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Original Article

Astaxanthin ameliorated adenine-induced renal tubulointerstitial lesions

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ABSTRACT

We examined the effect of astaxanthin (AST) on suppression of renal injury by improvement in intestinal flora in adenine-induced renal injury model. Histopathologically, moderate tubular damage and inflammatory cell infiltration were observed in 0.1% adenine-administrated group, and these changes were ameliorated by AST co-administration. In 0.2% adenine-administered group, renal injury became severer than 0.1% adenine-administered group and this was slightly ameliorated by AST co-administration. Positive reaction for HNF4α in the nuclei of tubular epithelial cells decreased in injured tubules which was recovered by AST co-administration. The number of CD3- and F4/80-positive cells increased by adenine administration and decreased by AST co-administration in both dose groups. Intestinal flora did not change by AST co-administration. The mRNA expression of inflammatory cytokines did not change by AST co-administration. In the present study, AST had some role in the anti-inflammatory effect, but not in the intestinal environment. Moreover, recovery of HNF4α in injured tubules indicated the amelioration of tubular injury by AST and the utility of HNF4α as a tubular injury marker. Based on the results of this experiment and other reports it was suggested that amelioration effect of AST on renal injury might be different according to the degree and quality of injury.

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KEYWORDS: astaxanthin, intestinal flora, renal injury.

INTRODUCTION

Chronic kidney disease (CKD) is a pathological condition associated with diverse renal diseases such as glomerular nephritis, and is a worldwide problem with high prevalence rate. Complications include cardiovascular disturbance, cognitive decline, anemia, fracture and high mortality [1]. Renal tubular damage and subsequent interstitial fibrosis are common findings in CKD and their development rate is closely related to a decline in renal function, and hence understanding the mechanism of onset and progress of interstitial fibrosis is important for treatment of renal failure. Therefore, a lot of studies using experimental models induced by unilateral ureteral obstruction (UUO), ischemia reperfusion, and drugs, such as cisplatin and mercury chloride, have been conducted to investigate the mechanisms of tubulointerstitial fibrosis. Adenine, a type of nucleic-acid base, is one of the inducers for renal injury and is reported to produce clinical state similar to human CKD [2]. Excess adenine is translated into 2,8-dihydroxyadenine through the action of adenine phosphoribosyl transferase (APRT) and xanthine dehydrogenase (XDH) [3]. However, extremely low solubility of 2,8-dihydroxyadenine results in precipitation and deposition of crystals in proximal and/or distal tubules, and leads to renal failure anchored by renal tubular damage [4, 5].

Under normal conditions, intestinal flora influences energy metabolism, and nitrogen and micronutrient

homeostasis, but alteration in function and interaction of intestinal bacteria causes a variety of disorders [6]. Uremic toxin belongs to the family of the metabolite generated from colonic fermentation of carbohydrate and protein by intestinal flora. These compounds are metabolized in liver and excreted by the kidney in normal condition, but in accordance with the progression of CKD, their blood concentrations increase along with alteration in systemic metabolism and impaired excretion of metabolic products into urine as the disorder progresses [7]. Uremic toxin is classified into 104 types by the European Uremic Toxin Work Group [8], of which serum protein-binding uremic toxins belong to the subfamily that is very difficult to remove through dialysis. Indoxyl sulfate and *p*-cresol sulfate are included in this subfamily. An increase in the blood concentrations of these factors in CKD causes the deterioration of renal dysfunction [9, 10].

Astaxanthin (AST) is a carotenoid found in marine organisms such as salmon, crustaceans and algae. Most animals cannot produce AST by themselves and gain it by the intake of AST produced by microalgae like *Heamatococcus*. AST is known as a strong antioxidant and also have some biologically important effects such as protection from ultraviolet rays, improvement of immunity, effect as the hormone precursor, and provitamin A [11, 12]. Moreover, Yonei *et al*. reported that AST had an inhibitory effect on disturbance of the intestinal flora in high-fat diet feeding mice [13]. Thus, AST is also inferred to be effective as a prebiotics. In the present study, we examined the effect of AST on suppression of renal injury by decreasing the production of uremic toxin in accordance with improvement in intestinal flora by use of an adenine-induced renal injury model.

MATERIALS AND METHODS

Chemicals

Adenine (6-aminopurine) was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan) and AST was distributed from Fuji Chemical Industries Co., Ltd. (Toyama, Japan). All other chemicals were of analytical grade and obtained commercially.

Experimental design

Fifty-five male ICR mice aged 5 weeks were purchased from Japan SLC, Inc. (Shizuoka, Japan). Mice were housed in cages and allowed *ad libitum* access to tap water and powdered CRF-1 basal diet (Oriental Yeast Co., Tokyo, Japan). All procedures of this study were conducted in compliance with the Guidelines for Proper Conduct of Animal Experiments (Science Council of Japan, June 1, 2006) and according to the Guide for Animal Experimentation of Tokyo University of Agriculture and Technology. All efforts were made to minimize animal suffering. Mice were handled under standard conditions (room temperature, 23 ± 3 °C; relative humidity, 50 ± 20%; 12 h light and dark cycle), and were acclimatized for 1 week and randomly allocated to groups.

In experiment 1 (Exp. 1), thirty-five mice were divided into 7 groups namely 1) the control group, 2) 0.1% adenine-administrated group, 3) 0.2% adenine-administrated group, 4) 0.1% adenine + 0.05% AST co-administrated group, 5) 0.1% adenine + 0.1% AST co-administrated group, 6) 0.2% adenine + 0.05% AST co-administrated group, and 7) 0.2% adenine + 0.1% AST co-administrated group, and examined for 4 weeks. In experiment 2 (Exp. 2), 0.2% adenine-containing diet was administered to fifteen mice for 6 weeks, then 5 mice were fed AST (0, 0.05 or 0.1%)-containing diet for 2 weeks. As the control group, 5 mice were fed basal diet for 8 weeks. These doses were determined according to the result of preliminary examination by others [14, 15]. During experimental periods, food and water consumption was measured once in three days, and body weight was measured once in a week. At the end of the respective experiments, all mice were sacrificed by exsanguination from the abdominal aorta under isoflurane anesthesia. Kidneys were excised and weighed, and relative weight was calculated. Rectal feces was also excised.

