FULL PAPER *Ethology*

Effect of Ovary Lipid of Skipjack Tuna on Rat Serum Components after Stress Application

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ABSTRACT. Lipid extracted from the ovary of skipjack tuna by the method that we developed is rich in phospholipid-type docosahexaenoic acid. The ovary lipid of skipjack tuna (OLS) was studied for its anti-stress activity in male Wistar rats, focusing on stress-related blood components: recovery from stress was examined after application of water immersion restraint stress. As a result, serum corticosterone (CORT) secretion was inhibited and decreased rapidly after stress application in rats given OLS compared with control rats. As CORT acts as a glucocorticoid, non-esterified fatty acid (NEFA) is expected to increase by stress application. However, the concentration tended to be lower in rats given OLS than in control rats. With respect to OLS concentration, OLS increased serum dehydroepiandrosterone, secretion concentration-dependently. In addition, as with the recovery study, it tended to inhibit the increase in NEFA. These results indicate that OLS may have an anti-stress activity against acute stress.

KEY WORDS: corticosterone, dehydroepiandrosterone, docosahexaenoic acid, Ovary lipid, stress.

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Ovaries removed from skipjack tuna as a processing by-product in Shizuoka Prefecture contain 6–7% of lipid. Hiratsuka *et al.* have established a method of efficiently extracting oil and fat from the ovary of skipjack tuna (JP Laid-Open No. 2004–2663). Analysis using this method showed that 34% of oil and fat extracted from the ovary is phospholipid mainly consisting of phosphatidylcholine, phosphatidylethanolamine, and lysophosphatidylcoline. As for fatty acids, the docosahexaenoic acid (DHA) content is the highest and DHA accounts for about 35% of the total. DHA, contained in fish oil at a high level, has been studied for a variety of functions including improvement of brain function, anti-inflammatory effects, antiatherogenic effects, cancer suppressing effects, retina reflection increasing effects, blood triglyceride decreasing effects, antiallergic effects, and antidiabetic effects [6, 18].

In today's so-called stress society, human is regularly exposed to various stresses. Companion animals are also exposed to various stresses. And, the stress can induce all kinds of mental disorder. There are attempts to reduce such stresses by taking foods and food ingredients (JP Laid-Open No.2005–82490, JP Laid-Open No.2005–97145, JP Laid-Open No. 2005–104877, JP Laid-Open No.2005–213216, and JP Laid-Open No.2004–2383, *et al.*). Reactions after electric shock were observed in a study on the function of DHA [17]. Findings have been obtained concerning anti-stress activities such as improvement of brain function and antiinflammatory effect. However, there are few reports from the viewpoint of anti-stress.

We have performed experiments using ovary lipid of skipjack tuna, which is rich in DHA combined with phospholipids, to study anti-stress. In this study, we examined recovery from stress applied in rats using the levels of stress compounds in serum. We also examined the relationship between the concentration of ovary lipid of skipjack tuna and anti-stress.

Corticosterone (CORT) is synthesized on the hypothalamic-pituitary-adrenal axis (HPA axis), which is one of the stress responses in rats, by stress in the adrenal glands and secreted into the blood. Secretion of CORT forms ulcers in the stomach through the mechanisms of increased gastric acid secretion, decreased gastric mucosal secretion, and impaired gastric mucosa regeneration as negative effects [10]. In test I, inhibition of CORT secretion was considered an anti-stress activity and CORT was used as a major indicator to examine whether OLS inhibits CORT secretion by stress application. Because the presence of CORT in the blood for a long time after stress release prolongs the symptoms as described above, rapid decrease in CORT was considered one of the anti-stress activities and its recovery was observed. Observation of the decrease in CORT after stress release is also significant because it confirms that the ingestion of OLS does not adversely affect the negative feedback of the HPA axis.

