

Continuous Subcutaneous Administration of Miso Extracts Attenuates Salt-Induced Hypertension in Dahl Salt-Sensitive Rats

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Abstract

Objective: Long-term intake of Miso attenuates hypertension in Dahl salt-sensitive (Dahl S) rats through an increased urinary sodium excretion. We examined whether a bolus injection into the peritoneal cavity (i.p.) or a continuous subcutaneous infusion of a Miso extract attenuates hypertension in Dahl S rats. **Materials and Methods:** We investigated the effects of a bolus, i.p. injection of 50 mg Miso extract in 0.5 mL on hypertension in Dahl S rats, and examined whether a long-term subcutaneous infusion of the Miso extract (50 mg Miso/day), using an osmotic mini-pump working for 14 days, attenuates hypertension in Dahl S rats. **Results:** A bolus, i.p. injection of 50 mg Miso extract decreased SBP in 2 hrs. The reduction was significant at 4 hrs, and SBP returned to the baseline at 24 hrs. The SBP reduction at 4 hrs after the injection was increasingly greater each day during the 4 days. The SBP reduction by the Miso extract was dose-dependent and the antihypertensive activity occurs in a <5 kDa fraction of the extract. The subcutaneous infusion of 50 mg Miso extract/day for 14 days significantly attenuated hypertension in Dahl S rats. The SBP reduction was associated with significant decreases in the heart and kidney weights. Urinary protein excretion significantly decreased in the Miso group. The SBP reduction was not associated with increases in either urinary sodium excretion or fractional excretion of sodium. **Conclusions:** SBP reduction by very low-dose of the Miso extract may be mediated partly by mechanisms other than renal sodium handling.

Keywords

Miso, Salt Induced Hypertension, Dahl Rats

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1. Introduction

Miso is a traditional Japanese food that was originally described in Taiho Ritsurei in 701 AD. Miso is manufactured using soybeans along with malted rice and salt; a significant amount of salt is needed for complete fermentation. One serving of Miso soup generally contains 1 - 1.5 g salt which supports the long-standing belief that traditional Miso soup is to blame for the high incidence of salt-sensitive hypertension and subsequent cerebral stroke in Japan.

Interestingly, however, we and investigators from other laboratory have reported in the previous study that long-term intake of Miso soup or Miso attenuates development of salt-induced hypertension in Dahl salt-sensitive (Dahl S) rats when compared with rats given the same amount of saline solutions as the salt contents in Miso soup [1]-[3]. The reduction in blood pressure in the Miso group corresponds to approximately 30% reduction in salt intake. The mechanisms for the blood pressure reduction are uncertain; however, several hypotheses are proposed. First, Miso inhibits salt absorption in the intestine. Second, the constituent(s) of Miso soup increase urinary excretions of sodium, thereby decreasing salt loading. Third, the functions of Miso constituents are similar to vasodilators.

In our preliminary study using metabolic cages, sodium absorption in the intestine was not influenced by the long-term intake of Miso in normotensive Wistar rats (unpublished data) [4]. In addition, the long-term intake of 5% Miso enhanced natriuresis and diuresis in association with increased urinary dopamine excretion, suggesting that oral intake of 10 - 20 g Miso/day attenuates salt-induced hypertension in Dahl S rats through enhanced excretion of sodium in the kidneys [2].

In the present study, we proposed the hypothesis that Miso extract attenuates hypertension through vasodilator effects on vascular walls via the circulation. To test this hypothesis, we injected a Miso extract into the abdominal cavity or subcutaneous tissue and we then examined the blood pressure in Dahl rats with salt-induced hypertension.

2. Materials and Methods

2.1. Acute Effects of Bolus, Intra-Peritoneal Injection of Miso Extract

Protocol of the study

We purchased 4-week-old male Dahl salt-sensitive (Dahl S/Iwai) rats from Sankyo Laboratories, Inc., Tokyo, Japan. The Dahl S rats were fed a high-salt (4%NaCl) diet to develop salt-induced hypertension. Dahl S rats aged 7 - 8 weeks old were used for assessing the antihypertensive activity of a bolus intra-peritoneal (i.p.) injections of Miso extract.

Manufacture of Miso

Miso is manufactured from soybeans with malted rice and salt. A significant amount of salt is needed for complete fermentation. One serving of Miso soup contains 1 - 2 g salt. A representative Japanese meal is composed of rice, the main dish, several side-dishes, e.g., vegetables, etc., and Miso soup. Therefore, when Miso soup is served 3 times a day, 3 g of salt or more is consumed in addition to the usual salt intake from other foods.