For histopathology and immunohistochemistry, the kidney obtained from each mouse was coronaly cut at the hilus and was fixed by the PLP (periodate-lysine-paraformaldehyde)-AMeX (acetone, methyl benzoate, and xylene) [16]. In brief, specimens were immersed in PLP fixative

(containing 4% paraformaldehyde) for 7 hours at 4 °C, and then were washed with phosphate-buffered saline (PBS; 0.01 M, pH 7.4) for 2 hours at 4 °C. Then, the tissues were dehydrated in acetone overnight at 4 °C and for 1 hour twice at room temperature, cleared in methyl benzoate for 30 minutes twice, and in xylene for 30 minutes twice, soaked in paraffin for 40 minutes three times at 60 °C, and embedded in paraffin. The paraffin blocks prepared by the PLP-AMeX method were kept at 4 °C. The kidney tissues for polymerase chain reaction (PCR) were collected by cutting renal cortex into small pieces, were frozen in liquid nitrogen, and then were preserved at -80 °C.

Histopathology

Two-μm paraffin sections were stained with hamatoxylin and eosin (HE) for histopathological examination.

Immunohistochemistry

For immunohistochemistry, two-μm sections were deparaffinized, and soaked in methanol containing 0.3% hydrogen peroxide for 30 minutes to block endogenous peroxidase activity. Then, the sections were treated with 10% normal goat serum for 30 minutes to block non-specific reaction. Anti-hepatocyte nuclear factor (HNF) 4α antibody (rabbit monoclonal, Cell Signaling Technology, Inc., USA), anti-CD3 antibody (rabbit polyclonal, Abcam Plc., UK), and anti-F4/80 antibody (rat monoclonal, Bio-Rad Laboratories Inc., USA) were applied as the primary antibodies overnight at 4 °C. As for HNF4α and CD3, the sections were incubated with Envision solution (Dako, Denmark) against rabbit immunoglobulin (Ig) G for 30 minutes at room temperature. In the case of F4/80, each section was incubated with anti-rat IgG (rabbit, Vector Laboratories Inc., USA) for 30 minutes and then with Vectastain ABC kit (Vector Laboratories Inc., USA) for 30 minutes. Antibody-binding was visualized with 3,3-diaminobenzidine (DAB) chromogen and counterstained with Mayer's hematoxylin. Tris-buffured seline (0.15 M NaCl, 0.05 M Tris-HCl, pH7.6) was used for rinsing. With regard to CD3 and F4/80, the number of positive cells was counted at 400-fold magnification, and an average number per field was calculated.

Real-time reverse transcription polymerase chain reaction (Real-time RT-PCR) analysis

Total RNA was extracted from the renal cortical tissue of each mouse using RNeasy Mini Kit (Qiagen, Netherlands). The concentrations of total RNA samples were measured using Gen5 2.0 (BioTek Instruments, Inc., USA). After that, cDNA was prepared from 500 ng of total RNA using PrimeScript RT Master Mix (Takara Bio Co., Ltd, Shiga, Japan) in LifeECO Thermal Cycler (Bioer Technology Co., Ltd, Hangzhou, China) (37 °C for 15 minutes and 85 °C for 5 seconds). Real-time PCR was performed using SYBR Premix Ex Taq II (Takara Bio Co., Ltd, Shiga, Japan) in Thermal Cycler Dice Real Time System II (Takara Bio Co., Ltd, Shiga, Japan) (95 °C for 30 seconds, 40 cycle of 95 °C for 5 seconds and 60 °C for 30 seconds, 95 °C for 15 seconds, 60 °C for 30 seconds, and 95 °C for 15 seconds). Eight targets for oxidative stress-related factors (NADPH oxidase (NOX) 2, NOX4, glutathione peroxidase (GPx) 1, glutathione reductase (GSR), catalase (CAT), superoxide dismutase (SOD) 1, SOD2, peroxiredoxin (Prdx) 1) and four targets for inflammatory cytokine (tumor necrosis factor (TNF) α, interleukin (IL)-1β, IL-6, monocyte chemotactic and activating factor (MCP) -1) were analyzed. Primer sequences are listed in Table 1. The relative value of gene expression was calculated using standard carve values that were normalized to those of the β-actin gene, the endogenous control, in the same sample, and then relative to a control value.

Real-time PCR for intestinal flora

Intestinal floral DNA extraction from feces was performed according to the QIAamp DNA stool kit methods (Qiagen, Netherlands). Real-time PCR was performed using SYBR Premix Ex Taq II in Thermal Cycler Dice Real Time System II (95 °C for 30 seconds, 40 cycle of 95 °C for 5 seconds and 60 °C for 30 seconds, 95 °C for 15 seconds, 60 °C for 30 seconds, and 95 °C for 15 seconds). Six targets for intestinal flora (*Bifidobacterium*, *Lactobacilli*, *Escherichia coli*, *Bacteroides*, *Clostridium* XIV, *Clostridium* IX) were analyzed. Primer sequences are listed in Table 1. The relative value was calculated using standard carve values that were normalized to those of the total bacteria in the same sample, and then relative to a control value.

Table 1. Sequences of Primer used for real-time PCR.

