Title	Protective Effects of Japanese Soybean Paste (Miso) on Stroke in Stroke-Prone Spontaneously Hypertensive Rats (SHRSP)
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Protective effects of Japanese soybean paste (miso) on stroke in stroke-prone spontaneously hypertensive rats (SHRSP)

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Running title: Protective effects of miso on stroke in SHRSP

Key words: SHRSP, stroke, brain protection, miso

The authors declare no conflicts of interest.

Abstract

BACKGROUND

According to epidemiological reports, soybean has been proposed to reduce the risk of cerebral infarctions. However, it is unknown whether miso can reduce the incidence of stroke in animal models. 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 only; final NaCl content 0.3%).

RESULTS

Survival in the high salt group was significantly lower than in the miso and low salt groups ($p<0.01$). Large hemorrhagic macules were found in the cerebrum in the high salt group, whereas none were found in the other two groups. There were also fewer histological and immunohistochemical changes in the brain and kidneys in the miso group compared to the high salt group.

CONCLUSIONS

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

INTRODUCTION

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 (ACF) and colon tumors in mice and rats[1]. We also reported that miso can prevent the induction of hypertension in Dahl salt sensitive hypertensive rats despite its high salt content[2]. These findings were further supported by similar studies conducted by Yoshinaga et al.[3]

Kokubo et al.[4] 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 hypertensive rats (SHR)[5-11], and excessive salt intake in this model increases the rate of fatal strokes. Sepehrdad et al.[9] reported that all SHRSP provided with a 1% NaCl drinking solution died by 16.4 weeks. Camargo et al.[10] reported that survival of SHRSP at 12 weeks was 26% in the 4% NaCl diet group and Kim-Mitsuyama et al.[11] 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).

A total of 36 4-week-old male SHRSP (SHRSP/Izm) were purchased from Nihon SLC, Ltd. (Hamamatsu, Japan) and divided into three groups: low salt diet (normal diet, Oriental Yeast Co., 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 Co. Ltd., Tokyo, Japan).

Rats were maintained as previously reported[2]. Normal tap water was provided *ad libitum*. Animals were observed three 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 Co. Ltd., Tokyo)[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 (PAS)-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.[12], 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. The scores of the four classifications were summed to generate a total score (minimum, 0; maximum, 4), and the mean total score ± SD was calculated for each group.

Kidney sections (3-μm thick) were treated for 30 min at room temperature with 2% BSA and incubated with primary antibodies against CD68 (diluted 1:200; Serotec

MCA341R), and monoclonal mouse anti-α smooth muscle antigen (αSMA, dilute 1:1000, Sigma-Aldrich A2547) antibodies overnight at 4℃. 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_2O_2 and diaminobenzidine for 30 min. At the final step, αSMA-stained sections were counterstained with PAS. CD68-positive cells were counted per 10 sites at 200x magnification, and αSMA-positive areas were measured using an image analyzer (Win Roof, Mitani Co, Fukui Japan).

Statistical significance was determined with Dunnett's method, multiple linear regression, Cox proportional hazards model, and the χ^2-test.

RESULTS

Body weight

Body weight at 56 days after initiation of the diets did not differ between the three groups (Supplementary Table 1).

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±7.4 ml/animal/day) was significantly higher than in the miso group (38.3±4.7 ml/animal/day), which was greater than in the low salt group (29.8±4.4 ml/animal/day). There were no significant differences in amount of food consumed between groups (Supplementary Table 2).

Blood pressure

At 28 days after the initiation of diets, systolic blood pressure (SBP) in the high salt group was significantly increased ($p<0.01$) compared to the miso and low salt groups. This difference was maintained until 42 days ($p<0.05$, Figure 1A). Although

diastolic blood pressure (DBP) was significantly increased in both miso and high salt groups at 14 days ($p<0.05$), only the high salt group showed significantly higher DBP at 42 days ($p<0.05$).

Animal survival

Of the 36 total rats, we observed 18 events of paralysis and one 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 seven (64%) 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 only in the high salt group ($p=0.0008$, Figure 1B).