Preparation of Miso extract

Miso utilized in the present study was offered by the Central Miso Research Institute (Tokyo, Japan). One thousand kilogram Miso is regularly made of 293 kg soybeans, 144 kg rice (malted), 130 kg NaCl, and water (Kome Miso full-boiled and colored light-yellow). Miso was fermented for 3 months [2]. We dissolved 100 g of Miso in 1000 mL of water and homogenized the mixture with an ultrasonic POLYTRON (KINEMATICA GmbH, Luzern, Switzerland) blender for 5 min [1] [2]. The homogenate was autoclaved at 120°C for 40 min and centrifuged at 1.5×10^4 g for 30 min. The supernatant was passed through Millipore membranes (0.25 μ m pore size) for sterilization. The salt concentration of the Miso extract was 1.3% (w/v). We prepared newly the Miso extract every time we tested the antihypertensive activity.

Assay of antihypertensive activity

We injected 0.5 mL of the extract from 50 or 25 mg Miso into the peritoneal cavity of hypertensive Dahl S rats aged 7 - 8 weeks and administered 0.5mL of 1.3% or 0.65% saline solutions corresponding to the concentrations of salt in the Miso extract to the control hypertensive rats. The systolic blood pressure (SBP) was determined using the tail-cuff method of Freedman and Freed with a slight modification in the detection system (Natsume Seisakujo Model KN-210-1; Tokyo, Japan) [5]-[7].

2.2. Long-Term Subcutaneous Infusion of the Miso Extract Using an Osmotic Mini-Pump in Rats with Salt-Induced Hypertension

Sixteen 4-week-old Dahl S rats were fed a high-salt diet (4% NaCl, w/w) for 4 weeks and six 4-week-old Dahl S rats were fed a low-salt diet (0.3% NaCl, w/w) diet for 4 weeks (low-salt DS). The Dahl S rats fed the high-salt diet (4% NaCl, w/w) were divided into 2 groups at the age of 6 weeks; 1) 8 Dahl S rats fed the high-salt with sham-operation (DS-high), and 2) 8 Dahl S fed the high-salt with a subcutaneous continuous infusion of the Miso extract (50 mg/day) using an osmotic mini-pump (ALZET model 2ML2; 5.0 μ L/h working for 14 days, Cupertino, CA) (DS-miso) [8]. The osmotic mini-pump filled with 5.4% saline solution was implanted to the sham Dahl S rats (DS-high). The concentration corresponded to that in the Miso extract. Chow and water were available *ad libitum* throughout the experiment.

At the end of the study, each rat was placed in a metabolic cage for 3 days consecutively, and 24-h urine samples were collected on the last day for testing. Urine samples were stored at -80°C until assayed. A single investigator measured the blood pressure of all animals in a quiet warm room from 13:00 to 16:00. The blood samples and organs of interest were obtained from rats under pentobarbital anesthesia (75 mg/kg body weight).

We used the osmotic mini-pump ALZET model 2ML2 for subcutaneous administration of the Miso extract [8]. The pump was preconditioned in a normal saline solution overnight before the use. This pump continuously infuses 2.0 mL of the Miso extract solution (416 mg/mL) at 5.0 ± 0.75 μ L/h. Theoretically, this pump is used for 13 to 17 days. The daily dose of the Miso was 50 ± 7 mg/day. This dose was much lower than the bolus, i.p. injection of the 50 mg Miso extract in the present study and the doses utilized in our previous studies on oral intake of Miso (around 20 g Miso/day) [1] [2].

2.3. Determination of Variables

Plasma and urinary concentrations of creatinine were measured using an autoanalyzer (Model Hitachi 736; Hitachi Co., Ltd., Tokyo, Japan). Concentrations of Na were determined by an Na^+ -sensitive electrode and Na METER TS-999i determination system (Tokyo Chemical Laboratories, Co, Ltd., Tokyo) [1] [2]. Urinary protein concentrations were measured using a protein assay kit (BioRad, Hercules, CA) [6] [7]. Plasma and urinary osmolality were measured by the standard method, *i.e.*, molecular depression of freezing point [2]. Investigators who were blind to the rat assignment determined the various parameters.