Target	Forward	Reverse
Intestinal flora		
Total bacteria	CGGTGAATACGTTCCCGG	GGWTACCTTGTTACGACTT
Bifidobacterium	CGGGTGAGTAATGCGTGACC	TGATAGGACGCGCGACCCCA
Lactobacilli	TGGATGCCTTGGCACTAGGA	AAATCTCCGGATCAAAGCTTACTTAT
Escherichia coli	CATGCCGCGTGTATGAAGAA	CGGGTAACGTCAATGAGCAAA
Bacteroides	CCTWCGATGGATAGGGGTT	CACGCTACTTGGCTGGTTCAG
Clostridium XIV	GACGCCGCGTGAAGGA	AGCCCCAGCCTTTCACATC
Clostridium IX	CCTTCCGTGCCGSAGTTA	GAATTAAACCACATACTCCACTGCTT
Oxidative stress-related factor		
NOX2	GACCATTGCAAGTGAACACCC	AAATGAAGTGGACTCCACGCG
NOX4	TCATTTGGCTGTCCCTAAACG	AAGGATGAGGCTGCAGTTGAG
GPx1	CAATCAGTTCGGACACCAGGAG	TCTCACCATTCACTTCGCACTTC
GSR	GGATTGGCTGTGATGAGATG	CTGAAGAGGTAGGATGAATGG
CAT	CAGGTGCGGACATTCTAC	TTGCGTTCTTAGGCTTCTC
SOD1	GGTGAACCAGTTGTGTTGTC	CCGTCCTTTCCAGCAGTC
SOD2	GACCTGCCTTACGACTATG	GAAGAGCGACCTGAGTTG
Prdx1	TTCTTTTACCCTCTTGACTTT	TCTTGGGTGTGTTAATCC
Inflammatory cytokine		
TNFα	CCCTCACACTCAGATCATCTTCT	GCTACGACGTGGGCTACAG
IL-1β	GCAACTGTTCCTGAACTCAACT	ATCTTTTGGGGTCCGTCAACT
IL-6	TAGTCCTTCCTACCCCAATTTCC	TTGGTCCTTAGCCACTCCTTC
MCP-1	TTAAAAACCTGGATCGGAACCA	GCATTAGCTTCAGATTTACGGG
House keeping gene		
β-actin	CCCTGGCTCCTAGCACCAT	AGAGCCACCAATCCACACAGA

Statistical analysis

Obtained data were expressed as mean and standard deviations. Statistical differences between groups were evaluated using the Steel-Dwass test.

RESULTS

Macroscopic findings and kidney weight

All 0.1% adenine-administrated groups in Exp. 1 had similar finding as control group, but renal shrinkage and color degradation were observed in all 0.2% adenine-administrated groups both in Exp. 1 and Exp. 2.

As for relative kidney weight, it increased by 0.1% adenine administration, but returned to control level in AST co-administration groups. In contrast, the relative kidney weight in 0.2% adenine-administered groups was similar as the control group. In Exp. 2, compared with the control group, the relative kidney weight decreased, but here was no significant difference among adenine-administrated groups (Table 2).

Table 2. Absolute and relative kidney weight.

Exp. 1							
	Control	0.1% Adenine			0.2% Adenine		
		alone	ASTL	ASTH	alone	ASTL	ASTH
Absolute	0.718 ± 0.064	0.756 ± 0.068	0.738 ± 0.074	0.666 ± 0.030	0.344 ± 0.084*	0.434 ± 0.073*	0.446 ± 0.090*
Relative	1.370 ± 0.093	1.708 ± 0.085	1.772 ± 0.152*	1.499 ± 0.096	1.081 ± 0.176	1.660 ± 0.196	1.460 ± 0.155

Exp. 2							
	Control				0.2% Adenine		
					alone	ASTL	ASTH
Absolute	0.718 ± 0.064				0.268 ± 0.034*	0.254 ± 0.041*	0.220 ± 0.043*
Relative	1.370 ± 0.093				0.687 ± 0.087*	0.710 ± 0.120*	0.659 ± 0.053*

Data represents as average + SD.
* $p < 0.05$ compared with control group.

Histopathology

In Exp. 1, moderate cell damage was observed at proximal tubules in which adenine was crystallized and deposited in lumen, and inflammatory cell infiltration was observed around the damaged tubules in 0.1% adenine-administrated groups (Figure 1 A, B). In AST co-administrated groups, similar findings were also observed, but the severities were reduced (Figure 1 C, D). In 0.2% adenine-administrated groups, elution of adenine crystal in tubular lumen, necrosis, dilation and reproduction of renal tubules, inflammatory cell infiltration, interstitial fibrosis, and localized calcification were observed. Inflammatory cell infiltration tended to be alleviated by administration of AST (Figure 1 E, F).

In Exp. 2, the similar findings of 0.2% adenine-administrated groups in Exp. 1 were observed, but not ameliorated by AST co-administration.

Immunohistochemistry

In Exp. 1, positive reaction for HNF4α was observed in the nuclei of tubular epithelial cells in control group (Figure 2 A, B). In accordance with the severity of tubular injury, these reactions gradually decreased in necrotic and/or regenerative tubules in adenine-administered groups. In addition, the decreased reaction was recovered by AST co-administration in 0.1% adenine-administered groups, but not in 0.2% adenine-administered groups (Figure 2 C-F). Also in Exp. 2, positive reaction for HNF4α decreased in necrotic and/or regenerative tubules in accordance with the severity of tubular injury, and was not recovered by AST co-administration.

