=3.4–22.7  $\mu mol/L$ ), which was higher than that in mice fed a normal concentration polyamine diet at 5.2 $\pm$ 0.9  $\mu mol/L$  (range=3.1–12.4  $\mu mol/L$ ) (*p* $<0.05$ ) while mice fed a low polyamine diet had a mean level of 4.7 $\pm$ 0.5  $\mu mol/L$  (range=3.4–22.7  $\mu mol/L$ ) (*p* $<0.05$ ) (Fig. 1). The mean spermine concentration in mice fed the test diet with a low polyamine concentration did not differ from the concentration in mice fed with test diet with a normal polyamine concentration. There was a somewhat wide variability in the individual spermine concentrations, and the variability was noted to be greater in mice fed the high polyamine test diet.

Similar to spermine concentrations, the blood spermidine concentration variability was augmented in mice fed test diets with a high polyamine concentration (mean=52.7 $\pm$ 8.1  $\mu mol/L$ ; range=29.1–100.3  $\mu mol/L$ ). The mean spermidine concentration in mice fed a high concentration polyamine diet was higher than mice fed test diets with a normal polyamine concentration (mean=34.1 $\pm$ 2.2  $\mu mol/L$ ; range=24.6–45.0  $\mu mol/L$ ) (*p* $<0.05$ ) and with a low polyamine concentration (mean=31.2 $\pm$ 2.2  $\mu mol/L$ ; range=21.8–39.7  $\mu mol/L$ ) (*p* $<0.05$ ) (Fig. 1). The blood spermidine/spermine ratio was similar among the three groups (Fig. 1).

### *Polyamine levels in human blood*

Individual whole blood concentrations of spermine and spermidine varied widely. Volunteers in the control group reported that they almost completely eliminated soybean products and fermented foods from their daily

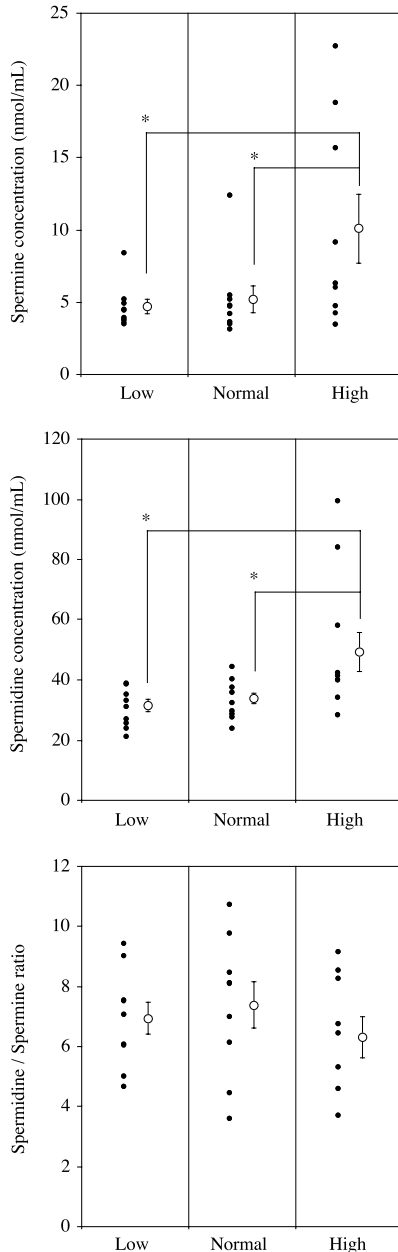


Fig. 1. High polyamine chow increased blood polyamine concentrations significantly, but variably. Mice were fed experimental chow for 26 wk. Blood was collected at 50 wk of age. Whole blood spermine concentrations were measured by HPLC. Filled circles indicate the individual values for 9 mice and open circles indicate the mean values with SE (bars). \* $p < 0.05$ . Low: mice fed a low polyamine concentration chow, Normal: mice fed a normal concentration, High: mice fed a high concentration. Upper: spermine concentration, Middle: spermidine concentration, Lower: spermidine/spermine ratio.

intake. The mean daily consumption of natto by volunteers in the experimental group was  $66.4 \pm 3.7$  g ( $n = 10$ , range = 46.4–89.3 g).

