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Letter

In vitro and *in vivo* evaluation of mutagenicity of fucoxanthin (FX) and its metabolite fucoxanthinol (FXOH)

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ABSTRACT — Mutagenicity of fucoxanthinol (FXOH), the major compound after oral ingestion of fucoxanthin (FX), was evaluated by *in vitro* Ames test, and of FX by *in vivo* micronucleus test. In *in vitro* Ames test, bacterial reverse mutation was examined by using *Salmonella typhimurium* strains TA98, TA100, TA1535 and TA1537, and *Escherichia coli* WP2*uvr*A/pKM101, with or without metabolic activation by S9 mix in the preincubation method, and mutagenicity of FXOH was found to be negative in all cases. In *in vivo* micronucleus test, mice were orally administered with FX at doses of 500, 1,000 and 2,000 mg/kg, and the bone marrow cells were taken 24 hr after the administration to observe the incidence of micronucleus cells, and mutagenicity of FX was found to be negative at all doses. Based on the data of the present study it can be presumed that orally administered FX is a safe compound in terms of mutagenicity under the experimental conditions employed here.

Key words: FX, Marine carotenoid, Ames test, Micronucleaus test

INTRODUCTION

Fucoxanthin (FX) (Fig. 1), a xanthophyll derivative, is an orange-colored pigment and is present specifically in edible brown algae such as wakame (*Undaria pinnatifida*). It has been reported that FX shows wide variety of beneficial activities such as antiobesity activity (Maeda *et al*., 2005), anticancer activity against different kinds of cancer cells (Das *et al*., 2008; Ishikawa *et al*., 2008; Konishi *et al*., 2006; Satomi and Nishino, 2007, 2009), antiinflammatory activity (Shiratori *et al*., 2005), and antioxidant activity (Sachindra *et al*., 2007). When FX is orally administered in mice, it is metabolized to fucoxanthinol (FXOH) (Fig. 1), which is further converted into amarouciaxanthin A (Asai *et al*., 2004; Sugawara *et al*., 2002). It was also reported that FXOH is a major gastrointestinal metabolite of dietary fuxoxanthin in humans, indicating that the major compound in circulations after FX intake is FXOH (Asai *et al*., 2008).

There are many reports on FX in terms of its effect on physiological and pharmacological implications, whilst few studies have been conducted on the safety evaluation of purified FX. Kadekaru *et al.* (2008) reported the repeated oral dose toxicity study of FX in rats, and we have recently reported the single and repeated oral dose toxicity study of FX in mice as a part of safety evaluation (Beppu *et al.*, 2009). We report here the evaluation of mutagenicity of FXOH, the major compound after oral ingestion of FX, by Ames test, which is the bacterial reverse mutation test developed by Ames *et al.*, (1972, 1973), and of FX by *in vivo* micronucleus test, which has been the commonly used assay for the screening of potential genotoxicants (MacGregor *et al.*, 1987).

MATERIALS AND METHODS

The studies reported in this work have been conduced in accordance with the guidelines for animal experiment and animal care adopted by Sunny Health Holdings Co., Ltd..

Test substances

FX and FXOH were prepared according to the meth-

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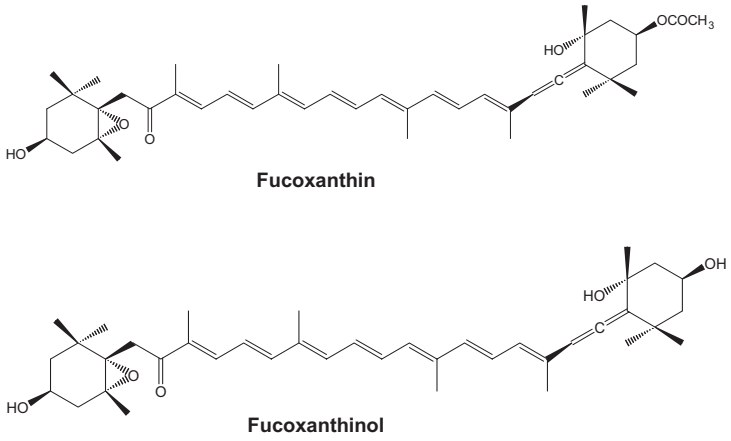

Fig. 1. Chemical structures for FX and FXOH.

od described previously (Maeda *et al*., 2007; Tsukui *et al*., 2007). The purities of FX and FXOH as checked by high performance liquid chromatography (HPLC) analysis were 96.9 and 96.8%, respectively.

