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審査学位論文
（博　士）

Effect of astaxanthin on cataract formation induced by glucocorticoids in the chick embryo

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Abstract

Purpose: To examine whether astaxanthin (AST) prevent the cataract formation induced by glucocorticoid in chick embryo.

Materials and Methods: Hydrocortisone hemisuccinate sodium (HC) (0.5 μmol / egg) was administered directly into the air chamber in the egg shell of chick embryo day 15. The eggs were then kept in an incubator at same conditions and administered 100 μL of 50 (HC + AST50 group), 80 (HC + AST80 group), 100 (HC + AST100 group) mg/ml of AST solutions dissolved in dimethyl sulfoxide (DMSO) 3 hr after administration of HC. In addition, non-HC treated group (treated with physiological saline without HC and 100μL of DMSO), HC-alone group (treated with 0.5 μmol of HC and 100μL of DMSO), and AST100 group (treated with physiological saline without HC and 100μL of DMSO) were also incorporated. After 48 hr of treatment, lenses were removed from embryo

and classified into five stages according to developed opacity. The amounts of reduced glutathione in the lenses and the blood glucose levels were measured.

Results: The average scores of lens opacitiy were 2.63 ± 1.02 (HC-alone), 2.78 ± 0.97 nmol/lens (HC + AST50), 2.22 ± 1.20 nmol/lens (HC + AST80), and 1.84 ± 0.83 (HC + AST100; $p < 0.05$) respectively. Administration of AST decreased the lens opacity dose-dependently. The amounts of reduced glutathione in lenses were 11.6 ± 2.8 nmol/lens (HC-alone), 11.3 ± 2.7 nmol/lens (HC + AST50), 13.4 ± 2.4 nmol/lens (HC + AST80), and 13.7 ± 3.1 nmol/lens (HC + AST100; $p < 0.05$) respectively. Higher levels of AST prevented loss of reduced glutathione from the lens.

Conclusion: These findings support that AST protects glucocorticoid-induced cataract in chick embryo.

Key words: Cataract, glucocorticoid, astaxanthin, oxidative stress, chick embryo

Introduction

Cataract development affects the opacity of the ocular lens, is associated with reduced visual acuity, and can be caused by various factors, such as aging, diabetes mellitus, use of steroids, and trauma. While patients with cataract in developed countries can avail of surgical treatment that can improve visual acuity, patients in developing countries may not have access to such treatments, and in many, the condition progresses to blindness. Thus, cataracts still remain the leading cause of blindness in the world.[1]

According to recent epidemiological studies, exposure to ultraviolet rays,[2,3]

smoking,[4-9] and insufficient vitamin C/E[10,11] intake are risk factors for developing cataract, while oxidative stress is also known to be involved in pathogenesis of the disease. Since oxidative stress develops when the production of reactive oxygen species (ROS) overwhelms antioxidant defenses,[12] it is thought that antioxidants may control cataract development by scavenging ROS, thereby decreasing oxidative stress.

Astaxanthin (AST) is one of the most common xanthophylls found in the red pigment of crustacean shells, salmon, and asteroideans.[13,14] AST has several biological actions, including antioxidant,[15-18] anti-inflammatory,[19-22] anti-tumor,[23] and anti-diabetic activities.[24] Indeed, AST appears to be a more powerful antioxidant than either β-carotene or α-tocopherol,[25,26] and as a result of this strong antioxidant activity, it may be useful in controlling cataract development.

The chick embryo model of steroid-induced cataract is one of the best established animal models of the disease in vivo.[27] In this model, a white ring develops around the periphery of the lens nucleus within 20 hr after injection of HC (a representative glucocorticoid) into the fertilized egg (15 days after the egg is laid). Within 48 hr after injection, the lens nucleus becomes opaque. According to previous studies, lens opacity is not attributable to local glucocorticoid effects,[28] but rather to the stimulation of hepatic peroxidation reactions and elevations in lipid peroxide levels. [29] Lipid peroxides then enter the lens via the circulation and aqueous fluid to induce oxidative stress.[30] When the oxidative stress levels exceed the inherent antioxidant potential of the lens, lens opacity may develop. Furthermore, glutathione levels in the lens decrease, which enhances the development of opacity. Therefore, the chick embryo

model of steroid-induced cataract formation is considered to be an excellent experimental model that is easy to develop, readily enables drug treatment, and is useful for screening drugs with antioxidant activity. To date, several antioxidant substances, such as vitamin C,[31,32] pyrroloquinoline quinone,[33] tiopronine,[33,34] and cysteamine[33,35] have been reported to suppress cataract formation in this model.