2.4. Statistical Analysis

All statistical analyses were performed using STATISTICA software (StatSoft, Tulsa, OK). Values were expressed as means \pm SD. Differences were assessed by one-way factorial analysis of variance (ANOVA) followed by the post-hoc least significant difference (LSD) test. p-values less than 0.05 were considered statistically significant.

2.5. Guidelines for Handling Rats for Experiments

We followed the guidelines for experimental animal handling, and our study was approved by the Animal Care Committee of the Kyoritsu Women's University (No. 13003, 2013 and No. 14001, 2014). The experiment was conducted in accordance with the guidelines of the National Institutes of Health.

3. Results

3.1. Acute Changes in SBP in Response to I.P. Injection of Miso Extract

A bolus i.p. injection of 50 mg Miso extract in 0.5 mL decreased SBP of Dahl S rats after 2 hrs. The reduction was statistically significant at 4 hrs and the SBP was equal to the baseline after 24 hrs (Figure 1).

The bolus injection once a day was continued for 4 days. The SBP values were determined at 4 hrs after the injection on day 2 and day 4 (Figure 2). The repeated injection of the Miso extract significantly decreased SBP on day 2 and day 4. The reductions in SBP were increasingly greater as the injections were repeated. Moreover, the repeated injection of Miso extract lowered the nadir SBP, compared with saline injection by 7.9% ($p < 0.05$) on day-3 and 9.6% ($p < 0.05$) on day-5. These suggested that an increase in dose of Miso extract attenuates SBP for 24 hrs.

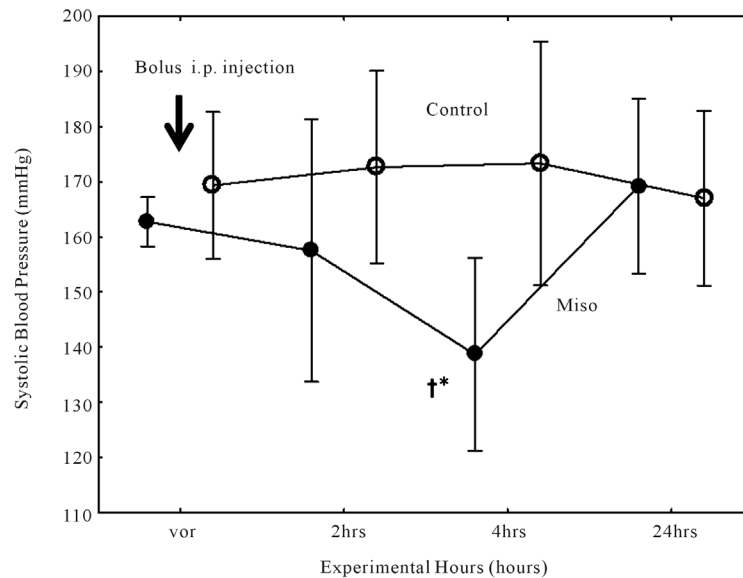


Figure 1. Changes in the SBP in response to a bolus i.p. injection of the Miso extract. Dahl salt-sensitive (Dahl S) rats fed a high-salt diet were given the Miso extract and changes in SBP were noted. The SBP was measured at before the injection (vor), after 2 (2 hrs), 4 (4 hrs) and 24 hours (24 hrs). The drawing module avoided the overlay of the SDs bars and the marks were described apart for each test period. Open circle, Dahl S rats given 0.5 mL 0.65% saline solution corresponding to the salt concentration in the Miso extract (Control); closed circle, Dahl S rats given 0.5 mL from 50 mg Miso (Miso). * $p < 0.05$ vs the value in 0.65% saline solution group, † $p < 0.05$ vs the value before injection of the Miso extract (vor). Group differences were assessed by one-way factorial analysis of variance (ANOVA) followed by a post-hoc least significant difference (LSD) test.