The number of CD3- and F4/80-positive cells was investigated only in Exp. 1. The number of CD3-positive cells moderately increased by 0.1% adenine administration (6.46 ± 4.41) compared with control (2.44 ± 0.64), and not significantly decreased by AST co-administration (2.02 ± 0.41 and 1.95 ± 0.31 in low and high doses of AST, respectively). In 0.2% adenine-administered groups, the number of CD3-positive cells obviously increased by adenine alone (33.01 ± 12.26) and significantly decreased by high dose of AST co-administration (10.20 ± 4.02) (Figure 3). The number of F4/80-positive cells moderately increased

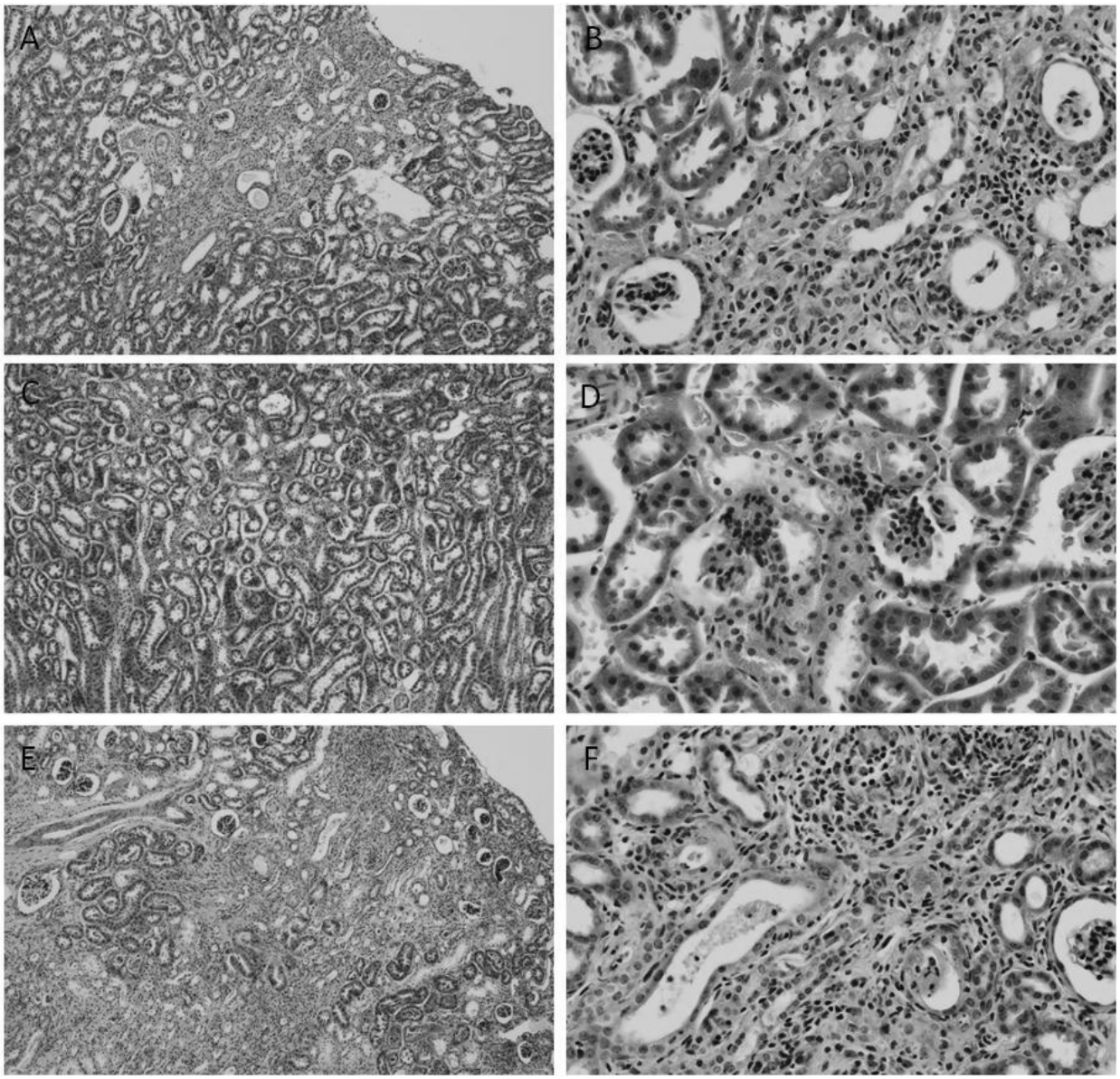

Figure 1. Histopathological changes by 0.1% adenine administration (A, B), AST co-administration with 0.1% adenine (C, D) and 0.2% adenine administration (E, F). HE stain, A, C, E : × 100, B, D, F : × 400.

by 0.1% adenine administration (7.51 ± 2.41) compared with control (1.53 ± 0.37), and significantly but not dose-dependently decreased by AST co-administration (2.14 ± 1.21 and 0.96 ± 0.76 in low and high doses of AST, respectively). In 0.2% adenine groups, the number of F4/80-positive cells severely increased by adenine alone (108.97 ± 22.93), and significantly but not dose-dependently decreased by AST co-administration (49.74 ± 14.09 and 65.64 ± 18.66 in low and high doses of AST, respectively) (Figure 4).

Changes in intestinal flora

E. coli tended to decrease by AST co-administration compared with 0.1% adenine administration, and *Clostridium* XIV significantly increased by AST co-administration compared with 0.2% adenine administration. In Exp. 2, *Lactobacillus* increased