In the present study, we used the chick embryo model of steroid-induced cataract formation to evaluate the protective effects of AST.

Materials and Methods

Establishment of a chick embryo model of steroid-induced cataract

Chick embryos were treated in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Based on a method described previously,[36] fertilized eggs laid by hens were incubated at a temperature of 37°C and relative humidity of 68%. On Day 15 (Day 1 = day of incubation), HC (Sigma-Aldrich, St. Louis, MO) was injected at a dose of 0.5 μmol/egg into the air chamber of each fertilized chicken egg to establish chick embryo models of steroid-induced cataract.

Administration of AST

AST was purchased from Wako Pure Chemical Industries (Osaka, Japan). AST was dissolved in dimethyl sulfoxide (DMSO) (Wako Pure Chemical Industries, Osaka, Japan) at the following concentrations: 50 (HC + AST50 group; n = 9), 80 (HC + AST80 group; n = 9), and 100 (HC + AST100 group; n =

19) mg/ml. AST solution was added to the fertilized chicken eggs at a volume of 100 μL at 3 hr after the injection of HC. In addition, non-HC group (treated with physiological saline without HC and 100μL of DMSO; n = 16), HC-alone group (treated with 0.5 μmol of HC and 100μL of DMSO; n = 16), and AST100 group (treated with physiological saline without HC and 100μL of 100 mg/ml AST; n = 16) were also incorporated.

Evaluation of lens opacity

Autopsy examination of each embryo was carried out 48 hr after the HC treatment. The chick embryo was removed from the egg, followed by incision of the corneal limbus and removal of the lens. The removed lens was observed macroscopically, with the severity of the opacity being scored on a 5-grade scale: I, no lens opacity; II, faint white ring in the periphery of the lens nucleus; III, clear white ring in the periphery of the lens nucleus; IV, opacity of the lens nucleus not spreading to the center of the nucleus; V, opacity of the lens nucleus spreading to the center of the nucleus as described previously.[37]

Measurement of the reduced glutathione levels in the lens

The removed lens was immediately frozen at -80°C and stored at this temperature until the measurement of the reduced glutathione level. Two lenses from each chick embryo served as one sample. The sample was taken out of the deep freezer and bathed in cool distilled water (0.4 ml), followed by ultrasonic crushing within ice. The crushed sample was immediately mixed with 0.1 ml of 20% cooled trichloroacetic acid. The mixture was centrifuged at

10,000 rpm for 5 minutes. The supernatant (100 µl) was harvested and applied to a 96-well microplate. 1M Tris buffer (pH 8, 95 µl) and 5 mM 5,5'-diobis(2-nitrobenzoic acid) dissolved in methanol (5 µL) were added to each well, followed by 15-minutes' incubation at room temperature. Absorbance at 415 nm was then measured. The microplate was treated with cysteine (1, 2, 5 and 10 nmol/well) in the same way, and the data from these microplates were used to construct a calibration curve.

Measurement of the blood sugar level

Blood was sampled from each embryo 48 hr after the HC treatment, and blood sugar level was measured with BREEZE 2 (Bayer, Indiana, USA).

Statistical analysis

The data is presented as the means ± SD. Mann-Whitney U test was used for comparisons of lens opacity. One-way ANOVA and Fisher's least significant difference as post hoc test were used for comparisons of reduced glutathione levels in the lens. A $p < 0.05$ was regarded as indicating statistical significance. The statistical analysis was performed with SPSS software version 20 (SPSS Inc, Chicago, IL).

Results

Lens opacity

No lens opacity was noted in the non-HC group and in the AST100 group. HC treatment induced lens opacity. The lens opacity scores more than score III

was seen in 63 % (HC-alone), 67 % (HC + AST50), 33 % (HC + AST80), and 26 % (HC + AST100) respectively. The score for lens opacitiy at 48 hr after the HC treatment was significantly lower in the HC + AST100 group (1.84 ± 0.83, $p = 0.04$) than in the HC-alone group (2.63 ± 1.02) (Table 1). Thus, AST suppressed partially the HC-induced cataract formation in a dose dependent manner.

Reduced glutathione levels in the lens

The lens glutathione levels were 15.1 ± 0.7 nmol/lens in the non-HC group and significantly lower in the HC-alone group (11.6 ± 2.8 nmol/lens, $p < 0.001$). The amounts of reduced glutathione in lenses were 11.3 ± 2.7 nmol/lens (HC + AST50), 13.4 ± 2.4 nmol/lens (HC + AST80), 13.7 ± 3.1 nmol/lens (HC + AST100; $p < 0.05$), and 16.4 ± 2.6 nmol/lens (AST100) respectively (Figure 1). Thus, AST recovered partially the HC-induced reduction in the lens glutathione level dose-dependently.