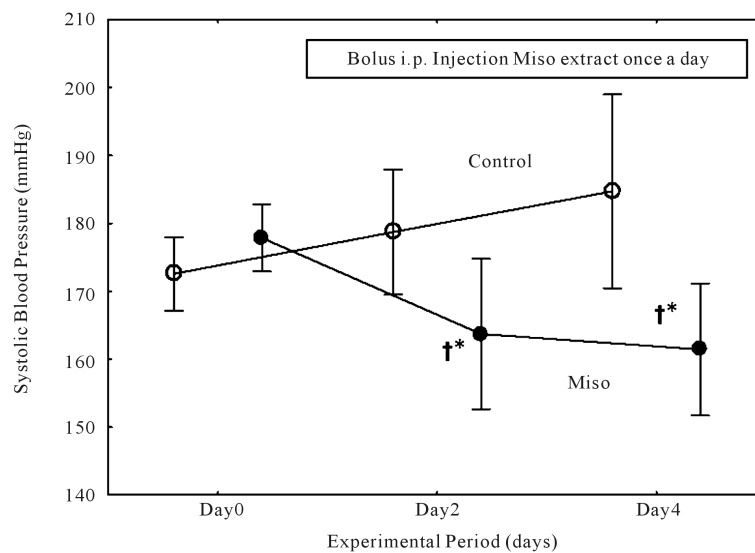


Figure 2. Changes in the SBP in response to an i.p. injection of the Miso extract for 4 days. Dahl salt-sensitive (Dahl S) rats fed a high-salt diet were given the Miso extract and the SBP changes were measured. The SBP was measured at before the injection (Day 0), after 2 days (Day 2) and 4 days (Day 4). The drawing module avoided the overlay of the SDs bars and the marks were described apart for each test period. Open circle, Dahl S rats given 0.5 mL 0.65% saline solution corresponding to the salt concentration in the Miso extract (Control); closed circle, Dahl S rats given 0.5 mL extract from 50 mg Miso (Miso). * $p < 0.05$ vs value in 0.65% saline solution group, † $p < 0.05$ vs the value before injection of the Miso extract (Day 0). Group differences were assessed by one-way factorial analysis of variance (ANOVA) followed by a post-hoc least significant difference (LSD) test.

The changes in SBP after administration of different doses of the Miso extract were shown in **Figure 3**. The SBP was measured 4 hrs after the bolus i.p. injection of the Miso extract. The Miso extract decreased the SBP of

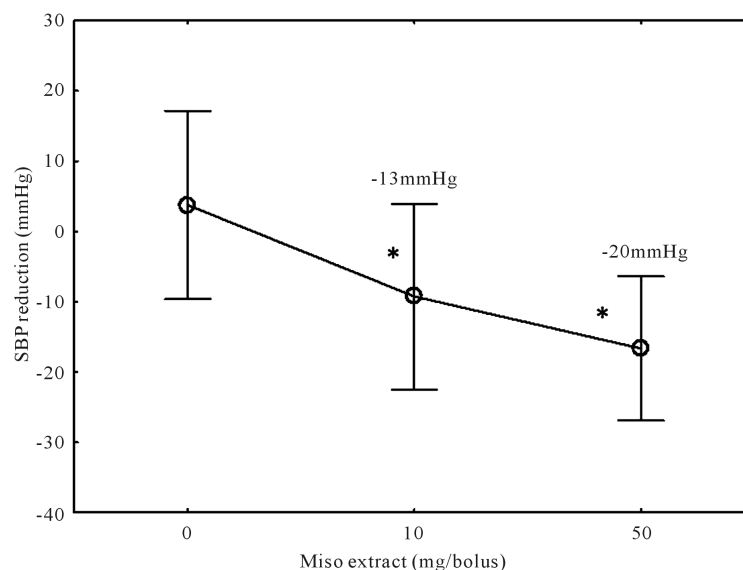


Figure 3. Dose-dependency of the Miso extract. The values are expressed as the difference between the SBP reduction in rats given the Miso extract and Dahl S rats given a saline solution. The marks showed the difference between BP in Dahl S rats that were injected Miso extract and that in each control (saline solution). The figures represented the difference from the zero Miso extract. * $p < 0.05$ vs value in Dahl S rats given a saline solution. Group differences are assessed by one-way factorial analysis of variance (ANOVA).

Dahl S rats in a dose-dependent manner.

The crude Miso extract was passed through the membrane sieves (cutoff point <5 kDa) and we obtained <5 kDa fraction of the Miso extract. At 4 hrs after the i.p. injection, we measured and compared the antihypertensive activity between the crude (50 mg Miso) and fractionated extract (<5 kDa). There was no difference in the antihypertensive activity between the 2 samples, suggesting that the antihypertensive factor(s) was expected to be present in the <5 kDa fraction (Figure 4).