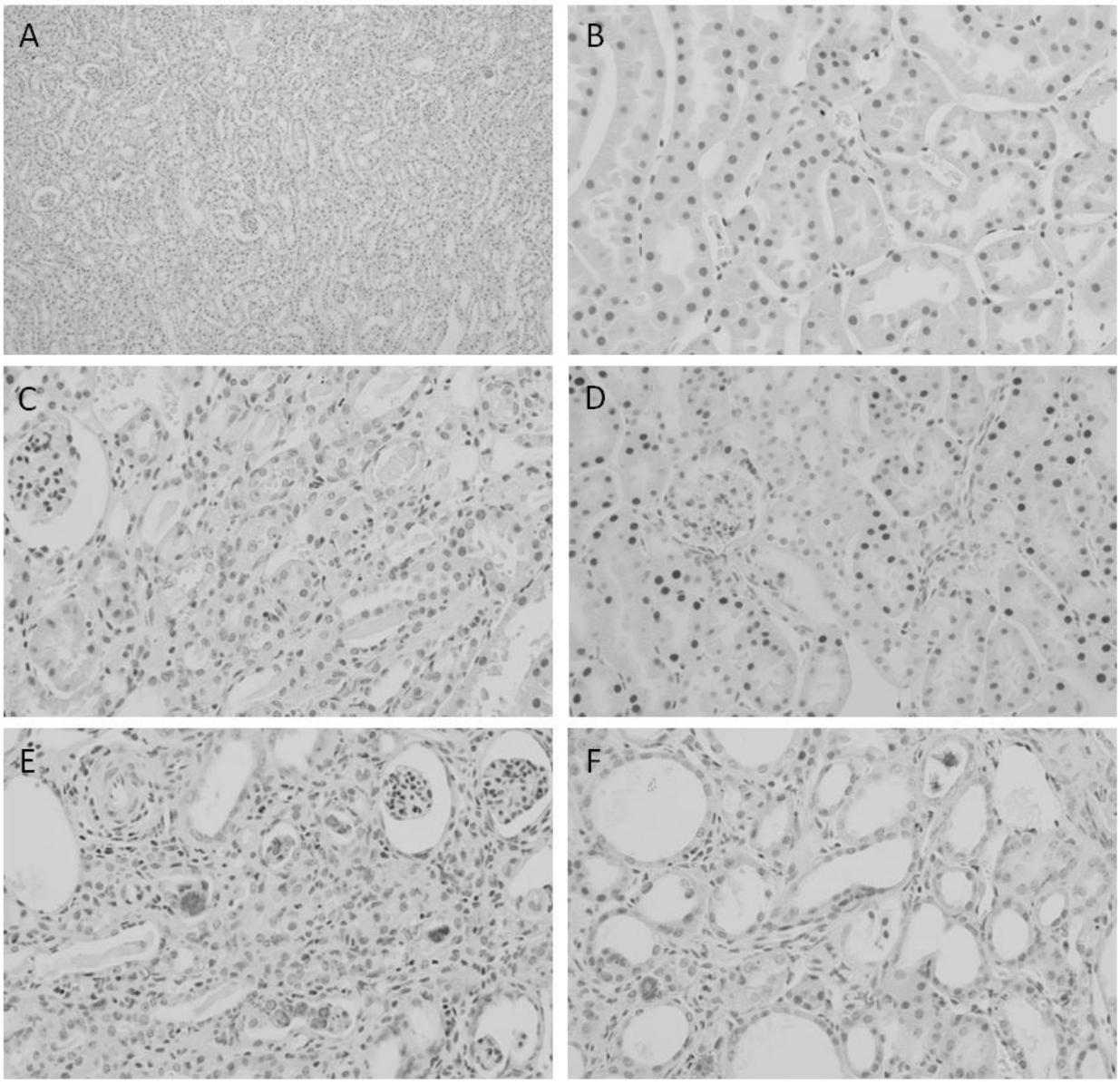

Figure 2. Immunohistochemistry for HNF4α in control (A, B), and 0.1% (C) and 0.2% (E) adenine-administered group and AST co-administration with 0.1% (D) and 0.2% (F) adenine administration. A : × 100, B-F : × 400.

by adenine administration, but not changed by AST co-administration (Table 3).

Changes in mRNA expression

The mRNA expression of oxidative stress-related factor was similar to control in 0.1% adenine groups. In 0.2% adenine groups, in Exp. 1 and Exp. 2, mRNA expressions of many oxidative stress-related factors were decreased. In contrast, Nox2 mRNA expression increased by adenine administration, and this increase was not reduced by AST co-administration.

The mRNA expression of inflammatory cytokine was similar to control in 0.1% adenine groups. In 0.2% adenine groups, in Exp. 1 and Exp. 2, the mRNA expression of inflammatory cytokine increased by adenine administration, and this increase was not reduced by AST co-administration (Table 4).

