

Communication to the Editor**Daily Meal Supplemented with Astaxanthin-Enriched Yolk Has Mitigative Effects against Hypertension in Spontaneously Hypertensive Rats**

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The aim of this study was to investigate the effects of egg yolk powder enriched with astaxanthin (ASX-E) on blood pressure in spontaneously hypertensive rats (SHR) and to verify the benefits of ASX-E as a functional food. To investigate the antihypertensive effect, SHR were fed with an ASX-E mixed diet before hypertension development. Blood pressures were determined periodically during the study by the tail-cuff method. At the end of the study, animals were euthanized, and their thoracic aortas were collected to determine vascular conductance. The thoracic aorta tension was measured with a force displacement transducer. Concentration-dependent response relationships were determined by cumulative addition of 10^{-9} – 10^{-4} M Carbamoylcholine (Cch). Blood pressures of the SHR in the ASX-E mixed diet group were ASX-dose-dependently lower than that of those in the control group. In SHR fed with an ASX-E mixed diet, Cch induced vasorelaxation in the thoracic aorta with endothelium lining but not without endothelium. However, the antihypertensive effect of ASX-E was not observed on blood pressures in SHR that were fed with ASX-E only after the development of hypertension. Results suggest that ASX-E protects endothelial function and thereby prevents the development of hypertension. Hence, the results of our research indicate that daily consumption of ASX-E has a potential benefit on human health.

Key words astaxanthin-enriched egg yolk; hypertension; spontaneously hypertensive rat

INTRODUCTION

At present, the prevalence of high blood pressure (BP) in adults is increasing, and the resulting economic loss arising from lifestyle-associated diseases is a major issue.^{1,2)} High BP is an important risk factor associated with cardiovascular morbidity and mortality. Cardiovascular disease is associated with vascular and functional changes.³⁾ Vascular endothelial cells (ECs) play an important role in the regulation of vascular tone and cardiovascular homeostasis through various relaxation systems such as nitric oxide (NO) production. It is, however, difficult to identify how hypertension develops because of its multifactorial nature. Recently, some researchers have reported that dietary polyphenols and carotenoids may help in maintaining normal BP.⁴⁾ In most instances, the human

body has an adequate supply of antioxidants such as carotenoids and polyphenols obtained from various foods. Hence, it is considered that a daily diet containing polyphenols or carotenoids has public health benefits in the prevention of cardiovascular disease.

Epidemiological research and clinical data have indicated that dietary carotenoids have a protective effect against lifestyle-associated diseases.^{5,6)} Astaxanthin (ASX) is a red carotenoid nutrient present in a wide variety of living organisms and is commonly ingested by humans as an ingredient in meals. ASX has biological and pharmacological effects, including antioxidant and anti-inflammatory properties, because it has strong free radical scavenging capability. It is also known to protect cells and tissues against lipid peroxidation and oxidative damage.^{7–9)} Several studies have reported the beneficial effects of ASX to treat patients with hypertension. The development of new, effective, and easy-to-take forms of ASX is required to prevent hypertension because they are currently difficult to take as a daily supplement. So far, the antihypertensive effect of ASX has been well evaluated, but the health benefits of chicken eggs containing ASX on hypertension has not been verified.

Hence, the aim of this study was to evaluate the beneficial and mitigative effects of yolk enriched with ASX (ASX-E) on hypertension *in vivo* and to verify its use as a functional food taken daily, as this has not been reported to date.

MATERIALS AND METHODS

Preparation of ASX-E ASX-E was prepared from eggs obtained from hens fed a *Phaffia* yeast mixed diet daily. Egg yolks were separated from the white and freeze-dried because the ASX was found to be richer in the yolk. The concentration of ASX in the yolk powder was determined (58.2 μg ASX/g yolk powder in this study) before use.

Animals and Feeding The Animal Experiment Committee of Okayama University approved this experimental facility (permission No. OKU-2013359, permission date: 20131029), and rats were managed in line with the Guidelines for Care and Use of Laboratory Animals. Male spontaneously hypertensive rats (SHR, 6 weeks old) were purchased from Charles River Laboratories Japan, Inc. (Kanagawa, Japan) and used in this experiment. The animals were maintained at $23 \pm 1^\circ\text{C}$ with constant humidity (60%) and lighting (12 h light/dark cycle, light 08:00–20:00). The rats were housed for 1 week before the experiments and fed a normal diet (MF, Oriental Yeast Co., Ltd., Chiba, Japan).

