# **ORIGINAL ARTICLE**

# Protective Effects of Japanese Soybean Paste (Miso) on Stroke in Stroke-Prone Spontaneously Hypertensive Rats (SHRSP)

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# BACKGROUND AND HYPOSESIS

Soybean isoflavones have been shown to reduce the risk of cerebral infarction in humans according to epidemiological studies. However, whether intake of miso can reduce the incidence of stroke in animal models remains unknown. In this study, we investigated the effects of soybean paste (miso) in an animal model of stroke.

#### METHODS

Stroke-prone spontaneously hypertensive rats (SHRSP) were fed a miso diet (normal diet 90%, miso 10%; final NaCl content 2.8%), a high salt diet (normal diet and NaCl 2.5%; final NaCl content 2.8%), or a low salt diet (normal diet; final NaCl content 0.3%).

## RESULTS

Kaplan–Meier survival curves revealed a significantly lower survival rate in the high salt group compared to the miso group (P = 0.002)

Miso is a traditional Japanese food fermented from soybeans. It is used regularly as a flavoring in soup and is an essential ingredient in Japanese-style cooking. We previously reported that miso can suppress the development of liver tumors, gastric tumors, lung tumors, and aberrant crypt foci and colon tumors in mice and rats.<sup>1</sup> We also reported that miso can prevent the induction of hypertension in Dahl salt-sensitive hypertensive rats despite its high salt content.<sup>2</sup> These findings were further supported by similar studies conducted by Yoshinaga *et al.*<sup>3</sup>

Kokubo *et al.*<sup>4</sup> reported an inverse association between isoflavone intake and risk of cerebral and myocardial infarctions in Japanese women. In this context, we thought that it would be interesting to examine the association between brain infarction and nutrition; more specifically, whether administration of miso can reduce the incidence of stroke in animal models. Stroke-prone spontaneously hypertensive rats (SHRSP) are generated from spontaneously

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and the low salt group ( $P \le 0.001$ ). Large hemorrhagic macules were found in the cerebrum in the high salt group, whereas none were found in the other 2 groups. There were also fewer histological and immunohistochemical changes in the brain and kidneys in the miso group compared to the high salt group.

#### CONCLUSION

Our results suggest that miso may have protective effects against stroke despite its high salt content.

*Keywords:* blood pressure; brain protection; hypertension; miso (Japanese soy bean paste); SHRSP; stroke.

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hypertensive rats (SHR),<sup>5-11</sup> and excessive salt intake in this model increases the rate of fatal strokes. Sepehrdad *et al.*<sup>9</sup> reported that all SHRSP provided with a 1% NaCl drinking solution died by 16.4 weeks. Camargo *et al.*<sup>10</sup> reported that survival of SHRSP at 12 weeks was 26% in the 4% NaCl diet group and Kim-Mitsuyama *et al.*<sup>11</sup> reported that all SHRSP died by 42 days when provided a 8% NaCl diet. In this study, we examined the effects of miso intake on the incidence of stroke in SHRSP.

# MATERIALS AND METHODS

This study was carried out in accordance with guidelines of the Institute of Laboratory Animal Science, Hiroshima University. The experimental protocols were approved by the ethics committee on animal experiments of Hiroshima University (Permission Number: A-14–114).

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A total of 36 4-week-old male SHRSP (SHRSP/Izm) were purchased from Nihon SLC (Hamamatsu, Japan) and divided into 3 groups: low salt diet (normal diet, Oriental Yeast, Tokyo, Japan; final NaCl content 0.3%), miso diet (normal diet 90% and miso 10%; final NaCl content 2.8%), and high salt diet (normal diet supplemented with 2.5% NaCl; final NaCl content 2.8%). We used red rice miso, which was fermented for 180 days and freeze-dried by the supplier (Miyasaka Jozo, Tokyo, Japan).