Spermine concentrations in blood increased after 2 mo of supplementation with the high polyamine diet. Among the 10 volunteers in the polyamine rich diet group, the mean spermine concentration before polyamine supplementation was  $5.3 \pm 0.4$   $\mu\text{mol/L}$  (range =

3.5–6.8  $\mu\text{mol/L}$ ), and after supplementation was  $7.2 \pm 0.6$   $\mu\text{mol/L}$  (range = 5.1–10.8  $\mu\text{mol/L}$ ) ( $p < 0.01$ ), a mean increase of 1.39-fold (individual range 0.96–1.83). In all volunteers except one, high oral intake of polyamines increased blood spermine concentrations. High polyamine intake slightly augmented the variability of individual spermine concentrations (Fig. 2). In contrast, among volunteers in the control group, who excluded polyamine-rich foods from their diet, the mean blood spermine concentration before intervention was  $6.7 \pm 3.2$   $\mu\text{mol/L}$  (range = 3.4–12.8  $\mu\text{mol/L}$ ), and did not change after 2 mo ( $6.9 \pm 3.2$   $\mu\text{mol/L}$ ; range = 4.5–13.7  $\mu\text{mol/L}$ ) ( $p = 0.72$ ) (Fig. 3).

Although the blood spermine concentration increased after a polyamine-rich diet, the mean blood spermidine concentration ( $12.3 \pm 1.4$   $\mu\text{mol/L}$  before supplementation) did not change after 2 mo of supplementation ( $12.8 \pm 2.4$   $\mu\text{mol/L}$ ) ( $p = 0.58$ ). Increased spermidine concentration was seen in four of 10 individuals, while six of them had decreased levels (Fig. 2). Among the control volunteers, blood spermidine concentrations also did not change (from  $10.6 \pm 1.8$   $\mu\text{mol/L}$  before, to  $11.0 \pm 1.4$   $\mu\text{mol/L}$  after) ( $p = 0.55$ ) (Fig. 3).

The blood spermidine/spermine ratio showed a significant change after eating a polyamine-rich diet. Among those in the high polyamine intake group, the blood spermidine/spermine ratio decreased in all subjects; the mean blood spermidine/spermine ratio decreased from  $2.4 \pm 0.5$  to  $1.8 \pm 0.4$  ( $p < 0.01$ ) (Fig. 2). However, among those in the control group, the blood spermidine/spermine ratio did not change during the observation period ( $1.8 \pm 0.5$  to  $1.8 \pm 0.6$ ) ( $p = 0.85$ ) (Fig. 3).

The amount of natto intake did not correlate with changes in blood spermine concentration (spermine concentration after the intervention/spermine concentration before the intervention). Although not statistically significant ( $p = 0.06$ ), age did have a positive correlation ( $r = 0.62$ ) with changes in blood spermine concentration.

## DISCUSSION

This study demonstrates that long term oral intake of a polyamine-rich diet can increase blood polyamine levels. However, an increase in blood polyamine concentrations was not observed after a short-term intake of a polyamine-rich diet. Brodal et al. have shown that intake of a polyamine-rich (5 times higher than regular chow) diet for 20 d failed to increase blood polyamine levels in the rat (11). The mechanism and metabolic changes by which short-term intake fails to increase blood polyamine levels while long term intake increases blood polyamine concentration requires further study to be elucidated.

The concentration of intra-cellular polyamines is regulated both by the activities of metabolic and catabolic enzymes and by the export and import of polyamines through cell membranes. Therefore, an acute increase in intracellular polyamines may be regulated by enhanced degradations of spermine and spermidine or

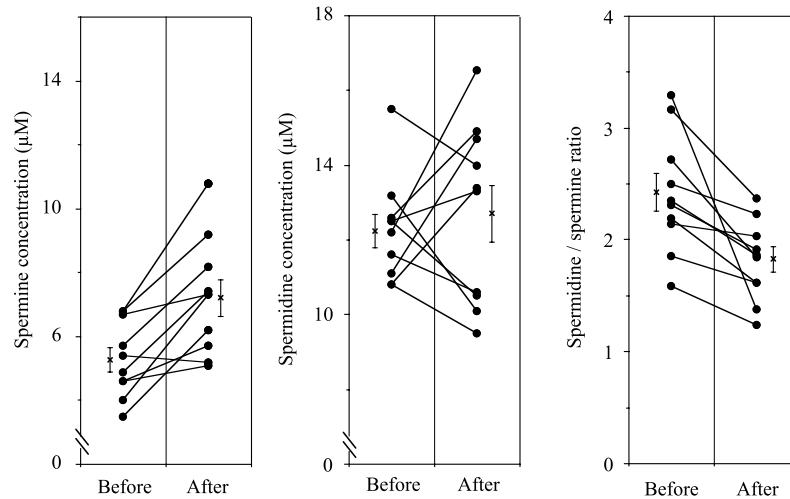


Fig. 2. Daily supplementation (50–100 g) for 2 mo with high polyamine food increased blood spermine concentrations (left) in human volunteers tested except one, but did not affect the blood spermidine concentrations (middle), resulting in decreased spermidine/spermine ratios (right). Filled circles indicate individual values and asterisks indicate mean values with SE (bars). The left column in each figure indicates values before polyamine supplementation, and the right column indicates values after 2 mo of supplementation.