Test diet was prepared using normal diet with freeze-dried ASX-containing yolk or normal yolk. The dose of feed was 20 g/d/rat. In order to keep the amount of chicken egg yolk powder containing in the diet constant, 17.5% of 20 g diet (3.5/20 g diet/d) was lyophilized egg yolk powder. To evaluate the protective effect of ASX-E on hypertension, prehypertensive SHR (7 weeks old) were randomized into four groups ($n = 4$ per group) and received the following dietary mix for 15 weeks to confirm any antihypertensive effect arising from ASX-E administration: diet mixed with freeze-dried normal egg yolk (control, ASX 0.00 mg/g), ASX 0.02 mg/g diet per

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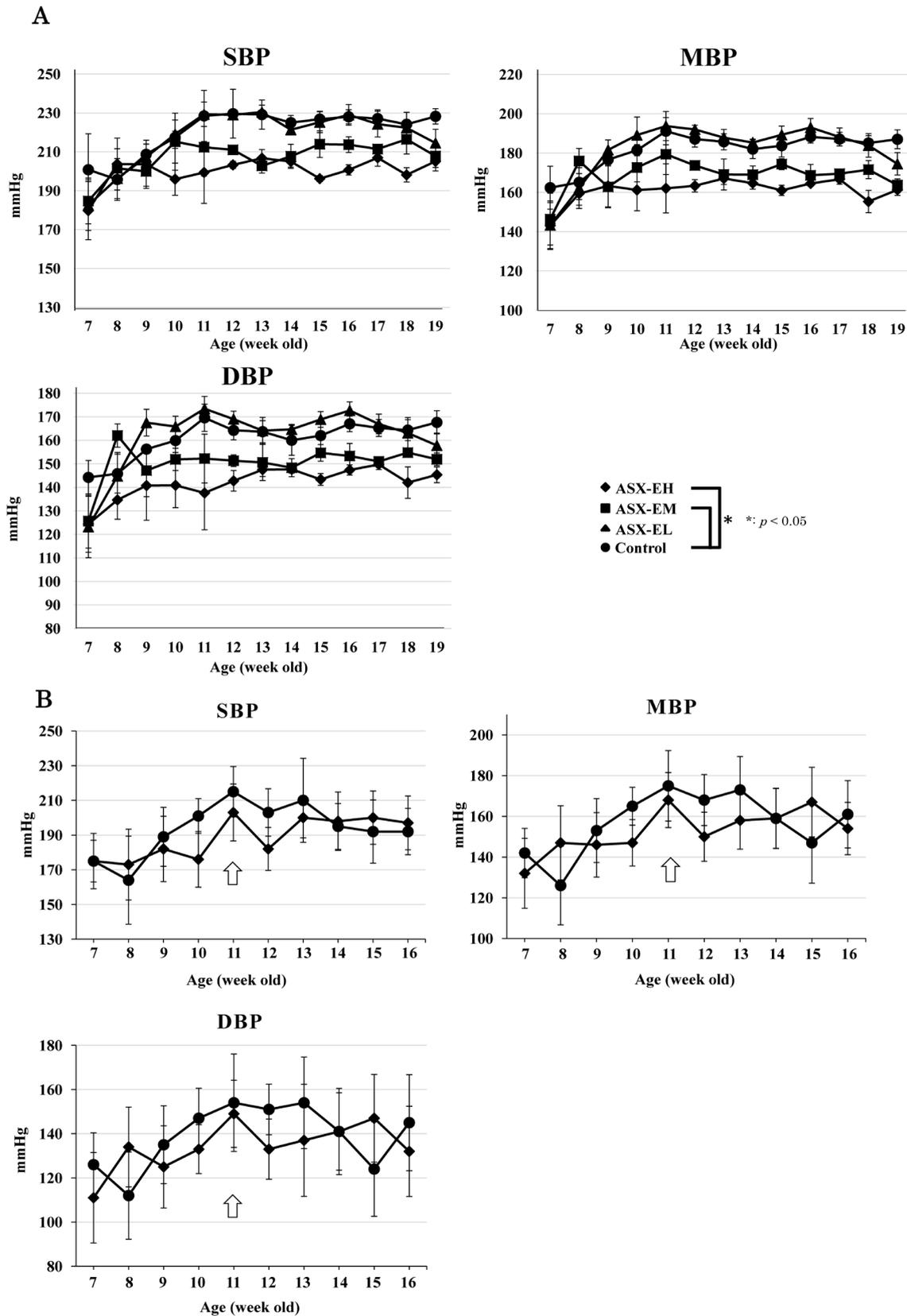