Rats were maintained as previously reported.<sup>2</sup> Normal tap water was provided ad libitum. Animals were observed 3 times per day and autopsied under ether anesthesia if they showed ataxic movements, were motionless, or were moribund. Animals alive at the end of experiment (63 days) were examined under ether anesthesia. Animal weight and food and water intake were measured.

Blood pressure was measured at 14, 28, and 42 days after initiation of the specific diets using the tail-cuff method (BP-98E, Softron, Tokyo, Japan).9 Bodies and major organs were weighed and fixed in phosphate-buffered 3% formalin. Histological evaluation was performed by routine procedures with H&E, periodic acid-Schiff-Alcian blue, and Azan-Mallory staining.

Using a cross-section containing the cerebral cortex, thalamus, third ventricle, and hippocampus at level II, as described by Solleld et al.,<sup>12</sup> we examined all arteries (A) and veins (V) on the surface (S) of the brain and referred to them as A-S and V-S, respectively. Similarly, arteries and veins inside (I) the brain were examined and referred to as A-I and V-I, respectively. Vessels in each classification (A-S, V-S, A-I, and V-I) were scored as zero or one. When no thrombus was observed, the score was zero. If one or more thrombi were identified, a score of one was assigned.

(a)

250

SRI

The scores of the 4 classifications were summed to generate a total score (minimum, 0; maximum, 4), and the mean total score  $\pm$  SD was calculated for each group.

Kidney sections (3-µm thick) were treated for 30 minutes at room temperature with 2% bovine serum albumin and incubated with primary antibodies against CD68 (diluted 1:200; Serotec MCA341R), and monoclonal mouse anti-a smooth muscle antigen (dilute 1:1000, Sigma-Aldrich A2547) antibodies overnight at 4 °C. Serial sections were used for negative controls (no primary antibody). All slides were exposed to a biotinylated secondary antibody and streptavidin-peroxidase using the Ultra Tech HRP kit (Ultra Tech HRP PN IM2391). Peroxidase activity was visualized by treatment with H<sub>2</sub>O<sub>2</sub> and diaminobenzidine for 30 minutes. At the final step,  $\alpha$  smooth muscle antigen-stained sections were counterstained with periodic acid-Schiff. CD68-positive cells were counted per 10 sites at 200× magnification, and a smooth muscle antigen-positive areas were measured using an image analyzer (Win Roof, Mitani, Fukui, Japan).

Statistical significance was determined with Dunnett's method, the  $\chi^2$ -test, fitted linear regression lines with the common intercept and different slopes on a scatter plot (multiple regression model), and Kaplan-Meier survival curves as assessed by log-rank test.

Body weight at 56 days after initiation of the diets did not

differ between the 3 groups (Supplementary Table 1).

# RESULTS

#### **Body weight**

(b) 100 Low salt (Normal diet) 60 Survival rate (%) Miso =0.062 40



Figure 1. Blood pressure and event-free survival of rats. (a) Scatter plot of systolic blood pressure (SBP) and diastolic blood pressure (DBP) in SHRSP with fitted linear regression lines showing the common intercept and different slopes. Animals were given a low salt diet (normal diet, 0.3% NaCl), miso diet (2.8% NaCl), or high salt diet (2.8% NaCl) for the indicated durations. (b) Kaplan-Meier survival curves in the low salt (normal diet), miso, and high salt groups, as assessed by log-rank test. The experimental span (from the first treatment of different diets to when the animal died or was sacrificed) of the high salt group was significantly shorter (P < 0.001) compared to the low salt group and the miso group (P = 0.002). There was no significant difference between the low salt group and the miso group (P = 0.062).

# Intake of water and food

After initiation of the diets, the amount of drinking water and food consumed by each group were measured for 56 days and mean values were calculated as ml/animal/day or gram/ animal/day, respectively. Water consumption in the high salt group ( $42.6 \pm 7.4$  ml/animal/day) was significantly higher than in the miso group ( $38.3 \pm 4.7$  ml/animal/day), which was greater than in the low salt group ( $29.8 \pm 4.4$  ml/animal/ day). There were no significant differences in amount of food consumed between groups (Supplementary Table 2).