In test II, the effects of the OLS concentration on CORT were examined and dehydroepiandrosterone (DHEA) and its excretion type DHEA-sulfate conjugate (DHEA-S) were also examined. DHEA and DHEA-S, like CORT, are produced from pregnenolone in the adrenal glands and have recently attracted attention as anti-stress substances [1]. If OLS can enhance the production of DHEA by stress application, this will be evidence of anti-stress activity. Thus, the amounts of DHEA and DHEA-S in the blood were used together as indicators of anti-stress activity.

MATERIALS AND METHODS

Materials: The ovary was removed from skipjack tuna (*Katsuwonus pelamis*), washed with water, boiled, freeze-dried, powdered, and subjected to extraction with ethanol. The resultant extract was filtered and concentrated to obtain ovary lipid of skipjack tuna (OLS).

Animal and administration: This study included test I, which examined recovery from stress applied (acute stress recovery study) and test II, which examined the effect of the concentration of OLS (OLS concentration study). OLS was mixed in feed and administered.

Three-week-old and 4-week-old male Wistar rats (purchased from Japan SLC) were used in test I and test II, respectively. They were raised in an animal room maintained at a temperature of 23 ± 1°C, humidity of about 55%, light/dark cycle of 12 hr (lighting 7:00–19:00, automatically controlled). For test I, rats after a 3-day acclimation period were divided into two groups of 30: the control and OLS groups. The rats in the OLS group were given OLS mixed in feed at 0.75%. For test II, rats were divided into 4 groups of 8: the control group and groups given OLS mixed in feed at 0.18%, 0.45%, and 0.90%. The feeds of test I and test II were prepared according to AIN93 and given *ad libitum*. The oil and fat content of feed was 4.2% for test I and 5.0% for test II. The contents were calculated to give the same energy percentages for n-6 and n-3 fatty acids and n-6/n-3 ratio (Table 1). Drinking water was given *ad libitum*.

This study was conducted after approval by the Animal Experiment Committee at the Shizuoka Industrial Research Institute, in compliance with the Guidance for Animal Experiments in the Shizuoka Industrial Research Institute.

Acute stress recovery study (test I): Rats in the control and OLS ingestion groups (30 per group) were raised for 15 days and after a 24-hr fast, were subjected to application of water immersion restraint stress (WIRS: 27°C, 7 hr) based on reports by Cheryl D. Conrad *et al.* and Padraig Kelliher *et al.* [3, 9]. They were decapitated 0 min (immediately) (n=6), 30 min (n=8), 60 min (n=8), and 240 min (n=8) after release from stress application to obtain serum.

OLS concentration study (test II): As with test I, rats were raised for 15 days, after a 24-hr fast were subjected to WIRS (27°C, 7 hr) application, and immediately decapitated to obtain serum.

Measurement of serum concentrations of CORT, DHEA and DHEA-S: CORT, DHEA and DHEA-S in serum were extracted with ethyl acetate and measured using liquid chromatography-tandem mass spectrometry (API3000, Applied Biosystems JAPAN Ltd.) under conditions shown in Table 2 [2, 8, 15].

Other serum components: Serum total cholesterol (Cho), triglyceride (TG), HDL-cholesterol (HDL), phospholipids (PL), and non-esterified fatty acid (NEFA) were measured using Cholesterol E-Test Wako, Triglyceride E-Test Wako, HDL-Cholesterol E-Test Wako, Phospholipid C-Test Wako, and NEFA C-Test Wako (purchased from Wako Pure Chemical Industries, Ltd.) as directed in the attached leaflets [12,14,16].

Statistical analysis: Data were expressed as a mean ± standard error (mean ± SE). Body weight, feed consumption, and feed efficiency in the raising period in test I were compared between the control and OLS ingestion groups using the two-tailed Mann-Whitney U-test. The results of blood component measurement in test I were analyzed at a significant level of 5% using the two-tailed Kruskal-Wallis H-test, and if a significant difference was noted, the data were analyzed using the one-tailed Mann-Whitney U-test with Bonferroni correction at a significant level of 5% (total time of comparison=4, 5%; $p<0.0125$) to make comparison at the same release time between the control and OLS ingestion groups. In test II, data were compared between all groups using Tukey's test at a significant level of 5%.