Fig. 1. Effects of Oral Administration of ASX-Enriched Yolk (ASX-E) Powder Mixed Diet to the Hypertension

(A) Effects of oral administration of ASX-E powder mixed diet on systolic blood pressure (SBP), mean blood pressure (MBP), and diastolic blood pressure (DBP) in pre-hypertensive SHR. Four groups were given the standard diet (control: ●), diet containing ASX 0.02 mg/g per day (ASX-EH: ◆), diet containing ASX 0.008 mg/g per day (ASX-EM: ■), and diet containing ASX 0.002 mg/g per day (ASX-EL: ▲). Each symbol and bar represent the mean \pm standard error of the mean (S.E.M.) ($n = 4$). Data in SBP, MBP, and DBP from 10 to 18 weeks old show statistical differences between ASX-EH, ASX-EM, and control. $*p < 0.05$. (B) Effects of oral administration of ASX-E powder mixed diet on SBP, MBP, and DBP in SHR after onset of hypertension. Arrows in each figure indicate the starting point which SHR fed with ASX-EH or control diet. The blood pressure of SBP in all SHR have reached to 200 mmHg in this time point and then SHR has been judged to develop the hypertension. SHR Two groups were given the control (●) and ASX-EH (◆). Each symbol and bar represent the mean \pm S.E.M. ($n = 4$). Data in SBP, MBP, and DBP from 11 to 16 weeks old did not show statistical differences between ASX-EH and control.

day (ASX-EH), ASX 0.008 mg/g diet per day (ASX-EM), and ASX 0.002 mg/g diet per day (ASX-EL). To observe the anti-hypertensive effect of ASX-E administration, arterial blood pressures in the SHR were also monitored by the tail-cuff method once a week.

To evaluate the antihypertensive effect of ASX-E, those SHR that developed hypertension received the standard ASX-E mixed diet. Briefly, male SHR (7 weeks old, $n = 10$) were fed a normal diet, and arterial blood pressures were monitored once a week until hypertension development. After achieving high blood pressure in 10-week-old rats (blood pressure reached to 200 mmHg), hypertensive SHR were randomized into two groups ($n = 5$ per group) and fed for 5 weeks: ASX 0.02 mg/g diet per day (ASX-EH) and standard diet (control).

Measurement of Blood Pressure and Heart Rate Arterial BP (diastolic, systolic, and mean BP) and heart rate were measured once a week by a tail-cuff system. The rats were gently supported in a mesh holder prewarmed to 37.5°C. Blood pressure in the tail artery was indirectly measured using a tail-cuff apparatus (BP-98, Softron, Tokyo, Japan). The average of three separate measurements was considered.

Relaxation Experiments Using the Thoracic Aorta of the Rat Animals were anesthetized using diethyl ether and euthanized by heart puncture. The thoracic aorta was isolated immediately and placed in a physiological salt solution (PSS), containing NaCl (136.9 mM), KCl (5.4 mM), CaCl₂ (1.5 mM), MgCl₂ (1.0 mM), NaHCO₃ (23.8 mM), and glucose (5.6 mM) saturated with 5% CO₂ in O₂. Helical aortic segments (1.5 mm wide) were prepared from the thoracic aorta, mounted on steel hooks in a Magnus chamber (Kishimoto UC-5TD, Kyoto, Japan), and attached to a force displacement transducer (Yokogawa 3134, Tokyo, Japan) and a recorder (Yokogawa LR 4220E, Tokyo, Japan). The Magnus chambers were filled with 10 mL PSS maintained at 37°C and bubbled continuously with 5% CO₂ in O₂ at pH 7.4. The specimen was loaded with a static tension of 1 g, and this tension was maintained until the end of the experiment. The specimens were left under basal active tension in oxygenated PSS for 60 min. The equilibration of segments was achieved by 3 times repeating the contraction with high K⁺ solution (65.4 mM) and relaxation with PPS. Nor-epinephrine (NE: Wako Pure Chemical Corporation, Osaka, Japan) was used as a contracting agent, and Carbamoylcholine (Cch: SIGMA, St. Louis, MO) was used as a relaxing agent. To measure the relaxation response, the specimens were contracted in advance with a concentration of NE (10⁻⁶ M) that caused maximum contraction, and Cch (10⁻⁹–10⁻⁴ M) was administered cumulatively when the tension became constant. NE induced contraction in the thoracic aorta segments with intact endothelium, whereas the relaxation disappeared in the denuded ones. The PSS solution was then replaced, and further experiments were carried out. Relaxation was presented as the percentage of the decrease in maximal tension obtained by NE-induced contraction.