Animals

Eight-week-old male and female ICR mice were purchased from Japan SLC Inc. (Shizuoka, Japan), and used after acclimatization for 1 week. During acclimatization and experimental periods, animals were kept under free access to food pellets (CRF-1, Oriental Yeast Co., Ltd., Tokyo, Japan) and tap water. Animals were housed at 22°C in a 12 hr light /12 hr dark cycle.

Ames test

The test strains used were *Salmonella typhimurium* TA100, TA98, TA1535, TA1537, and *Escherichia coli* WP2*uvrA*. Each test strain was pre-incubated in 2.5% Nutrient broth No.2 for 8 hr at 37°C with shaking at 100 strokes/min, and the resultant suspension was used for the assay. Male rat liver S9 (Sprague-Dawley) pretreated with phenobarbital and 5,6-benzoflavone was purchased from Kikkoman Corp. (Tokyo, Japan). S9 mix (1 ml) was prepared to contain 0.1 ml of S9, 8 mmol of $MgCl_2$, 33 mmol of KCl, 5 mmol of D-glucose-6-phosphate, 4 mmol of NADPH, 4 mmol of NADH and 100 mmol of sodium phosphate (pH 7.4). Top agar consisting of 0.5% NaCl and 0.6% Bacto-agar (Becton, Dickinson and Company, Franklin Lakes, NJ, USA) was sterilized by an autoclave. For the cultures of *S. typhimurium*, an aqueous mixure containing 0.5 mM L-histidine was added to the top agar solution (1/10th of agar solution). For the culture of *E. coli*, an aqueous solution of 0.5 mM L-tryptophan was added to the top agar solution (1/10th of agar solution). Ten-fold dilutions of FXOH in dimethylsulfoxide (DMSO) were prepared for dose-finding assay, and two-fold dilutions of FXOH in DMSO were for the final assay.

Ames test was conducted according to the standard protocol (preincubation) as described by Yahagi *et al.* (1977). A test tube containing 100 µl of a test substance (FXOH solution or a positive control solution), 500 µl of buffer (0.1 M sodium phosphate buffer, pH 7.4) with or without S9 mix, and 100 µl of test strain suspension was incubated for 20 min at 37°C with shaking at 120 strokes/min. Then, 2 ml of the top agar was added to the test tube and thoroughly mixed. The mixture obtained was poured onto the surface of a plate containing minimal glucose agar medium (Tesmedia AN, Oriental Yeast Co., Ltd., Tokyo, Japan). The plate was incubated for 48 hr at 37°C, and the colonies undergone reverse mutation was counted. Duplicate plates were used for each dose to calculate an average value. The positive controls for the cultures without S9 mix were 2-(2-furyl)-3-(5-nitro-2-furyl) acrylamide (AF-2, Wako Pure Chemicals, Osaka, Japan) for TA98, TA100 and WP2*uvr*A/pKM101 strains, sodium azide (NaN_3, Wako Pure Chemicals) for TA1535 strain, and 9-aminoacridine hydrochloride (9-AA, Sigma-Aldrich, St. Louis, MO, USA) for TA1537 strain. The positive controls for the cultures with S9 mix was 2-aminoanthracene (2-AA, Wako Pure Chemicals) for all test strains.