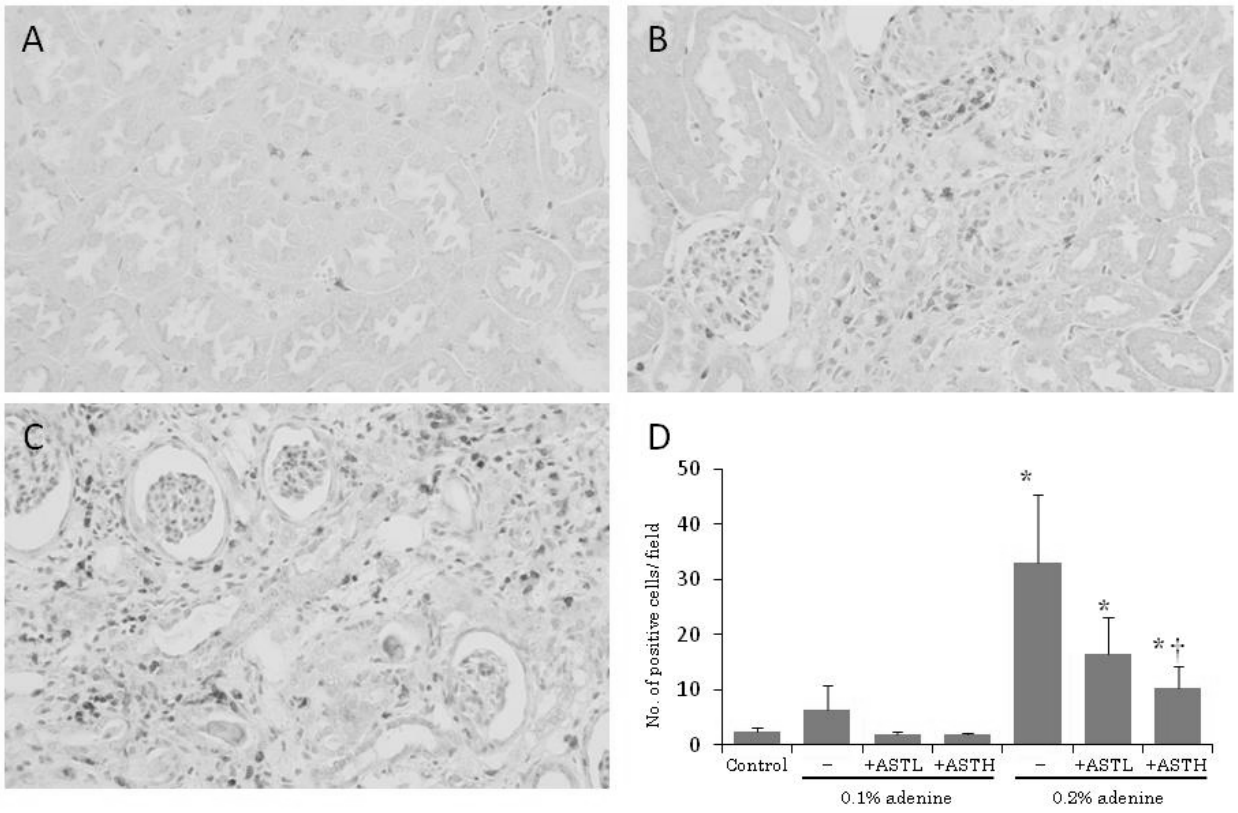

Figure 3. Immunohistochemistry for CD3 in control (A), and 0.1% (B) and 0.2% (C) adenine-administered group. × 400. The number of positive cells (D). Data represents average + SD. * $p < 0.05$ compared with control group. † $p < 0.05$ compared with adenine-administered group.

DISCUSSION

AST ameliorated the tubular injury in 0.1% adenine-administered groups, and slight amelioration was also observed in 0.2% adenine-administered groups. As for the effect of AST on renal injury, attenuating effects of kidney function and oxidative stress in mercuric chloride-treated rats and adriamycin-induced focal segmental glomerulosclerosis in mice have been reported [17, 18]. Therefore, it was speculated that AST showed different effect depending on region, quality and degree of injury. Immunohistochemically, the number of CD3- and F4/80-positive cells decreased by AST co-administration both in 0.1% and 0.2% adenine-administered groups. Because AST also has an anti-inflammatory effect, it may exert an anti-inflammatory effect on adenine-induced renal lesion [19, 20]. In contrast to the immunohistochemical results, mRNA expression of inflammatory cytokine was not significantly changed by AST co-administration. Because focal inflammatory cell infiltration was sparsely observed in cortical and medullary area, the piece of renal cortex used for total RNA extraction did not include such lesions. Therefore, future investigation including use of laser micro-dissection for capturing such regional alteration would be needed to clarify the discrepancy.

The expression of HNF4α in renal tubular epithelial cells decreased by adenine administration, and recovered by AST co-administration. HNF4α is a cell-specific transcription factor and seems to play a crucial role in selective gene expression in a variety of organs in the adult. In the kidney, HNF4α was abundantly expressed in the proximal convoluted and straight tubules, whereas the other sections lack significant expression [21, 22]. It was reported that Hnf4α could bind to the proximal promoter regions of some genes encoding transporters in the differentiating rat kidney, and