3.2. Long-Term Subcutaneous Infusion of Miso Extract in Rats with Salt-Induced Hypertension

We examined the long-term effects of the low-dose (50 mg/day) Miso extract on SBP in Dahl S rats with salt-induced hypertension using a continuous, osmotic mini-pump working for 14 days. As shown in Figure 5, there was no difference in the body weight between the DS-high and DS-miso groups.

Systolic blood pressure increased in a time-dependent manner in the DS-high group when compared with that in the DS-low group (Figure 6). In DS-miso, however, the SBP elevation was significantly attenuated at day 4 and day 14, as compared with that in the DS-high group (144 ± 4 mmHg vs 167 ± 6 mmHg at day 4, $p < 0.001$; 155 ± 13 mmHg vs 172 ± 8 mmHg at day 14, $p < 0.005$).

The heart weight of the DS-high group increased as compared with that of the DS-low group (Table 1). The increase in heart weight observed in the DS-high group was attenuated significantly in the DS-miso group. Similarly, the aortic weight increased in the DS-high group when compared with that in the DS-low group; this increase was attenuated with administration on the Miso extract. The difference, however, was not significant. The kidney weight was lower in the DS-miso group than in the DS-high group; additionally, the increase in urinary protein excretion in the hypertensive DS-high group was significantly decreased in the Dahl S rats that were given the Miso extract (Figure 7).

Next, to understand the role of the kidneys in sodium handling with respect to the reduction in blood pressure by the subcutaneous infusion of the Miso extract, we examined the parameters indicating renal sodium metabolism (Table 2). Urinary volume and urinary excretion of sodium significantly decreased in the DS-miso group compared with that in the DS-high group; neither the sodium clearance rate nor the fractional excretion of sodium (FENa) changed after the subcutaneous infusion of the Miso extract. These findings are supported by the fact that there were no differences in the osmotic clearance rate between the DS-high and DS-miso groups. On

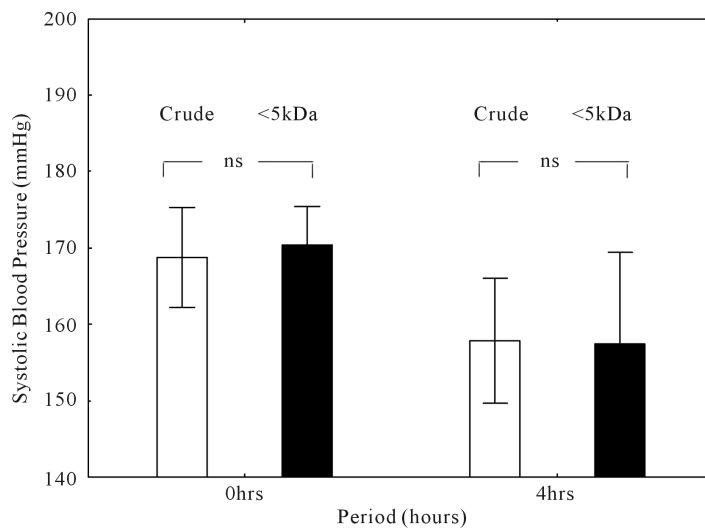


Figure 4. The hypotensive activity in the <5 kDa Miso extract. Crude, Miso extract; <5 kDa, fractionated Miso extract using membrane sieves. 0 hr, SBP before the i.p. injection; 4 hrs, SBP 4 hrs after the i.p. injection. No differences in SBP were noted between the Dahl S rats given crude and those given <5 kDa Miso extract. ns represents statistically not significant.