Micronucleus assay

Micronucleus assay was conducted according to the standard protocol as described by Schmid (1975) and

Krishna and Hayashi (2000). FX was suspended in 0.5% (w/v) carboxymethyl cellulose-Na (CMC-Na) solution containing 0.5% (v/v) Tween 80 to get a final concentration of 5, 10 and 20% (w/v). Animals were orally administered with FX suspension at a volume of 10 ml/kg (doses of FX were 500, 1,000 and 2,000 mg/kg). Control animals received the same amount of vehicle (CMC-Na solution containing 0.5% Tween 80) only. Cyclophosphamide (CPA, Sigma-Aldrich) used as a positive control was dissolved in the same vehicle, and was orally administered at 50 mg/kg. The bone marrow from both femurs of each animal was flushed out using 1 ml of fetal bovine serum (Equitech-Bio, Inc., Kerrville, TX, USA) followed by centrifugation at 220 x g for 5 min. The supernatant was discarded, and the precipitate was taken for spread smears. The slides with smears were fixed with methanol, and stained with Giemsa solution. Two thousands polychromatic erythrocytes (PCE) per animal were observed, and the number of micronucleus cells was counted under a light microscope. To check possible cytotoxic effects, the PCE: normochromatic erythrocytes (NCE) ratio in 2,000 erythrocytes was calculated.

Statistical analysis

Significant differences between treatment and the control group in the micronucleus assay were assessed by Dunnet's multiple comparison test.

RESULTS AND DISCUSSION

In the dose-finding study of Ames test, in any of the FXOH-treated tester strains regardless of the presence or absence of S9, the number of colonies was less than twice the number of colonies in the corresponding negative control (data not shown). Growth inhibition was observed in the plates of TA100 and TA98 without S9 due to the addition of FXOH at doses of 5-500 μg/plate, and of TA1537 without S9 due to the addition of FXOH at doses of 5-50 μg/plate. The results of the final assay are summarized in Tables 1-a and b. As is the case with

Table 1-a. Analysis of the mutagenicity of FXOH in four strains of *Salmonella typhimurium* and one strain of *Escherichia coli* without S9 mix

		Concentration (μg/plate)	Number of revertant colonies (colonies/plate)				
			Base-pair substitution type			Frameshift type	
			TA100	TA1535	WP2 *uvrA*	TA98	TA1537
Control	DMSO		117	13	29	30	7
FXOH		0.0763	113			19	5
		0.153	113			21	5
		0.305	114			19	6
		0.61	116			22	8
		1.22	106			19	9 *
		2.44	127			24	11 *
		4.88	112 *			23 *	9 *
		9.77	121 *			23 *	10 *
		19.5				20 *	6 *
		78.1 +	120 *				
		156 +	158 *	15	27		
		313 +	144	16	32		
		625 +	142	12	22		
		1,250 +	167	11	22		
		2,500 +	186	11	25		
		5,000 +	204	13	22		
Positive control	AF-2	0.01	640		157		
		0.1				785	
	NaN_3	0.5		716			
	9-AA	80					381

Each data represents the mean of duplicate cultures.
AF-2: 2-(2-Furyl)-3-(5-nitro-2-furyl) acrylamide; NaN_3: Sodium azide;
9-AA: 9-Aminoacridine hydrochloride.
+: Sediment of fucoxanthinol was observed.
*: Growth inhibition was observed.

Table 1-b. Analysis of mutagenicity of FXOH in four strains of *Salmonella typhimurium* and one strain of *Escherichia coli* with S9 mix

		Concentration (μg/plate)	Number of revertant colonies (colonies/plate)				
			Base-pair substitution type			Frameshift type	
			TA100	TA1535	WP2 *uvrA*	TA98	TA1537
Control	DMSO		102	11	27	39	33
FXOH		39.1	139				
		78.1 +	141				
		156 +	138	12	27	39	33
		313 +	166	13	25	38	30
		625 +	127	10	21	32	18
		1,250 +	150	17	21	32	16
		2,500 +	140	13	27	37	14
		5,000 +	149	7	27	28	17
Positive control	2-AA	0.5				534	
		1	975				
		2		498			169
		10			930		

Each data represents the mean of duplicate cultures.
2-AA: 2-Aminoanthracene.
+: Sediment of FXOH was observed.