RESULTS

Acute stress recovery study (test I): As shown in Table 3, in the raising period, feed consumption was not significantly different ($p=8.93 \times 10^{-2}$) and final body weight was significantly different ($p=1.29 \times 10^{-2}$) between the control and OLS ingestion groups; feed efficiency was higher in the OLS ingestion group than in the control group ($p=4.47 \times 10^{-5}$).

Figure 1 shows serum CORT concentration. The CORT concentration 0 min (immediately) after release from stress tended to be lower in the OLS ingestion group than in the control group ($p=6.78 \times 10^{-2}$). CORT concentration was significantly lower in the OLS ingestion group than in the control group 30 min ($p=3.12 \times 10^{-3}$), 60 min ($p=8.26 \times$

Table 1. Lipid composition of tested diets

Lipids		Test I		Test II			
		Control	OLS	Control	OLS		
					0.18%	0.45%	0.90%
Corn oil	(%(w/w))	–	1.00	–	0.24	0.60	1.20
Rapeseed oil	(%(w/w))	2.51	–	3.00	2.38	1.55	–
Sunflower oil	(%(w/w))	1.67	2.42	2.00	2.20	2.40	2.90
Ovary lipids of skipjack tuna	(%(w/w))	–	0.75	–	0.18	0.45	0.90
n-3 fatty acid	(energy%)	0.66	0.68	0.79	0.78	0.81	0.81
n-6 fatty acid	(energy%)	1.37	1.41	1.62	1.62	1.67	1.67
n-6/n-3		2.1	2.1	2.1	2.1	2.1	2.1

Table 2. HPLC & Mass Spectrometry

	CORT	DHEA	DHEA-S
Column	INERTSIL ODS(3) 3.0 mm × 150 mm (Gl science)		
Flow rate	0.3 m*l*/min		
Column Temp.	40°C		
Mobile phase			
0 min.	0.1%(v/v) Acetic acid soln.: Acetonitrile = 7:3		
10 min.	0.1%(v/v) Acetic acid soln.: Acetonitrile = 3:7		
15 min.	0.1%(v/v) Acetic acid soln.: Acetonitrile = 3:7		
Injection volume	10 μl		
Mass Spectrometry			
Polarity	Positive	Negative	Positive
Ion Source (ESI)	Turbo Spray	Turbo Spray	Turbo Spray
Q1 Mass (amu)	347.2C	367.2C	289.2C
Q3 Mass (amu)	121.3C	96.85C	253.3C
Ion-spray voltage (IS)	5500	–4500	5500
Tem.	550	550	550
Declistering Potential (DP)	31	–66	51
Collision Energy (CE)	35	–60	19
NEB	12	8	8
CUR	6	8	8
CAD	8	8	4

CORT: Corticosterone. DHEA: Dehydroepiandrosterone. DHEA-S: Dehydroepiandrosterone sulfate.

Table 3. Test I growth parameters of rats

Growth parameter	Control	OLS
Body weight (g)		
Initial	49.7 ± 0.8	49.7 ± 0.9
Final (after 15 days; before fasting)	117.5 ± 1.3	123.1 ± 1.4*
Food intake (g/day)	11.3 ± 0.1	11.6 ± 0.1
Food efficiency (%)	40.1 ± 0.3	42.1 ± 0.3**

Data is expressed as mean ± S.E for 30 male rats. *: Significantly different from the control group at P<0.05. **: Significantly different from the control group at P<0.01.

10^{-3}), and 240 min ($p=1.18 \times 10^{-2}$) after release from stress application.

Results for other blood components are indicated in Table 4.