Histological findings in the brain

Macroscopically, large hemorrhagic macules were observed on the surface of the cerebrum in six of 12 rats in the high salt group (Figure 2Aa), 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[12]. Small hemorrhagic sites were observed in both the miso and

high salt groups (Figure 2Ab), with no significant difference in the number of sites between the two groups (Figure 2B).

Thrombi were frequently observed in cerebral arteries and veins in the high salt group (Figure 2Cc). The thrombus score significantly differed between the three groups (low salt, 1.92±1.00; miso, 2.23±0.90; high salt, 3.25±1.06; Figure 2D).

Biochemical markers

Supplementary Table 3 summarizes the biochemical parameters of the three 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 two groups. Pale staining of columnar epithelium of renal tubules was detected in all groups (Figure 3A).

The numbers of CD68-positive cells and αSMA-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 3C).

DISCUSSION

The findings of the present study suggest that miso can suppress the incidence of stroke and injuries to the brain and kidneys in a rat model of stroke relative to high salt intake conditions. Alderman et al. proposed an interesting paradox that amongst people with high sodium consumption, Japanese people have a longer lifespan[13]. 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[14]. We previously reported that miso has potent anti-hypertensive effects in Dahl salt sensitive hypertensive rats[2]. In that study, the difference in blood pressure between the miso group and high salt group was substantial and reached 35 mmHg. Here, we

found that miso also has anti-hypertensive 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 mmHg 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[7]. Although the anti-hypertensive effect of miso was not so remarkable in SHRSP, miso clearly reduced the 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[16]. 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[17]. 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[4]. Given that miso contains a lower amount of isoflavones than beans (Nakano K, Central Miso Institute, Tokyo, Japan, personal communication), isoflavones may play a minor role in the anti-stroke effects of miso.

We recently found that the levels of 25 substances, including genistein, several anti-hypertensive substances, anti-diabetic 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.

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

Source of Funding

This work was supported by a grant-in-aid from the Central Miso Institute, Tokyo, Japan.

FIGURE LEGENDS

Figure 1. **Blood pressure and event-free survival of rats**

A. Mean systolic blood pressure (SBP) and diastolic blood pressure (DBP) in SHRSP. Data are presented as mean±SD. Animals were given a low salt diet (0.3%NaCl), a miso diet (2.8% NaCl), or a high salt diet (2.8% NaCl) for the indicated durations. *Significantly different from low salt group ($p<0.05$), ** Significantly different from low salt group ($p<0.01$), § Significantly different from miso group ($p<0.05$), †Significantly different from miso group ($p<0.01$).

B. Event-free survival in the low salt (●), miso (△), and high salt (■) groups, as assessed by Cox proportional hazards model analysis. Life span of the high salt group was significantly shorter ($P=0.0008$) compared to the miso and low salt groups.

Figure 2. Macroscopic and microscopic analysis of brain

A. Bleeding in the brain (macroscopic (2Aa) and microscopic (2Ab)).

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 (2Ca), miso (2Cb), and high salt (2Cc) groups. Thrombi in arteries and veins on the

surface of brain are indicated by arrows (2Cc). The bar indicates 100 μm.

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±SD. ** Significantly different from low salt group ($p<0.01$), * Significantly different from miso group ($p<0.05$).

Figure 3. Microscopic and immunohistological analysis of kidneys

A. Histological examination of kidneys in the low salt (3Aa), miso (3Ab), and high salt (3Ac) 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 μm, Azan Mallory staining.

B. Number of CD68-positive cells in the kidney cortex. Cell number per one microscopic field under 200x magnification was counted and expressed as mean±SD for each group.

C. αSMA-positive areas in the kidney cortex. αSMA-positive areas per field (200x magnification) were measured and expressed as mean±SD (μm^2) for each group.