To confirm the activity of endothelial cells, the endothelial lining of the strips was removed by pressing the segment slightly and rolling it gently onto a filter paper a few times. The removal of the endothelial lining was functionally confirmed by NE, as described above.

Statistical Analysis Data are shown as means ± standard errors ($n = 4$ or 5). When appropriate, the data were analyzed

by Student's *t*-test or Two-way ANOVA followed by Williams method, and differences in means were considered significant at $p < 0.05$.

RESULTS AND DISCUSSION

Blood Pressures, Heart Rate, and Relaxation of Thoracic Aorta We determined the protective effects of the daily administration of ASX-E against lifestyle-associated diseases such as hypertension. SHR were fed an ASX-E mixed diet for 15 weeks, and arterial BPs and heart rate were measured once a week. The heart rate during experimental periods was 421.2 ± 13.3 bpm (ASX-EH), 416.71 ± 16.1 bpm (ASX-EM), 421.8 ± 12.8 bpm (ASX-EL), and 417.9 ± 19.1 bpm (Control). The BPs in SHR in ASX-EH or ASX-EM group were significantly lower than those in the control ($p < 0.05$, Fig. 1A) in SHR aged from 8 to 18 weeks old.

These results indicate that hypertension development in SHR was inhibited by a daily diet with either ASX-EH or ASX-EM. To evaluate the recovering effect of ASX-EH after onset of hypertension, SHR fed with ASX-EH or control after systolic BP in all SHR has reached to 200 mmHg (11 weeks old). The BPs in both groups were not statistically different from 11 to 16 weeks old (Fig. 1B). These results indicate that ASX-E in a daily meal has a mitigative effect against hypertension development but not recovering effect on blood pressure after onset of hypertension.

The Cch-induced contraction of the thoracic aorta segments with endothelium was significantly reduced in both ASX-EM (E_{\max} , 62.24 ± 12.49%; pD_2 , $3.9 \times 10^{-8} \pm 2.2 \times 10^{-8}$) and ASX-EH (E_{\max} , 62.74 ± 11.67%; pD_2 , $5.38 \times 10^{-8} \pm 1.93 \times 10^{-8}$) treated SHR compared with the control (E_{\max} , 47.89 ± 21.83%; pD_2 , $3.70 \times 10^{-7} \pm 2.6 \times 10^{-7}$) (Fig. 2A). No differences were observed in the Cch-induced contraction of aorta specimens without endothelium (Fig. 2B), but the data in Fig. 2B has also shown the slight relaxation by Cch in ASX-EH. Chen *et al.* has reported that resveratrol, which is a member of polyphenol and has antioxidative capacity like ASX, induces the endothelium-independent vasorelaxation in rat superior mesenteric artery.¹⁰ They have shown the mechanism of this relaxation related with K⁺ channel and blockade of extracellular Ca²⁺ influx. The slight vasorelaxation in Fig. 2B may be induced by the similar mechanism. No differences were observed in the BPs, heart rate, and the Cch-induced contraction of thoracic aorta specimens with/without endothelium in SHR fed with the ASX-EH mixed diet during the 5 weeks after hypertension arose (data not shown). In addition, thoracic aortas were fixed with 10% formaldehyde to observe the vascular remodeling. Paraffin-embedded thoracic aorta specimens (4-μm thick) were made and stained with HE and Elastica van Gieson. However, no differences were observed in vascular elastin structures in the tissue sections of both ASX-EH and the control SHR (data not shown).