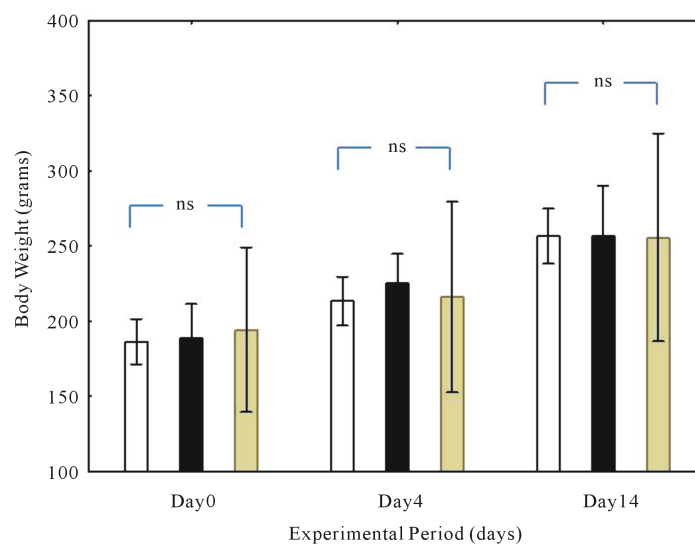


Figure 5. Body weights. Open column, control Dahl S rats with hypertension (DS-high); solid column, Dahl S rats given Miso extract (DS-miso); gray column, Dahl S rats fed a low-salt diet (DS-low). ns represents statistically not significant. Day 0 the data before the implantation of an osmotic mini-pump.

the other hand, free water clearance was significantly decreased in the DS-miso group compared with that in the DS-high group.

In addition, it is noted that Na/K in urine was significantly lower in Dahl S rats fed a low-salt diet than in Dahl S rats fed a high-salt diet, suggesting that Na/K exchange was enhanced in the low-salt Dahl S rats through up-regulated renin-angiotensin system on a low-salt diet. In DS-miso, however, the Na/K balance was not changed when compared with DS-high.

4. Discussion

Fermented Miso is an integral part of Japanese cuisine. Miso usually contains 10% - 13% salt (w/w) and 10 - 30 g Miso/day is served as Miso soup. This means that the intake of salt from Miso averages 1 - 3.9 g/day, because of which, in Japan, Miso is believed to be responsible for incidence of hypertension. To our knowledge, however,

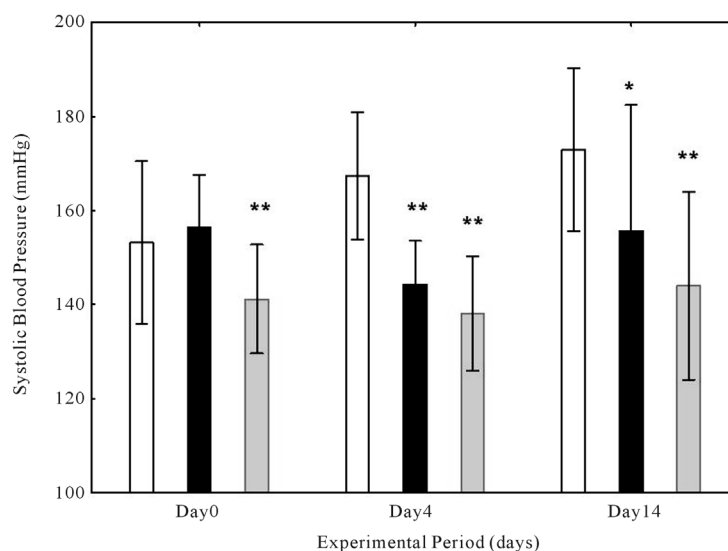


Figure 6. Systolic blood pressures in Dahl S rats. Open column, control Dahl S rats with hypertension (DS-high); solid column, Dahl S rats given the Miso extract (DS-miso); gray column, Dahl S rats fed a low-salt diet (DS-low). ns represents statistically not significant. Day 0, the data before the implantation of an osmotic mini-pump. * $p < 0.05$, ** $p < 0.001$ vs the DS-high group (open column). Group differences were assessed by one-way factorial analysis of variance (ANOVA) followed by a post-hoc least significant difference (LSD) test.