Blood glucose level

The blood glucose levels were 220 ± 19 mg/dl in the non-HC group and significantly higher in the HC-alone group (308 ± 38 mg/dl, $p < 0.001$). The blood glucose levels were 220 ± 36 mg/dl in the AST100 group and 312 ± 45 mg/dl in the HC + AST100 group. No significant difference was detected both between the non-HC group and the AST100 group, and between the HC-alone group and the HC + AST100 group (Figure 2). AST did not affect the serum glucose level in the embryo with or without treatment of HC.

Discussions

In this study, we examined whether AST prevents the cataract formation, using a steroid-induced cataract model in the chick embryo. To date, there are two studies reporting that AST prevented cataracts. One reported that cataract formation was prevented by administration of AST in Atlantic salmon. The authors, however, could not detect increase of lens GST in the fishes treated with AST and could not indicate that AST suppressed cataract related to the anti-oxidant defense of lens.[38] The other reported that selenite-induced cataractogenesis was prevented to a certain degree by oral administration of AST. This prevention may not be due to an anti-oxidant activity of AST but be due to a direct interaction of AST with selenite.[39] In the present study, administration of AST to a chick embryo model of steroid-induced cataract resulted in significant suppression of HC-induced lens opacity. Furthermore, treatment with AST also significantly suppressed reduction of the lens glutathione level, suggesting that the effect of AST in suppressing lens opacity involved reduction of oxidative stress.

Whereas, in an ultraviolet-induced cataract model, AST 0.1% eye drops was reported to have showed no preventive effects on lens opacification.[40] In our experiments, AST was administered to chicken eggs weighing about 60 g at the maximum dose of 100 μg, i.e., at a relatively high dose of 1.67 mg/kg. Thus, a sufficient dose of AST may be effective for prevention of cataracts.

In chick embryo HC enhances not only hepatic peroxidation reaction but also hepatic gluconeogenesis,[41] and insulin was reported to suppress cataract

formation in this model.[42] AST did not affect blood glucose levels in this study, although some researchers reported that AST increased the effect of insulin, thereby increasing glucose uptake into cells.[43] Therefore, in this study AST suppressed cataract formation not by AST's effect on sugar metabolism. And the present dose of AST might have been too low to affect sugar metabolism. More extensive studies using higher dose of AST seem to be needed.

Oral administration of AST reportedly increased the AST levels in the anterior chamber, resulting in an increase in antioxidant potential.[44] Thus, it may be possible that AST injected into the air chamber of a chicken egg and uptaken into the blood could have passed through a barrier into the eye, having exerted antioxidant actions. But, it is not clear whether AST acted in the eye, since AST levels were not determined in the eye in this study.

DMSO was used as a solvent of AST in this experiment. In the previous experiments with the same model, administration of HC 0.25 nmol/egg caused lens opacity scores to be IV or more in more than 90% of chicken embryos.[38] Whereas, in this experiment, HC 0.50 nmol/egg caused lens opacity scores to be IV or more in only 30% of eggs. It was reported that DMSO has antioxidant[45] and cytoprotective effects.[46] One of the reasons for this may be due to the suppression of lens opacification by DMSO. Since DMSO was administered in both the AST group and the control group at the same amount, suppression of lens opacification by DMSO is considered to be the same in the two groups. Therefore, the actual effect of AST must be the difference in the effects between the AST group and the control group. Moreover, the steroid-induced cataract model in the chick embryo can determine the

antioxidant effect of a poorly-water soluble substance by dissolving it in DMSO like in this study. This model is expected to be widely used for investigation of various substances.

In the present study, administration of AST to a chick embryo model of steroid-induced cataract resulted in significant suppression of HC-induced lens opacity and significant recover of the lens glutathione level, suggesting that the effect of AST in suppressing lens opacity involved reduction of oxidative stress.

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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Figure legends

Figure 1: Effect of HC and AST on the GSH in the lens

HC decreased lenticular GSH level from 15.1 ± 0.7 nmol/lens (non-HC) to 11.6 ± 2.8 nmol/lens (HC-alone). This decrease was partially recoverd by administration AST. The amounts of GSH in lenses were 11.3 ± 2.7 nmol/lens (HC + AST50), 13.4 ± 2.4 nmol/lens (HC + AST80), 13.7 ± 3.1 nmol/lens (HC + AST100; $p < 0.05$), and 16.4 ± 2.6 nmol/lens (AST100) respectively. *: $p < 0.01$ vs. non-HC, #: $p < 0.05$ vs. HC-alone (Fisher's least significant difference).