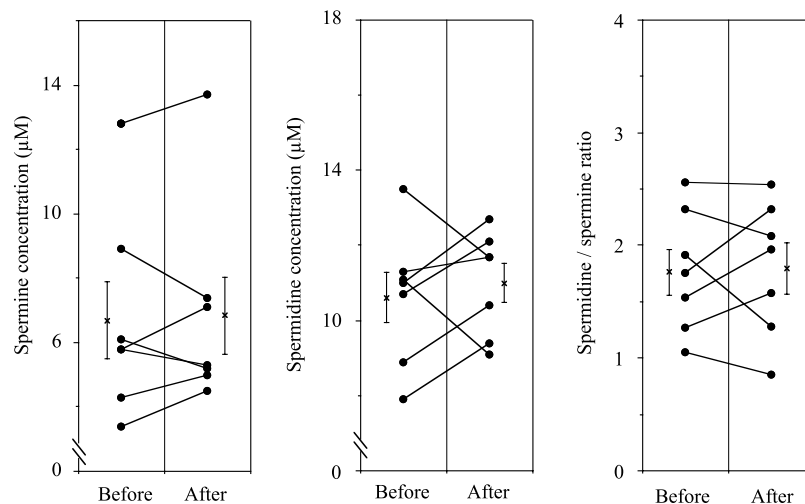


Fig. 3. Whole blood polyamine concentrations did not change in humans who excluded polyamine-rich food such as soybean products or fermented food from their diet. Filled circles indicate individual values and asterisks indicate mean values with SE (bars). The left column in each figure indicates values before intervention, and the right column indicates values after the intervention.

by increased transport out of the cells. A cellular mechanism to prevent acute changes in intracellular polyamine concentrations may partially explain why 8 to 16 wk of increased polyamine intake failed to elevate blood polyamine concentration. As shown by previous observations (15, 16), which show that blood polyamine concentrations in patients with malignancies whose polyamine supply is abundant from the tumors are often increased, a continuous supply from extracellular sources may help maintain the intracellular polyamine concentration and, as a result, gradually alter the blood polyamine concentration. Considering that polyamine synthesis decreases with age and the observation here that polyamine rich foods had a greater effect on blood spermine concentrations in older individuals than in young people, blood spermine levels

in older people may be more affected by the oral intake of polyamines. In the animal experiments, high polyamine chow was noted to elevate blood polyamine levels as the mice advanced in age, possibly for a similar reason.

In animal experiments, the concentrations of spermine and spermidine in chows were estimated based on the information published previously on polyamine concentrations in daily foods, and on the subacute toxicity of polyamines (3, 12–14, 17). High polyamine chow was prepared so that the concentrations of spermine and spermidine were approximately 2–3 fold higher than the concentrations in cheese and soybeans, and the polyamine concentrations in the experimental chows are far below toxic levels (14, 17).

The concentrations of spermine and spermidine in

foods vary considerably. Among natural products, soybeans are one food that contains a large amount of both spermine and spermidine (12, 13, 18). In addition, fermentation increases and modifies the concentration of polyamines; cheese contains abundant polyamines, whereas milk does not (3). Natto, being representative of fermented soy, contains abundant spermine and spermidine. Based on previous data (12), 100 g of natto contains approximately up to 80  $\mu\text{mol}$  of the polyamines spermine and spermidine. Therefore, the 66 g of natto consumed by volunteers is estimated to contain up to 54  $\mu\text{mol}$  of polyamines. The Japanese daily oral intake of polyamines including diamines is thought to be about 250–350  $\mu\text{mol}$ . Therefore, a daily intake of 66 g of natto contains about one sixth of the daily polyamine intake (3, 19).

Although the blood spermidine/spermine ratio in mice did not change after increased polyamine intake, this ratio did decrease in human volunteers. The difference in blood spermidine/spermine ratio between mice and human volunteers might be due to a species difference, differences in the ratios of spermine/spermidine loaded, and differences in the intestinal environment such as endogenous flora. Because enhanced oral polyamine intake augments the variability of blood polyamine concentrations and polyamine concentrations vary widely among foods, the wide individual variability in blood polyamine concentrations is considered to be, at least partly, due to the amount of oral polyamine intake. Further studies are necessary to elucidate the mechanisms of control of polyamine levels at both the cellular and organism level.

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