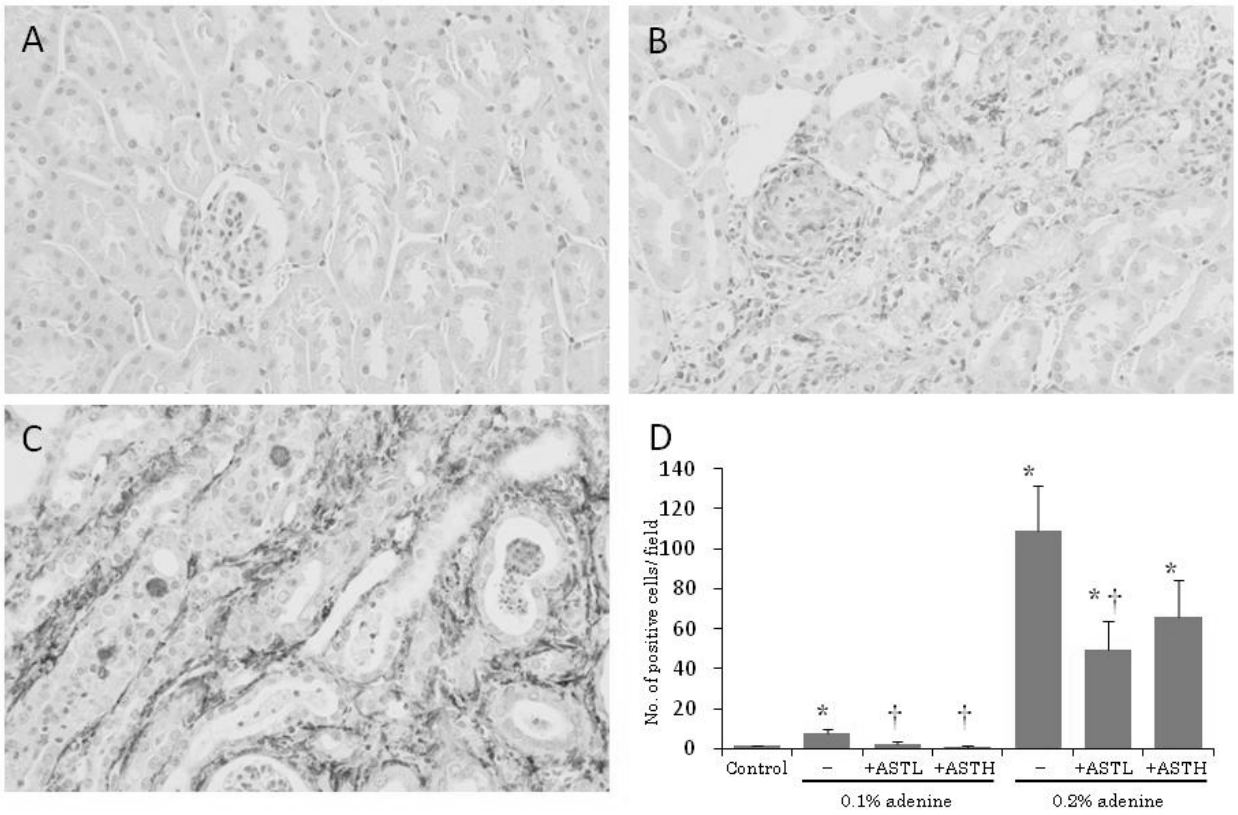

Figure 4. Immunohistochemistry for F4/80 in control (A), and 0.1% (B) and 0.2% (C) adenine-administered group. × 400. The average number of positive cells (D). Data represents average + SD. * $p < 0.05$ compared with control group. † $p < 0.05$ compared with adenine-administered group.

Table 3. Change in interstitial flora.

Exp. 1							
	Control	**0.1% Adenine**			**0.2% Adenine**		
		alone	**ASTL**	**ASTH**	**alone**	**ASTL**	**ASTH**
Bacteroides	1.00 ± 0.06	1.69 ± 0.96	1.29 ± 0.57	0.78 ± 0.27	0.35 ± 0.24	0.58 ± 0.34	0.72 ± 0.25
Clostridium IX	1.00 ± 0.27	1.23 ± 0.19	1.64 ± 0.57	1.18 ± 0.34	1.07 ± 0.23	1.06 ± 0.29	0.82 ± 0.82
Clostridium XIV	1.00 ± 1.41	4.83 ± 6.26	0.05 ± 0.03	0.07 ± 0.04	0.77 ± 1.41	16.77 ± 16.31†	10.62 ± 1.49*†
E. coli	1.00 ± 1.32	5.36 ± 4.91	1.18 ± 1.34	0.21 ± 0.17	0.24 ± 0.35	0.34 ± 0.53	0.16 ± 0.2
Bifidobacterium	1.00 ± 0.87	0.46 ± 0.23	0.73 ± 0.37	0.66 ± 0.28	0.61 ± 0.46	0.78 ± 0.29	1.22 ± 0.53
Lactobacillus	1.00 ± 1.18	0.49 ± 0.66	0.61 ± 0.42	0.26 ± 0.17	12.76 ± 10.99	31.86 ± 48.15	2.95 ± 3.07

Table 3 continued..

Exp. 2							
	Control				**0.2% Adenine**		
					alone	**ASTL**	**ASTH**
Bacteroides	1.00 ± 0.77				1.15 ± 0.48	1.25 ± 0.49	1.41 ± 0.81
Clostridium IX	1.00 ± 1.69				5.18 ± 4.36	32.79 ± 37.12	158.33 ± 308.72
Clostridium XIV	1.00 ± 0.28				0.82 ± 0.27	0.99 ± 0.26	0.76 ± 0.23
E. coli	1.00 ± 1.35				0.18 ± 0.13	0.07 ± 0.06	0.13 ± 0.07
Bifidobacterium	1.00 ± 1.01				0.92 ± 0.40	0.74 ± 0.29	0.71 ± 0.27
Lactobacillus	1.00 ± 0.92				52.22 ± 32.08*	66.36 ± 36.88*	14.26 ± 8.13*

Data represents as average + SD.
* $p < 0.05$ compared with control group.
† $p < 0.05$ compared with adenine-administrated group.

Table 4. Change in mRNA expression.

Exp. 1							
	Control	**0.1% Adenine**			**0.2% Adenine**		
		alone	**ASTL**	**ASTH**	**alone**	**ASTL**	**ASTH**
Oxidative stress related factors							
Nox2	1.00 ± 0.19	0.84 ± 0.34	1.00 ± 0.08	0.81 ± 0.16	6.25 ± 3.49*	9.24 ± 4.34*	7.80 ± 1.54*
Nox4	1.00 ± 0.11	0.92 ± 0.17	1.05 ± 0.22	1.07 ± 0.21	0.16 ± 0.09*	0.1 ± 0.04*	0.12 ± 0.01*
Cat	1.00 ± 0.18	0.91 ± 0.14	0.91 ± 0.12	0.96 ± 0.08	0.12 ± 0.08*	0.05 ± 0.03*	0.07 ± 0.01*
Gpx2	1.00 ± 0.2	0.99 ± 0.12	1.10 ± 0.03	1.1 ± 0.09	0.25 ± 0.14*	0.15 ± 0.07*	0.21 ± 0.04*
Gsr	1.00 ± 0.21	1.13 ± 0.14	1.03 ± 0.13	1.04 ± 0.14	0.29 ± 0.12*	0.24 ± 0.11*	0.26 ± 0.05*
Prdx1	1.00 ± 0.38	0.63 ± 0.10	0.67 ± 0.06	0.71 ± 0.11	0.14 ± 0.07*	0.11 ± 0.05*	0.11 ± 0.02*
Sod1	1.00 ± 0.17	0.76 ± 0.14	0.80 ± 0.08	0.78 ± 0.06	0.12 ± 0.07*	0.06 ± 0.06*	0.07 ± 0.02*
Sod2	1.00 ± 0.14	0.90 ± 0.11	0.86 ± 0.11	0.92 ± 0.04	0.13 ± 0.05*	0.08 ± 0.08*	0.09 ± 0.02*

Table 4 continued..