# **Blood pressure**

The fitted linear regression lines on a scatter plot revealed a significantly higher systolic blood pressure (SBP) in the high salt group compared to the low salt group (P < 0.001) and the miso group (P = 0.009). There was no significant difference in systolic blood pressure between the low salt group and the miso group (P = 0.457). Diastolic blood pressure was significantly higher in the high salt group compared to the low salt group (P = 0.006) and the miso group (P = 0.027). There was no significant difference in diastolic blood pressure between the low salt group and the miso group (P = 0.027). There was no significant difference in diastolic blood pressure between the low salt group and the miso group (P = 0.611).

#### Animal survival

Of the 36 total rats, we observed 18 events of paralysis and 1 death. Rats were autopsied immediately after these events. None of the rats in the high salt group survived for 64 days, while survival was noted for 7 (54%) in the miso group and 10 (83%) in the low salt group (Supplementary Table 3). The Cox proportional hazards model revealed that event-free time was significantly shorter in the high salt group compared to the low salt group (P < 0.001) and the miso group (P = 0.002) (Figure 1b). There was no significant difference between the low salt group and the miso group (P = 0.062).

#### Histological findings in the brain

Macroscopically, large hemorrhagic macules were observed on the surface of the cerebrum in 6 of 12 rats in the high salt group (Figure 2a, A), whereas no macules were observed in the miso and low salt groups (P < 0.01, Figure 2b). After macroscopic inspection, brains were sectioned at the caudal border of the mammillary body for microscopic analysis.<sup>12</sup> Small hemorrhagic sites were observed in both the miso and high salt groups (Figure 2a, B), with no significant difference in the number of sites between the 2 groups (Figure 2b).



**Figure 2.** Macroscopic and microscopic analysis of brain. (a) Bleeding in the brain (macroscopic (A) and microscopic (B)). (b) Incidence of bleeding in the brain (by macroscopic and microscopic observations). In the high salt group, the number of large hemorrhagic macules in the brain was significantly higher than that in the miso and low salt groups. (c) Microscopic analysis of arteries and veins on the surface of the brain in the low salt (A), miso (B), and high salt (C) groups. Thrombi in arteries and veins on the surface of brain are indicated by arrows (C). The bar indicates 100  $\mu$ m. (d) Thrombus scores for all vessels in the brain. Dots (•) indicate the score of each animal. At the bottom of the figure, scores for each group are expressed as mean  $\pm$  SD. \*\*Significantly different from low salt group (P < 0.01), \*Significantly different from miso group (P < 0.05).

Thrombi were frequently observed in cerebral arteries and veins in the high salt group (Figure 2c, C). The thrombus score significantly differed between the 3 groups (low salt,  $1.92 \pm 1.00$ ; miso,  $2.23 \pm 0.90$ ; high salt,  $3.25 \pm 1.06$ ; Figure 2d).

# **Biochemical markers**

Supplementary Table 3 summarizes the biochemical parameters of the 3 groups. Blood glucose and total cholesterol levels in the miso and high salt groups were significantly increased compared to the low salt group. Conversely, total protein, albumin, ALP, Na, and Cl levels were lower in the miso and high salt groups compared to the low salt group. It is noteworthy that creatinine and BUN in the high salt group was significantly higher than in the miso and low salt groups.

# Histological findings in kidneys

Protein casts and thickened adventitia of arteries were increased in the miso and high salt groups compared to the low salt group (Figure 3a), while the number of degenerated glomeruli was only increased in the high salt group (Supplementary Table 4). Area of collagen fibers was also significantly larger in the high salt group compared to the other 2 groups. Pale staining of columnar epithelium of renal tubules was detected in all groups (Figure 3a).

The numbers of CD68-positive cells and  $\alpha$  smooth muscle antigen-positive areas, both of which are markers of kidney

damage, were decreased in the miso group compared to the high salt group (Figures 3b and c).