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Fig. 1

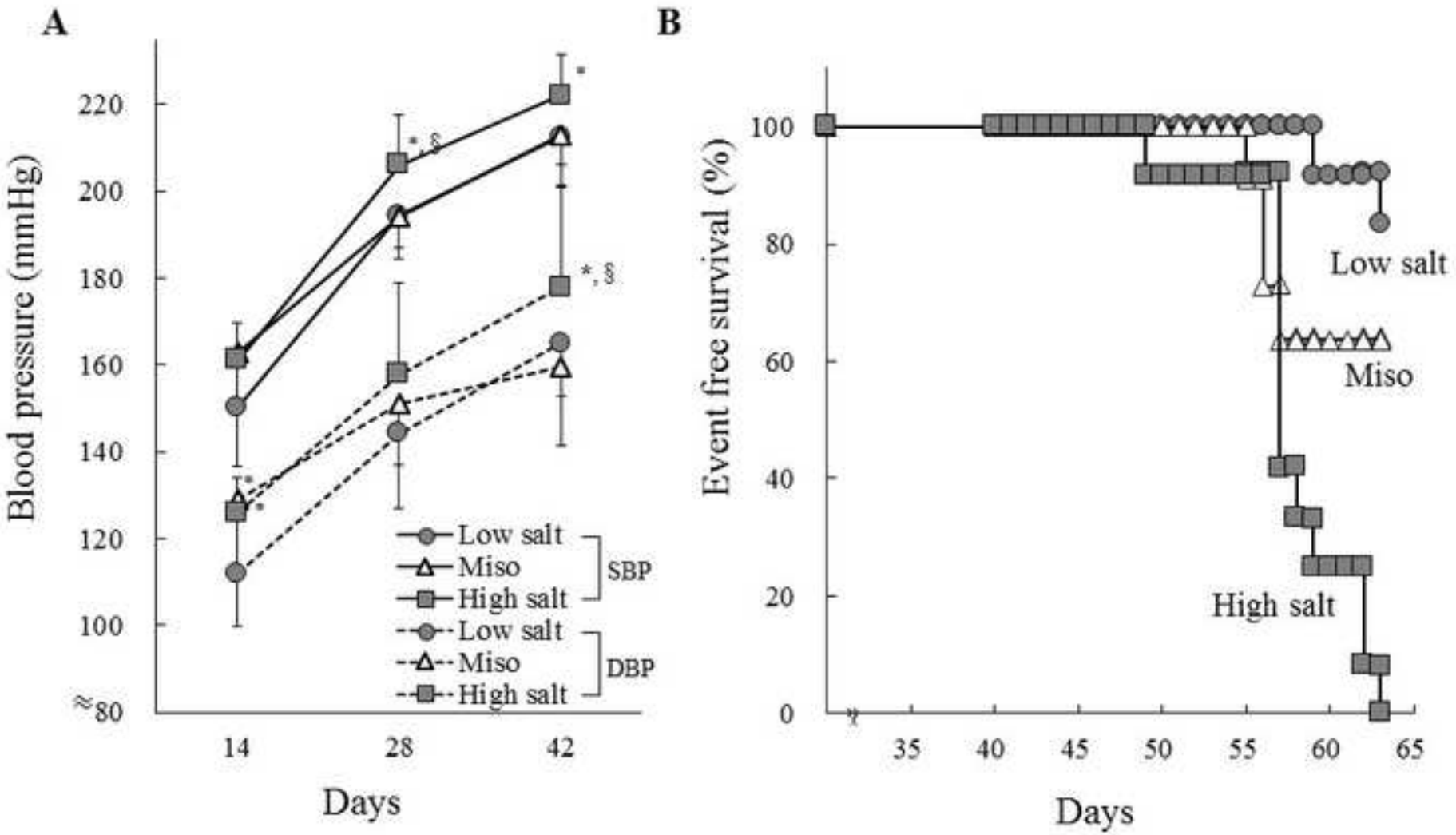


Fig. 2