ECs play an important role in regulating BP.^{3,11} NO from vascular ECs is an important compound involved in the induction of vascular relaxation. Hypertension is associated with the presence of reactive oxygen species (ROS) in blood vessels, and these ROS lead to the development of both vascular and endothelial dysfunctions.¹¹ EC damage occurs in many vascular beds during hypertension³; ROS damage the endothelium, and an imbalance of NO levels occurs. One sug-

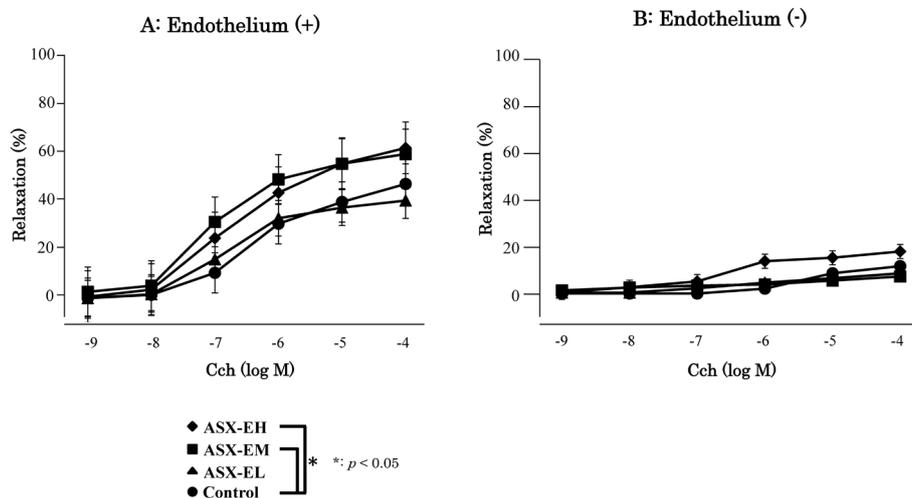


Fig. 2. Vascular Reactivity on SHR Thoracic Aorta Administrated with ASX-E Powder Mixed Diet for 15 Weeks

Endothelium-dependent (A) and endothelium-independent (B) relaxation induced by Cch using thoracic aorta specimens from SHR that are administrated with ASX-E powder mixed diet for 15 weeks. CCh at cumulative concentrations (10^{-4} – 10^{-9} M) showed endothelium-dependent vascular relaxation. Four groups were given the standard diet (control: ●), diet containing ASX 0.02 mg/g per day (ASX-EH: ◆), diet containing ASX 0.008 mg/g per day (ASX-EM: ■), and diet containing ASX 0.002 mg/g per day (ASX-EL: ▲). Values are shown as a percentage of decrease in the maximal tension contracted with norepinephrine. Data represent the mean \pm S.E.M. ($n = 4$). * $p < 0.05$.

gestion is that increased oxidative stress in hypertensive state leads to decreased availability of NO. Several researchers have reported that damage to endothelium-dependent relaxation was observed in the aortic vasculature of SHR or stroke-prone SHR.^{12–14} The result presented in Fig. 2A indicates that vasorelaxation in aortic vascular with endothelium was observed in SHR fed with ASX-E before hypertension development. Several researchers have reported that antioxidants improve endothelium function^{15–17}; however, the improvement of endothelium-dependent relaxation in SHR that started the diet with ASX-E after hypertension development was not observed in our study (Fig. 1B). The ASX showed mostly significant BP lowering activity. Mostly NO- and oxidative stress-related mechanisms have been suggested to explain ASX's antihypertensive effects *in vivo*. In fact, ASX has been reported to prevent oxidative stress in human ECs without cytotoxicity and reduce in the risk of oxidative-stress by its antioxidant capacity.⁹ These results may suggest that ASX-E administration before hypertension development decreases the ROS levels in the blood and maintains endothelial function.

In this study resistance arteries were not used because our aim of this study is to evaluate the vascular response in thoracic aorta of SHR fed with daily diet with ASX-E. Resistance artery such as mesenteric artery is important for the development of hypertension.^{18,19} The abnormalities such as hypertrophic and eutrophic remodeling of these arteries have contributed both the pathogenic and maintenance mechanisms of hypertension in SHR, but there is no report which verified the effect on hypertension using resistance arteries by ASX.²⁰ It is necessary to further investigate the prevention mechanisms of hypertension by ASX using resistance arteries.

In conclusion, we evaluated the potential benefit of ASX-E against hypertension by daily supplementation in the diet. The results in this research indicate that ASX-E has a mitigative effect against hypertension. Our results also suggest that a daily meal containing functional ingredients such as ASX has potential benefits and is an easy method maintaining good health.

Conflict of Interest The authors declare no conflict of interest.

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