HDL concentration was significantly lower in the OLS ingestion group than in the control group 60 min after release from stress ($p=9.19 \times 10^{-3}$) and showed a similar tendency at other time points.

The Kruskal Wallis H-test showed a significant difference in TG and NEFA (TG: $p=2.26 \times 10^{-6}$, NEFA: $p=4.87 \times 10^{-5}$). However, no significant difference was noted in comparison at the same time points after release from stress between the control and OLS ingestion groups using the Mann-Whitney U-test with Bonferroni correction.

Kruskal Wallis H-test revealed no difference in total cholesterol or phospholipids.

OLS concentration study (test II): As shown in Table 5, in the raising period, there was no difference in feed consumption or final body weight between the control and OLS ingestion groups.

Figure 2 shows measurements of serum CORT and DHEA concentrations. DHEA-S was almost under the detection limit. Serum CORT concentration was lower in the OLS ingestion groups than in the control group, support-

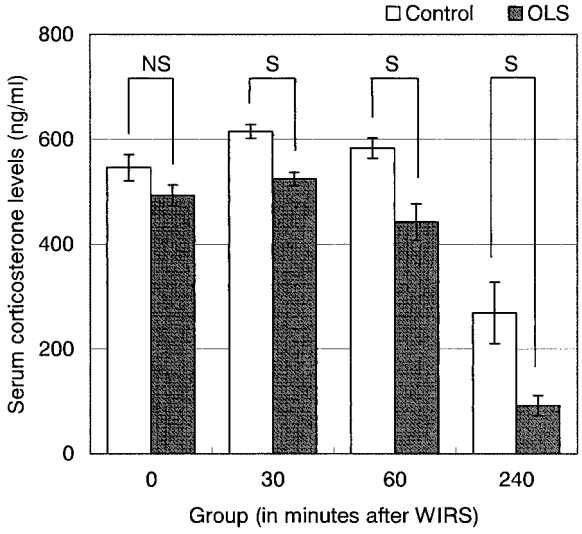

Fig. 1. Influence of time after water immersion restraint stress and extracts from ovary of skipjack tuna on serum corticosterone levels. Data is expressed as mean ± SE. NS: Not significantly different from the pair time control group at P<0.05. S: Significantly different from the pair time control group at P<0.05.

Table 4. Test I serum components of rats

Group (in minutes after WIRS)	Total cholesterol (mg/d*l*)	HDL-cholesterol (mg/d*l*)	Triglyceride (mg/d*l*)	Phospholipid (mg/d*l*)	Non esterified fatty acid (mEq/*l*)
Control					
0	87.2 ± 4.7	34.1 ± 1.2	37.6 ± 9.6	94.2 ± 7.1	1.46 ± 0.13
30	96.2 ± 6.3	32.1 ± 1.5	77.3 ± 18.1	100.0 ± 4.2	1.36 ± 0.07
60	92.0 ± 5.6	32.8 ± 2.1	21.0 ± 2.8	96.4 ± 7.0	1.19 ± 0.07
240	85.7 ± 4.6	38.7 ± 1.7	11.9 ± 2.3	90.3 ± 5.3	0.89 ± 0.06
OLS					
0	97.5 ± 6.9 NS	33.0 ± 2.7 NS	41.5 ± 8.0 NS	107.4 ± 9.6 NS	1.12 ± 0.04 NS
30	94.2 ± 5.4 NS	28.3 ± 1.5 NS	45.5 ± 8.2 NS	104.3 ± 9.6 NS	1.15 ± 0.05 NS
60	83.3 ± 5.7 NS	25.3 ± 1.6 S	22.2 ± 1.5 NS	91.1 ± 7.4 NS	0.95 ± 0.08 NS
240	81.5 ± 4.1 NS	35.5 ± 1.8 NS	17.0 ± 6.8 NS	85.3 ± 5.5 NS	0.89 ± 0.07 NS

Data is expressed as mean ± S.E. NS: Not significantly different from the pair time control group at $P<0.05$. S: Significantly different from the pair time control group at $P<0.05$.