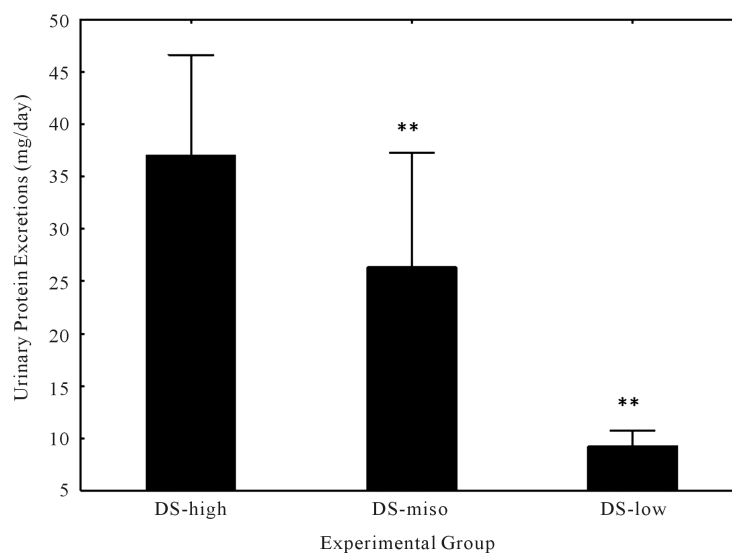


Figure 7. The effects of Miso extract on urinary protein excretion. DS-high, Dahl S rats fed a high-salt diet; DS-miso, Dahl S rats fed a high-salt diet and given Miso extract; DS-low, Dahl S rats fed a low-salt diet. Group differences were assessed by one-way factorial analysis of variance (ANOVA) followed by post-hoc least significant difference (LSD) test. ** $p < 0.001$ vs DS-high.

there are no studies showing that the intake of Miso soup induces hypertension in humans. Our preliminary, cross-sectional survey on subjects at an annual medical checkup found that the more frequent intake of Miso soup consuming 3.0 - 4.5 g salt is not associated with an elevation of blood pressure, compared with less than once a day intake of Miso soup consuming less than 1.5 g/day, suggesting that Miso contains some nutrients that attenuates blood pressure elevation due to higher salt intake (unpublished data).

To test the possibility that Miso contributes to hypertension, we examined the influence of long-term oral intake of Miso soup in salt-induced hypertension of Dahl S rats. We found that long-term intake of Miso prevents salt-induced hypertension as well as attenuates heart and kidney damage [1]. The reduction in blood pressure by

Table 1. The Effects of subcutaneous infusion of Miso extracts on organ weights.

Organ	Unit	DS-high	DS-miso	DS-low
Heart weight	g/100g BW	0.450 ± 0.025	0.392 ± 0.022*	0.420 ± 0.106
Aortic weight	mg/mm ²	0.327 ± 0.078	0.293 ± 0.036	0.260 ± 0.025*
Kidney weight	g/100 BW	0.592 ± 0.056	0.550 ± 0.013*	0.468 ± 0.015**

*p < 0.05, **p < 0.001 vs DS-high.

Table 2. The effects of subcutaneous infusion of Miso extracts on Na handling in the kidney.

Variables	DS-high	DS-miso	DS-low
UV	14.47 ± 1.46	9.69 ± 2.53**	2.64 ± 0.40**
U _{Na} V	4.45 ± 0.41	3.81 ± 0.99**	0.09 ± 0.01**
U _K V	1.10 ± 0.14	1.04 ± 0.32	0.90 ± 0.20
U _{Na} /U _k	4.0 ± 0.3	3.7 ± 0.6	0.1 ± 0.0**
C _{cr}	1.74 ± 0.18	1.46 ± 0.20*	1.00 ± 0.22**
C _{Na}	30.55 ± 3.18	27.65 ± 7.01	0.74 ± 0.10**
FENa	1.76 ± 0.24	1.89 ± 0.45	0.07 ± 0.01**
P _{Osm}	314 ± 4	310 ± 2*	307 ± 4**
U _{Osm}	15.3 ± 1.3	15.7 ± 3.4	6.3 ± 0.9**
C _{Osm}	48.9 ± 4.4	50.6 ± 11.0	20.7 ± 3.2**
C _{H₂O}	-34.4 ± 4.2	-40.9 ± 8.7*	-18.1 ± 3.3**

UV, urine volume (mL/100g BW/day); U_{Na}V, urinary excretion of sodium (mEq/100g BW/day); U_KV, urinary excretion of potassium (mEq/100g BW/day); U_{Na}/U_k, ratio of urinary sodium/potassium; C_{cr}, creatinine clearance (L/100g BW/day); C_{Na}, clearance of sodium (mL/100g BW/day); FENa, fractional excretion of sodium (%); P_{Osm}, plasma osmolality (mOsm/KgH₂O); U_{Osm}, urinary osmolality (Osm/KgH₂O); C_{Osm}, clearance of osmolality (mOsm/KgH₂O/100g BW/day); C_{H₂O}, free water clearance (mL/100g BW/day). p values indicate statistically significant between DS-high and DS-miso. *p < 0.05, **p < 0.001 vs DS-high.

the oral intake of 5 - 10 g Miso/day was associated with enhanced natriuresis/diuresis, likely mediated by the urinary dopaminergic system [2].