Exp. 1							
	Control	**0.1% Adenine**			**0.2% Adenine**		
		alone	**ASTL**	**ASTH**	**alone**	**ASTL**	**ASTH**
Inflammaotry cytokines							
Tnfa	1.00 ± 0.36	1.14 ± 0.74	1.01 ± 0.11	1.09 ± 0.46	16.49 ± 6.47*	27.18 ± 4.61*	21.53 ± 5.00*
Il-1b	1.00 ± 0.13	0.90 ± 0.44	0.95 ± 0.38	0.58 ± 0.06*	10.31 ± 2.24	8.85 ± 1.78*	7.77 ± 2.93*
Il-6	1.00 ± 0.34	1.43 ± 0.67	0.92 ± 0.30	0.59 ± 0.23	18.14 ± 9.07*	25.32 ± 5.66*	23.77 ± 9.08*
Mcp-1	1.00 ± 0.28	1.78 ± 1.32	0.98 ± 0.28	0.55 ± 0.08*	27.97 ± 4.37*	31.15 ± 5.10*	46.24 ± 11.53†

Exp. 2							
	Control				**0.2% Adenine**		
					alone	**ASTL**	**ASTH**
Oxidative stress related factors							
Nox2	1.00 ± 0.33				2.60 ± 0.67*	3.74 ± 1.39*	5.05 ± 1.95*
Nox4	1.00 ± 0.58				0.15 ± 0.06*	0.12 ± 0.05*	0.13 ± 0.08*
Cat	1.00 ± 0.29				0.24 ± 0.13*	0.21 ± 0.14*	0.20 ± 0.16*
Gpx2	1.00 ± 0.69				0.15 ± 0.05*	0.11 ± 0.05*	0.11 ± 0.06*
Gsr	1.00 ± 0.4				0.36 ± 0.08*	0.31 ± 0.11*	0.32 ± 0.10*
Prdx1	1.00 ± 0.95				0.14 ± 0.06*	0.12 ± 0.05*	0.12 ± 0.08*
Sod1	1.00 ± 0.52				0.15 ± 0.05*	0.12 ± 0.06*	0.11 ± 0.09*
Sod2	1.00 ± 0.51				0.17 ± 0.07*	0.14 ± 0.07*	0.13 ± 0.09*
Inflammaotry cytokines							
Tnfa	1.00 ± 0.36				9.25 ± 2.03*	12.52 ± 3.83*	12.70 ± 3.35*
Il-1b	1.00 ± 0.13				5.66 ± 4.32*	4.57 ± 3.86*	2.84 ± 0.96*

Table 4 continued..

Exp. 2							
	Control				**0.2% Adenine**		
					alone	**ASTL**	**ASTH**
Inflammaotry cytokines							
Il-6	1.00 ± 0.34				14.51 ± 9.63*	14.04 ± 6.75*	19.91 ± 15.95*
Mcp-1	1.00 ± 0.28				33.15 ± 15.98*	24.11 ± 7.62*	23.08 ± 6.08*

Data represents as average + SD.
* $p < 0.05$ compared with control group.
† $p < 0.05$ compared with adenine-administrated group.

suggested that a network of genes with Hnf4α at its center plays a role in regulating the capacity for drug and toxin handling by the nascent proximal tubule of the kidney [23]. In addition, it was also suggested that Hnf4α protein is associated with the mesenchymal epithelial transition forming the intercellular junction in early nephrogenesis [24]. According to these reports, HNF4a may have some effects on the loss of transport gene expression and on the induction of epithelial mesenchymal transition that were speculated as the phenomena occurring as a result of nephrotoxicity and renal insufficiency. As far as we know, the effect of HNF4α on the development of tubulointerstitial lesion has not been investigated. Therefore, our result suggested that HNF4α is a useful marker of tubular injury and new target for investigating the mechanism of renal dysfunction.

The actual condition of adenine-induced tubular obstruction involves persistent stimulus by crystallization of 2,8-dihydroxyadenine in renal tubules. Because the production of 2,8-dihydroxyadenine resulted from homeostatic redox system, the change of mRNA expression of oxidative stress-related factors was investigated. However, except NOX2, the mRNA expression did not change by 0.1% adenine administration and reduced by 0.2% adenine administration. It was speculated that oxidative stress caused by 0.1% adenine might be too weak to influence the mRNA expressions, and that the ability to produce anti-oxidative enzymes was lost by severe tubular injury induced by 0.2% adenine administration. As for NOX2, its mRNA expression increased by 0.2% adenine administration. NOX2 and NOX4 belong to NOX family, but their expression in kidney was different. NOX4 is the most abundant NOX homolog in the kidney, and is highly expressed in renal tubules [25]. In contrast, NOX2 is mainly expressed in phagocytes, and its expression was very low in the kidney [26]. Because tubular epithelial cells, NOX4 producer, were necrotized and regenerated in 0.2% adenine-administered groups, these cells might lose normal function. In contrast, F4/80-positive macrophages, NOX2 producer, increased in 0.2% adenine-administered groups. Therefore, it is speculated that the difference in cell type could influence the expression of the respective NOX.

Amelioration of the intestinal environment by AST was one of the aims of this study, but the expected result was not obtained. Although bacteria test of feces was generally used for investigating the alteration of intestinal flora, according to the reports by Yonei *et al*. [13], analysis by real-time PCR was selected. Hence, raising the precision of technique may help in the improvement of this result.

Mishima *et al*. reported that an anti-constipation drug, lubiprostone, ameliorates the progression of CKD and the accumulation of uremic toxins by

improving the gut microbiota and intestinal environment with 2-weeks lubiprostone administration after 6-weeks 0.2% adenine administration [27]. Although Exp. 2 was modeled on this report, amelioration of renal injury by AST was not observed. It is thought that AST has less effect on improvement of intestinal flora than lubiprostone.

CONCLUSION

In conclusion, AST had ameliorative effect on tubular injury and inflammatory cell infiltration in adenine-induced nephropathy, but might not have the effect on amelioration of intestinal flora and reduction of oxidative stress that we had expected. However, because the degree of tubular injury and inflammatory cell infiltration were varied among mice in the individual group, future research that repeat the same investigation to increase the data in respective groups would be required to clarify the results obtained in this investigation.

CONFLICT OF INTEREST STATEMENT

There are no conflicts of interest.

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