Table 5. Test II growth parameters of rats

	Control	OLS		
		0.18%	0.45%	0.90%
Body weight (g)				
Initial	86.5 ± 1.0	86.6 ± 0.9	86.5 ± 0.9	86.5 ± 0.9
Final (after 15 days; before fasting)	153.3 ± 1.6	148.9 ± 2.4	152.7 ± 2.3	155.9 ± 3.3
Food intake (g/day)	14.2 ± 0.2	13.8 ± 0.3	14.1 ± 0.3	14.0 ± 0.3
Food efficiency (%)	31.4 ± 0.3[a)b)]	30.1 ± 0.5[a)]	31.2 ± 0.3[a)b)]	32.9 ± 0.7[b)]

Data is expressed as mean ± S.E. for 8 male rats. Means within the same line that do not share a common superscript letter were significantly different ($P<0.05$).

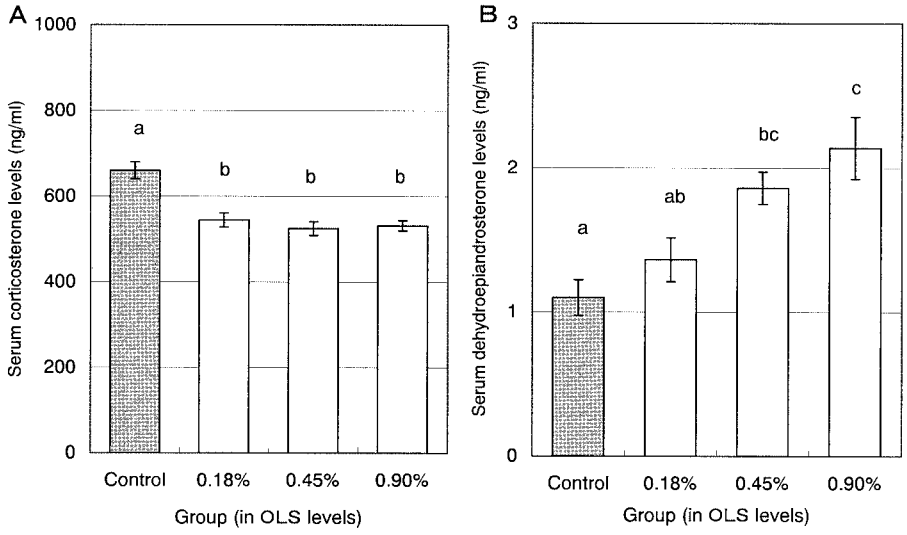

Fig. 2. Influence of extracts from ovary of skipjack tuna on serum corticosterone levels and serum dehydroepiandrosterone levels. A: Serum corticosterone levels, B: Serum dehydropeiandrosterone levels. Data is expressed as mean ± SE. Means within the same line that do not share a common superscript letter were significantly different ($P<0.05$).

ing the result of test I. However, there were no changes depending on OLS concentration. Serum DHEA concentration was higher in the OLS 0.45% and 0.90% ingestion groups than in the control group, with a significant difference. A significant difference was also noted between the 0.18% and 0.90% groups, with the DHEA concentration being higher in the 0.90% group.

Table 6 shows measurements of other blood components in test II. As with test I, a significant difference was observed in HDL. NEFA tended to be lower in the OLS ingestion groups than in the control group as in test I and to decrease depending on the OLS intake.