the dose-finding assay, the number of colonies in any of the FXOH-treated test strains with or without S9 was less than twice the number of colonies in the corresponding negative control. Growth inhibition was observed in the plates of TA98 without S9 due to the addition of FXOH at doses of 4.88 μg/plate or more and of TA1537 without S9 due to the addition of FXOH at doses of 1.22 μg/plate or more. In the plates of TA100, growth inhibition was observed from 4.88 to 156 μg/plate, but was not at doses of 313 μg/plate or more. Since sedimentation of FXOH was observed at doses of 78.1 μg/plate or more, effective concentrations seem to be reduced by the sedimentation in a range of high doses of FXOH (similar phenomenon was also observed in the dose-finding study). These results indicate that the mutagenicity of FXOH, a mother compound in the body after dietary FX intake, was found to be negative in all test strains.

In the micronucleus assay, preliminary study was conducted to determine the sampling time. In the preliminary study, two animals were allocated to each group. In one study, animals were orally administered with FX at a dose of 2,000 mg/kg, and the bone marrow cells were taken 24, 48 and 72 hr after the administration. In the other study, FX at dose of 2,000 mg/kg was orally administered to animals twice or three times at 24 hr intervals, and the bone marrow cells were taken 24 hr after the final administration. No clear differences were found in the incidences of micronucleus cells among the experimental groups. In addition, no growth inhibition of bone marrow cells was observed in any of the experimental groups, since no differences were found in the PCE:NCE ratio among the groups. From these, sampling time was determined. That is, the bone marrow specimens were taken 24 hr after the single administration of FX. The doses of FX in the final study were 500, 1,000 and 2,000 mg/kg, and CPA at 50 mg/g was used as a positive control. In the final study, five animals were allocated to each group. The results are summarized in Table 2. The incidence of micronucleus cells in the CPA treated group was significantly higher than that in negative control group. In the FX-treated groups, no such differences in the incidence of micronucleus cells were found as compared with that in the negative control group. The estimated PCE:NCE ratio in the bone marrow preparations of FX-treated groups also showed no significant differences from that in the negative control group, indicating no cytotoxic effects. These results indicate that FX has no genotoxic/mutagenic effects on the bone marrow cells of *in vivo* mice. In our previous study (Beppu *et al.*, 2009), FX at 2,000 mg/kg as the maximal dose was orally administered to mice, and neither mortality nor abnormal findings were observed. In this study, no significant difference in the incidence of micronucleus cells was induced by FX even at the maximal dose (2,000 mg/kg), indicating that safety evaluation of FX as a foodstuff has advanced.

FXOH is a major compound in the body after FX was ingested (Asai *et al*., 2008). Thus, we used FXOH in *in vitro* assay and FX in *in vivo* assay. The data in the

Table 2. Analysis of mutagenicity of FX in the micronucleus test in the bone marrow cells of mice

Group	Number of MNPCE per animal					Total (%)	
	1	2	3	4	5	MNPCE/PCE	PCE/Erythrocytes
Negative control	7	3	12	7	9	0.38 ± 0.16	52.96 ± 0.70
Fx 500 mg/kg	6	8	5	4	8	0.31 ± 0.08	51.53 ± 5.45
Fx 1,000 mg/kg	10	8	1	4	10	0.33 ± 0.18	50.79 ± 4.92
Fx 2,000 mg/kg	5	8	3	7	5	0.28 ± 0.09	53.59 ± 3.78
CPA 50 mg/kg	23	19	17	30	25	1.14 ± 0.23**	46.48 ± 6.50

The number of micronucleated polychlomatic erythrocytes (MNPCE) was evaluated in the bone marrow cells of mice 24 hr after the single administration.
Total (%) is expressed as the mean ± S.D..
PCE: Polychlomatic erythrocytes.
CPA: Cyclophosphamide used as a positive control.
** : Statistical significance from the negative control ($p < 0.01$).

present study permit us to presume that orally administered FX is a safe compound in terms of mutagenicity under the experimental conditions employed here.

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