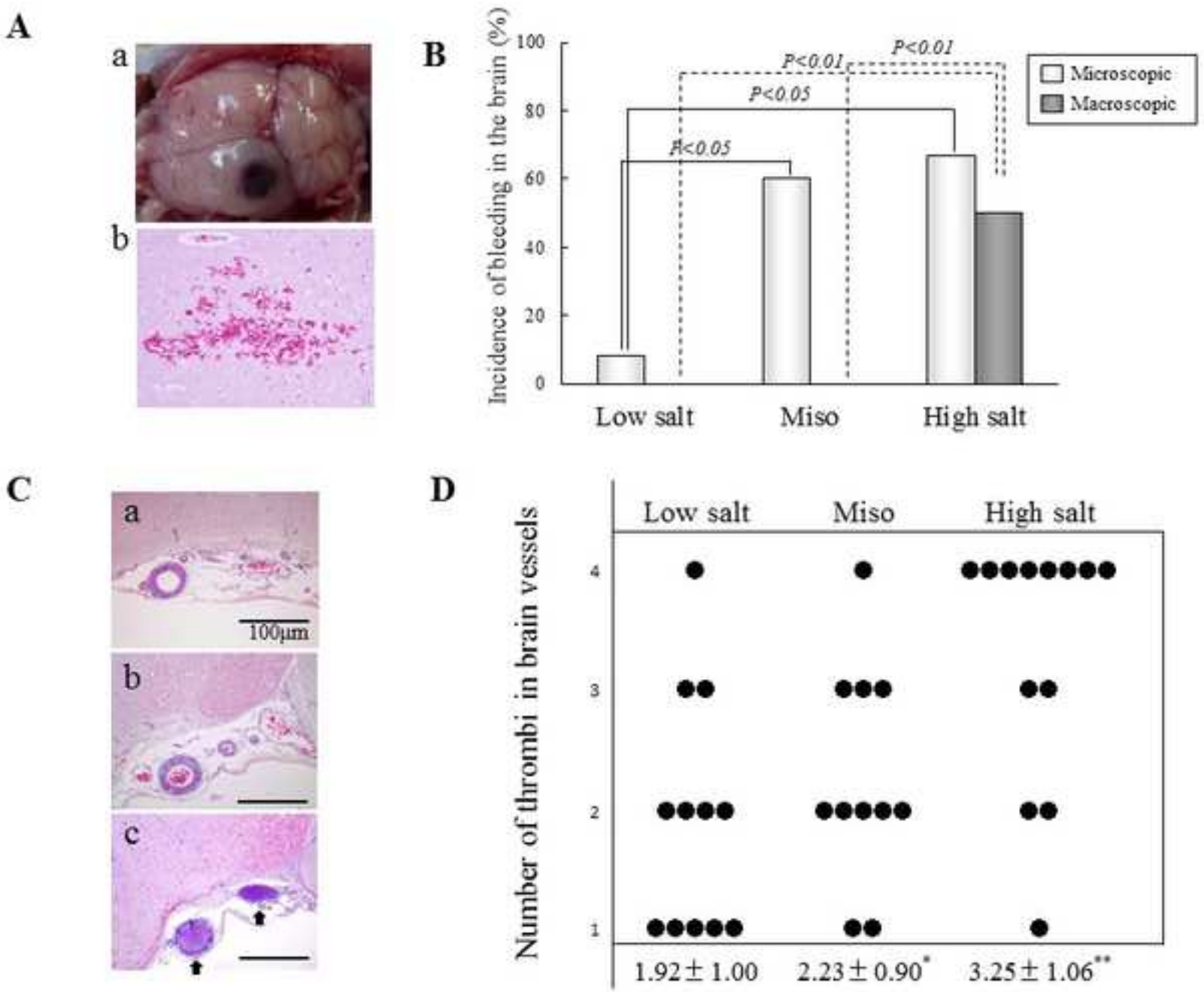
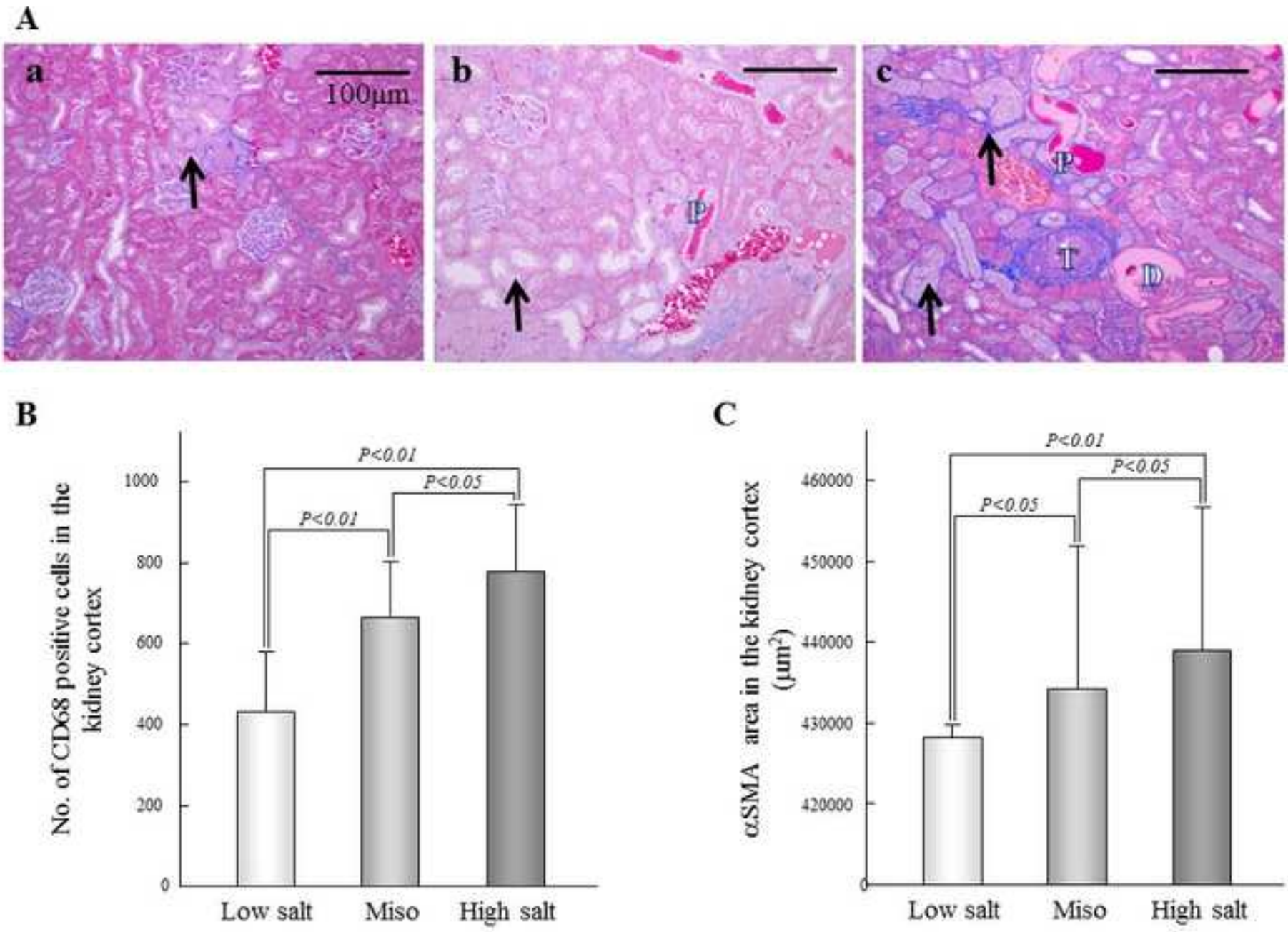