Figure 2: Effect of HC and AST on the blood glucose level

HC treatment increased the blood glucose level from 220 ± 19 mg/dl (n = 8) to 308 ± 38 mg/dl (n = 11). The blood sugar levels were 220 ± 19 mg/dl in the AST100 group (n = 11) and 308 ± 38 mg/dl in the HC + AST100 group (n =11). Significant differences were detected between all groups, except between the non-HC group and the AST100 group and between the HC-alone group and the HC + AST100 group (Fisher's protected least significant difference).

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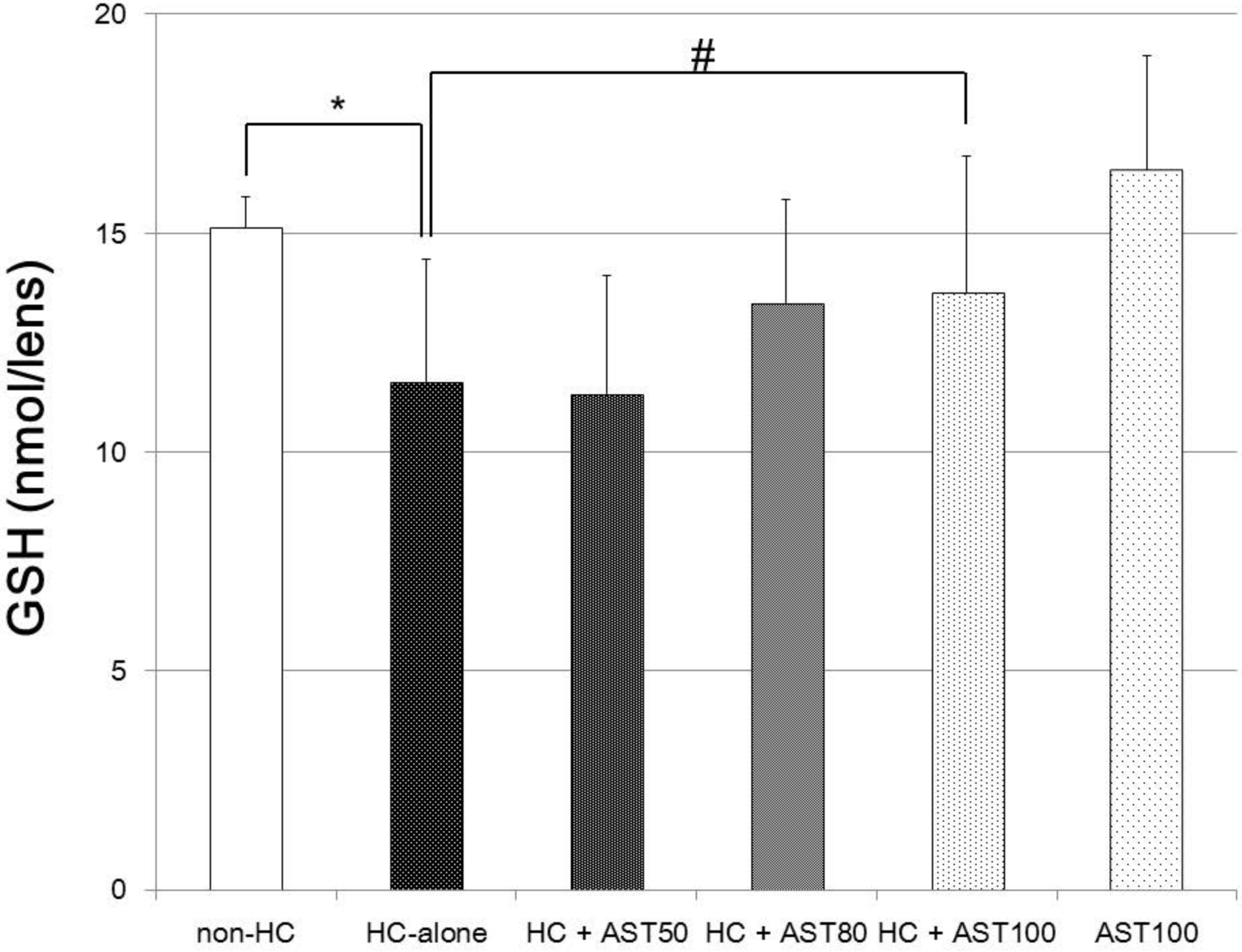
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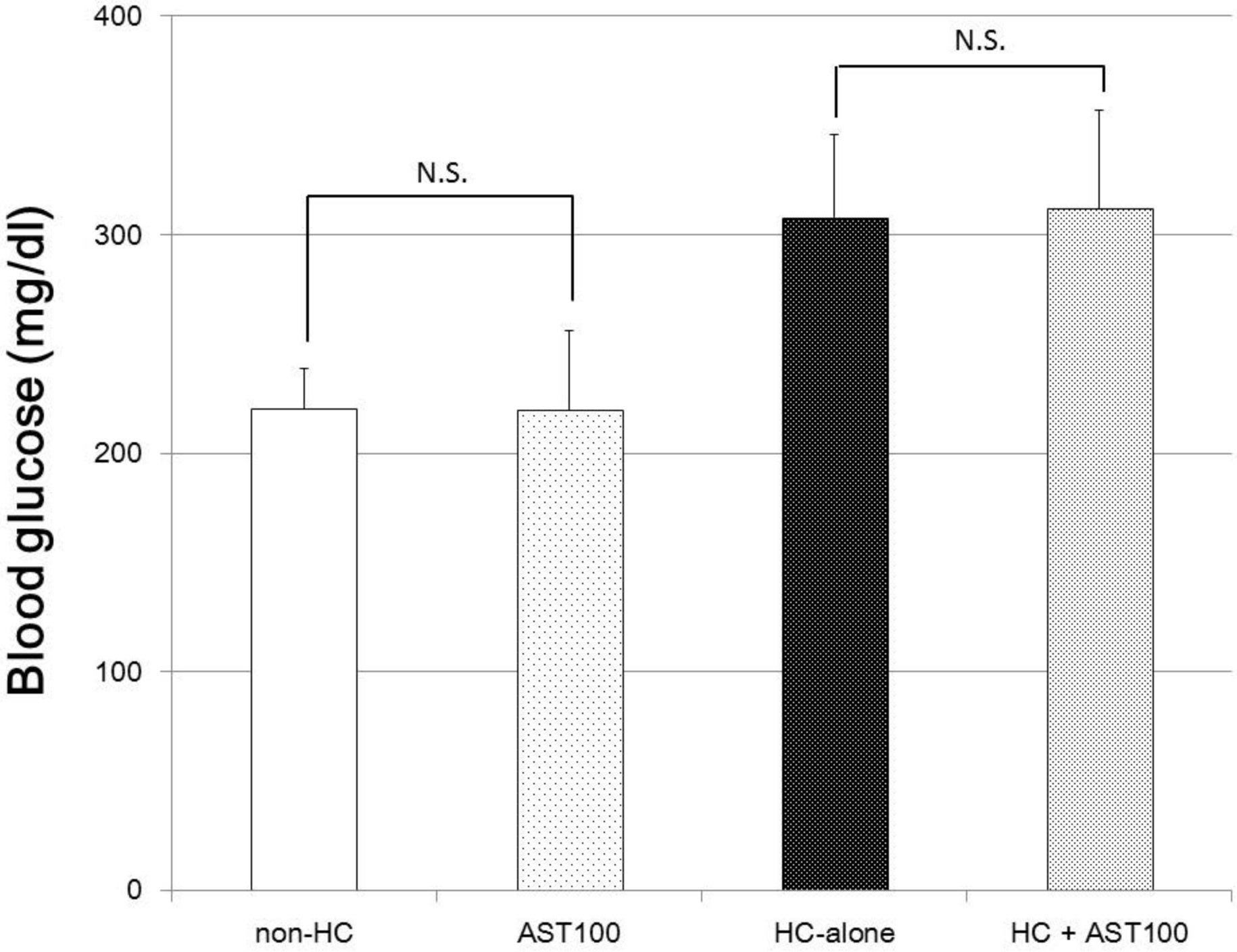
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Blood glucose (mg/dl)
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AST100
HC-alone
HC + AST100

Table 1: Incidence of cataract in HC-treated developing chick embryos after AST administration

	Stage of Lenses at 48 hours after HC Treatment				
	I	II	III	IV – V	Average
non-HC (n = 16)	100	0	0	0	1
AST100 (n = 16)	100	0	0	0	1
HC-alone (n = 16)	19	19	44	19	2.63 ± 1.02*
HC+AST50 (n = 9)	11	22	44	22	2.78 ± 0.97*
HC+AST80 (n = 9)	33	33	11	22	2.22 ± 1.20*
HC+AST100 (n = 19)	42	32	26	0	1.84 ± 0.83*, #

HC induced cataract formation. This cataract formation was partially inhibited by administration of AST. Data are percentages in parentheses. *: $p < 0.01$ vs. non-HC, #: $p < 0.05$ vs. HC-alone (Mann-Whitney U test).