Thus, the present study examined whether a Miso extract decreases blood pressure via the circulation. We demonstrated that a bolus i.p. injection of Miso extract decreases the SBP in salt-induced hypertension in Dahl S rats fed a high-salt diet. The finding suggests that the Miso extract contains a substance(s) that decreases SBP in Dahl S rats via the circulation. Furthermore, the antihypertensive substance(s) is heat-stable and less than 5 kDa in molecular weight. These data suggested that there are several substances to decrease blood pressure in Miso, and we provided evidence for the occurrence of antihypertensive substance(s) that are different from the activity shown in the previous studies.

We administered 0.5 mL of the extract prepared using 50 mg Miso (10% Miso solution). The amount of Miso given via the i.p. route is much lower than that given via the oral route as reported previously [1] [2]. This suggests that mechanisms for these effects may be different depending on the route of administration. To test this hypothesis, we examined the long-term antihypertensive action of Miso using an osmotic mini-pump placed in the subcutaneous tissue and investigated the sodium handling by the kidney using a metabolic cage. In the study where the administration was via the subcutaneous route, we infused an extract of 50 mg Miso/day. A continuous, 14-day subcutaneous infusion of 50 mg Miso extract/day attenuated the blood pressure. The blood pressure reduction was associated with decreases in the heart weight or kidney weight and tended to be associated with a decrease in the aortic weight, suggesting that the antihypertension is associated with attenuation of cardiovascular damage. This is also supported by the finding that proteinuria was lower in the group given the Miso extract than in the control Dahl S rats that were fed a high-salt diet.

Such a reduction in blood pressure was not accompanied by increases in natriuresis and diuresis that was observed for administration via the oral route. Additionally, neither osmotic nor free water clearance changed, suggesting that the Miso extract does not affect urinary excretion of solutes. These data indicate that the mechanism of antihypertension via the circulation is different from the mechanism via the oral route. The mechanism via the subcutaneous route is not clear; however, some reports indicate that peptides from soybeans may suppress the renin-angiotensin system, thereby decreasing the blood pressure [9]-[11]. In our preliminary study, however, we found that the antihypertensive substance(s) does not occur in the soybean homogenate per se and the activity was more marked in 2 - 3 months' fermented rather than 1 - 2 years' matured Miso (unpublished data). These might suggest that the substances may be peptides produced from soybeans by fermentation. It is unclear whether the Miso extract inhibits renin-angiotensin system. However, the Na/K balance in urine was not altered in Dahl S rats given the Miso extract when compared with Dahl S rats with hypertension (**Table 2**). If renin-angiotensin system was inhibited by the Miso extract, the secretion of aldosterone would have declined and this decreases the aldosterone-sensitive Na/K exchange in the distal tubules in the kidneys, thereby increasing the Na/K balance in urine. We did not directly measure the status of renin-angiotensin system; however, these data appeared to be against suppression of renin-angiotensin system by the Miso extract. To address these points directly, we are required to investigate the pharmacological properties of the current antihypertensive activity in the Miso extract. We are under way to purify and identify the antihypertensive activity via the subcutaneous route.

We did not infuse the Miso extract directly into vascular vessels mainly because the influence by the operation was minimized. We believe that the direct determination of blood pressure was the next step in studying pharmacological properties of the antihypertensive activity along with purification and identification.

The Dietary Approaches to Stop Hypertension (DASH) diet has been reported to decrease the blood pressure compared with an American style diet [12] [13]. The DASH diet is rich in vegetables (plant fibers, potassium, and calcium) and low in fat. The blood pressure reduction noted with the DASH diet is independent of the salt-intake (intake range, 4 to 8 g/day), suggesting that blood pressure is related not only to salt-intake, but also to intake of various nutrients in a balanced manner. We believe that this is true for Miso extract as well, because some substances from fermented soybeans and molted rice attenuate salt dependent hypertension through several mechanisms.

5. Conclusion

In conclusion, the present study shows that treatment with very low doses of Miso extract via the i.p. or subcutaneous routes decreases blood pressure in rats with salt-induced hypertension. This blood pressure reduction is not mediated by increases in sodium excretion from the kidneys. The blood pressure reduction via the subcutaneous route is associated with the attenuation of cardiovascular stress following hypertension. Miso contains several substances beneficial for health [1]-[3] [14]-[18]. These factors involved in the antihypertensive effects of Miso through integrated mechanisms. However, further study is required to identify these substances.

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Declaration of Interest

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

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