Fig. 3



Cover Letter

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Apr 7th, 2017

Dear Sir:

Attached paper and files are the manuscript entitled **"Protective effects of Japanese soybean paste (miso) on stroke in stroke-prone spontaneously hypertensive rats (SHRSP)"** myseli Sasatani M, Doi T, Masaki T and Yoshizumi M.

We would appreciate its consideration for publication in the **American Journal of Hypertensiont** contributions to the content of the paper. This manuscript has not been previously published nor are they under consideration for publication in another journal. Animals were maintained under the guidelines set forth in the "Guide for the Care and Use of Laboratory Animals" by the Hiroshima University. And also English was checked by a native English speaking scientist. All authors have seen and approved of the study submitted. The authors declare no conflicts of interest.

Thank you very much for your consideration.

Sincerely yours,

Information of recommended individuals to peer review the submitted manuscript

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SUPPLEMENTAL DATA

Supplementary Table 1. Body weight (g)

Days	Low salt	Miso	High salt
0	67.0±3.1 (12)	66.9±3.7 (12)	63.4±4.6 (12)
14	145.5±5.5 (12)	142.8±6.0 (12)	142.6±5.9 (12)
28	207.3±8.6 (12)	207.6±11.0 (12)	215.2±9.3 (12)
42	237.1±11.7 (12)	238.6±13.0 (12)	243.5±9.5 (12)
56	254.8±14.4 (12)	243.3±37.2 (10)	234.50±28.4 (12)
58	NA	NA	232.7±16.5 (6)
63	257.2±28.4 (10)	232.7±36.6* (6)	NA

Data are presented as mean±SD

( ) Number of animals

* Significantly different from 63 days in low salt group ($p<0.05$)

Supplementary Table 2. Water and food intake

	Low salt	Miso	High salt
Drinking water (ml/animal/day)	29.8±4.4	38.3±4.7**	42.6±7.4**,§
Food (g/animal/day)	15.7±0.6	15.7±0.8	16.3±1.2

Data are presented as mean±SD

** Significantly different from low salt group ($p<0.01$)

§ Significantly different from miso group ($p<0.01$)

Supplementary Table 3. Number of surviving rats 64 days after treatment

Group	Total animals	Surviving animals
Low salt	12	10 (83%)
Miso	11	7 (64%)
High salt	12	0* (0%)

* Significantly different from low salt and miso groups ($p<0.05$)

Supplementary Table 4. Biochemical markers

	Low salt	Miso	High salt
Total protein (g/dl)	6.61±0.27	6.17±0.42**	6.19±0.45**
Albumin (g/dl)	4.58±0.26	4.19±0.30**	4.15±0.28**
A/G ratio	2.30±0.30	2.16±0.31	2.06±0.27*
Blood glucose (mg/dl)	174.5±40.7	267.3±132.5*	241.4±67.7*
TG (mg/dl)	110.3±61.6	95.9±29.6	127.2±61.1
Total cholesterol (mg/dl)	74.6±15.4	93.6±19.7*	114.1±26.2**, §
HDL cholesterol (mg/dl)	34.9±2.7	38.4±7.6	41.6±8.4*
LDL cholesterol (mg/dl)	4.25±1.96	5.21±1.85	8.78±5.46**, §
AST (U/L)	924.0±294.3	593.1±285.1	493.0±155.2
ALT (U/L)	56.5±11.3	51.6±16.7	51.9±22.8
LDH (U/L)	683.0±406.2	595.4±285.5	626.2±348.9
ALP (U/L)	924.0±294.3	593.1±285.1**	493.0±155.2**
γ-GTP (U/L)	1.50±0.52	1.79±0.58	1.78±0.55
Choline esterase (U/L)	4.42±1.56	5.07±2.30	5.17±1.98
Amylase (U/L)	1005.5±284.4	1040.6±438.4	967.2±147.9
Creatinine (mg/dl)	0.22±0.05	0.25±0.08	0.28±0.04**
Uric acid (mg/dl)	2.00±1.11	2.51±1.06	3.33±2.89
BUN (mg/dl)	21.8±6.3	25.9±6.4	26.0±3.8*
Na (mEq/L)	143.1±3.3	138.6±7.0*	138.4±5.6*
K (mEq/L)	3.49±0.35	3.59±1.08	3.43±0.62
Cl (mEq/L)	99.6±3.6	93.9±6.7*	92.9±5.4**

Data are presented as mean±SD

* Significantly different from low salt group ($p<0.05$)

** Significantly different from low salt group ($p<0.01$)

§ Significantly different from miso group ($p<0.05$)

Supplementary Table 5. Kidney histology

	Low salt	Miso	High salt
Pale staining columnar epithelium	12/12 (100)	10/10 (100)	12/12 (100)
Protein cast	6/12 (50)	10/10 (100)	12/12 (100)
Number of animals with glomerular degeneration	1.8±0.5	2.6±1.7	5.5±1.2*,§
Thickened adventitia in kidney arteries	4/12 (33)	10/10 (100)	12/12 (100)
Area of collagen fiber cortex (μm^2)	88176±2294	104016±8464**	141641±4831**,§

Data are presented as mean±SD

* Significantly different from low salt group ($p<0.05$)

** Significantly different from low salt group ($p<0.01$)

§ Significantly different from miso group ($p<0.05$)