# DISCUSSION

In the present study, miso intake reduced the incidence of stroke in a rat stroke model despite its high salt content, and also suppressed injuries to the brain and kidneys. Alderman et al. proposed an interesting paradox that among people with high sodium consumption, Japanese people have a longer lifespan.<sup>13</sup> Anderson et al. showed that, when comparing Japan, the United States, the United Kingdom, and China, people in Japan had the highest sodium intake, of which approximately 30% was from fermented foods such as miso and soy sauce; however, blood pressure was the lowest in Japan.<sup>14</sup> We previously reported that miso has potent antihypertensive effects in Dahl salt-sensitive hypertensive rats.<sup>2</sup> In that study, the difference in blood pressure between the miso group and high salt group was substantial and reached 35 mm Hg. Here, we found that miso also has antihypertensive effects in a different model of hypertension. However, the effects of miso were somewhat weaker in this model, with a difference in blood pressure of only about 10 mm Hg between the miso and high salt groups.

Yamori *et al.* reported that inclusion of supplemental proteins such as fish or soybean protein, calcium, potassium, magnesium, and fiber in food can effectively prevent stroke in SHRSP.<sup>7</sup> Although the antihypertensive effect of miso was not so remarkable in SHRSP, miso clearly reduced the



**Figure 3.** Microscopic and immunohistological analysis of kidneys. (a) Histological examination of kidneys in the low salt (A), miso (B), and high salt (C) groups. Pale staining columnar epithelium (arrows), protein cast in renal cortex (P), degeneration of glomeruli (D), and thickening of adventitia of arteries (T). Bar indicates 100  $\mu$ m, Azan Mallory staining. (b) Number of CD68-positive cells in the kidney cortex. Cell number per one microscopic field under 200× magnification was counted and expressed as mean ± SD for each group. (c)  $\alpha$ SMA-positive areas in the kidney cortex.  $\alpha$ SMA-positive areas per field (200× magnification) were measured and expressed as mean ± SD ( $\mu$ m<sup>2</sup>) for each group. Abbreviation:  $\alpha$ SMA, anti- $\alpha$  smooth muscle antigen.

incidence of fatal stroke, suggesting that miso components may have a direct effect on stroke prevention.

Isoflavones found in soybeans are known to have cholesterol-lowering effects.<sup>15</sup> Liu *et al.* reported in a meta-analysis of 11 randomized controlled trials that ingestion of 65–153 mg of soy isoflavones per day lowered blood pressure in hypertensive subjects.<sup>16</sup> Furthermore, Kokubo *et al.* reported an inverse association between dietary intake of isoflavones, miso, and beans and cerebral and myocardial infarctions in Japanese women without cardiovascular disease.<sup>4</sup> Given that miso contains a lower amount of isoflavones than beans (Nakano K, Central Miso Institute, Tokyo, Japan, personal communication, 2010–2017), isoflavones may play a minor role in the antistroke effects of miso.

We recently found that the levels of 25 substances, including genistein, several antihypertensive substances, antidiabetic substances, and some antioxidants, increase during the fermentation/maturation of miso (unpublished data). This raises the possibility that miso may contain compounds that are directly protective against stroke. Further studies will be needed to identify the effective factors in miso that confer protection against stroke and to elucidate the mechanisms underlying this effect.

As for the strength of the present study, our findings may encourage Japanese people—who are accustomed to foods with salty taste—to use more miso instead of salt for seasoning dishes, given that the Japanese diet is generally high in salt. However, there is a limitation to our study: we used an animal model. Therefore, further investigation will be necessary to verify our results in humans.

In conclusion, our findings suggest that a miso-containing diet, regardless of its high salt content, may be protective against stroke.

### SUPPLEMENTARY MATERIAL

Supplementary data are available at *American Journal of Hypertension* online.

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#### DISCLOSURE

The authors declared no conflict of interest.

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