Table 6. Test II serum components of rats

		Control	OLS		
			0.18%	0.45%	0.90%
Total cholesterol	(mg/d*l*)	90.4 ± 4.2	99.8 ± 8.6	79.9 ± 6.4	82.1 ± 5.5
HDL-cholesterol	(mg/d*l*)	37.9 ± 2.0[a)]	25.8 ± 0.9[b)]	22.5 ± 2.0[b)]	28.5 ± 3.2[b)]
Triglyceride	(mg/d*l*)	62.4 ± 6.7	64.3 ± 4.7	70.7 ± 4.7	60.6 ± 5.9
Non esterified fatty acid	(mEq/*l*)	1.15 ± 0.07	1.15 ± 0.08	1.10 ± 0.04	0.93 ± 0.07

Data is expressed as mean ± S.E for 8 male rats. Means within the same line that do not share a common superscript letter were significantly different ($P<0.05$).

DISCUSSION

Acute stress recovery study (test I): Changes in CORT, a stress hormone specific to rodents, after release from stress showed the same tendency as reported by Conrad *et al.* [3] in both control and OSL ingestion groups. When the CORT concentration immediately after release from stress was taken as 100%, changes in CORT concentration 30, 60, and 240 min after release from stress were 113%, 107%, and 49% for the control group, and 106%, 90%, and 19% for the OLS ingestion group. These results suggested that OLS inhibits excessive secretion of the stress hormone CORT and makes a rapid recovery from stress.

HDL concentration was significantly lower in the OLS ingestion group than in the control group 60 min after release from stress and showed a similar tendency at other time points. This was attributable to DHA contained in OLS because it is known that DHA ingestion causes HDL to decrease [13].

An animal in the control group had an abnormal concentration of TG 30 min after release from stress. Even if this was excluded, a significant difference was still observed ($p=7.41 \times 10^{-6}$). In the one-tailed Mann-Whitney U-test with Bonferroni correction, high p values were obtained: 0.25, 0.11, 0.34, and 0.46 at 0, 30, 60, and 240 min after release from stress. This suggests that the significant difference in TG by Kruskal Wallis H-test represents the changes over time after release from stress.

As with TG, the significant difference in NEFA is also considered to represent the changes over time after release from stress. Excepting 0.46 at 240 min in the one-tailed test, however, low p-values were obtained for NEFA: 3.94×10^{-2}, 2.64×10^{-2}, and 2.64×10^{-2} at 0, 30, and 60 min, being lower in the OLS ingestion group than in the control group at any time point.

CORT has glucocorticoid activity and is the strongest glucocorticoid in rodents. Glucocorticoid is reported to "decrease the uptake and use of glucose by adipose tissue, resulting in decreased glycerol production. As glycerol is necessary for reesterification of fatty acids, the decreased glycerol production causes fatty acid release into plasma [11].

In test I, CORT changed depending on the time of release from stress, which may have influenced TG and NEFA. In addition, it is considered that the lower CORT concentration in the OLS ingestion group than in the control group was responsible for the tendency of lower NEFA.

OLS concentration study (test II): The feed efficiency for the 0.45% group was between those for the 0.18% and 0.90% groups. This, combined with the results of test I suggests that OLS increases feed efficiency concentration-dependently. Correlation analysis of the total OLS intake in the raising period and feed efficiency in each OLS ingestion group showed that Spearman's rank correlation coefficient was 0.76 (n=24, two-tailed $p=1.83 \times 10^{-5}$) with a positive correlation.

The results of serum DHEA concentration suggest that the OLS intake correlates with serum DHEA concentration after stress application. Correlation analysis of the total OLS intake in the raising period and serum DHEA concentration in each rat revealed a positive correlation (n=32, Spearman's rank correlation coefficient: 0.70, tow-tailed $p=6.82 \times 10^{-6}$), suggesting that OLS concentration-dependently increases DHEA secretion under stress application.

DHEA and DHEA-S are known to have various biological actions such as antiatherogenic, antiobesity, glucose metabolism improving, immunomodulatory, and malignant tumor development/growth inhibiting effects [5]. They are also being watched as an anti-stress substances [1]. The inhibition of serum CORT secretion and concentration-dependent increase of serum DHEA suggested that OLS enhances the physical ability to reduce acute stress.

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