FULL PAPER *Ethology*

Effect of Ovary Lipid from Skipjack Tuna (*Katsuwonus pelamis*) on Brain Monoamines in Rats

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ABSTRACT. In our previous experiments with rats, ovary lipid from Skipjack Tuna (*Katsuwonus pelamis*) (OLS) was shown to have a mitigating effect on anxiety and/or fear in elevated T-maze tests. This suggests that OLS has some effect on the central nervous system (CNS) of rats. Thus, we performed experiments to examine the status of CNS in rats given OLS. The effect of OLS on chronic stress was also examined at the same time. The feed for control rats used oil and fat that have the same energy percentages for n-6 and n-3 fatty acids and the same n-6/n-3 ratio as OLS. As a result, rats given OLS for 28 days had lower serotonin levels in various brain areas regardless of stress application, showing that OLS affected the serotonin nervous system. From this, it was inferred that the ability of OLS to mitigate anxiety and/or fear resulted from its action on CNS, especially the serotonin nervous system. Substances other than the essential fatty acids may have been responsible for the action of OLS on monoamines and the metabolites. The effect of OLS on CNS, especially the serotonin nervous system, suggests that OLS may suppress anxiety.

KEY WORDS: anxiety and/or fear, 5-Hydroxytryptamine, 5-Hydroxy-3-indoleacetic acid, monoamine, ovary lipid.

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Ovary lipid from Skipjack Tuna (*Katsuwonus pelamis*) (OLS), extracted and purified using our patented method (JP Laid-Open No. 2004–2663), contains a large amount of docosahexaenoic acid (DHA) in the form of phospholipids such as phosphatidylcholine, phosphatidylethanolamine, and lysophosphatidylcholine. (The DHA content of OLS is 34.9%.) We hypothesized that the ingestion of OLS mixed in feed could improve and prevent stress-induced problem behavior in animals. In previous experiments with rats, we found that OLS inhibited the increase in corticosterone (CORT) production under acute stress, stimulated the secretion of dehydroepiandrosterone (often referred to as the anti-stress hormone) [1, 6], and accelerated the decrease in CORT levels following relief from stress [14]. In addition, elevated T-maze tests [5, 7, 16, 21] demonstrated that OLS had the ability to mitigate anxiety and/or fear [15].

Stress and anxiety increases secretion of 5-hydroxytryptamine (5-HT) in the central nervous system (CNS) [22] and an appropriate amount of 5-HT is automatically released in some fear responses [4]. The concentration of 5-hydroxy-3-indoleacetic acid (5-HIAA), a metabolite of 5-HT, in cerebrospinal fluid is low in animals that display aggressive behavior [8]. Norepinephrinergic (NE) neurons in the locus coeruleus control a wide variety of behavior and physiological responses related to anxiety and/or fear [3]. In this way, brain monoamines play an important role in stress, anxiety, and in the physiological responses to these conditions.

In the present study, we investigated the effect of OLS on brain monoamines under normal conditions and determined how the brain monoamines change under chronic stress. We also investigated the association between the CNS and the results of our previous experiments. We focused on monoamines in the prefrontal area (cortex), hippocampus, hypothalamus, and locus coeruleus, which are involved with emotion. In addition, we studied the state of stress-related organs under chronic stress and determined the levels of serum components, including CORT secreted from the hypothalamic-pituitary-adrenal axis. Water immersion restraint stress (WIRS), based on a report by Mizoguchi *et al.* [9–11], was applied for 28 days as a chronic stressor.

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 from skipjack tuna (OLS).

Animal and administration: Three-week-old male Wistar rats (purchased from Japan SLC) were used in the experiments. They were raised in an animal room maintained at a temperature of 23 ± 1°C, with humidity of about 55%, and a light/dark cycle of 12 hr (lighting 7:00–19:00, automatically controlled). A red crawl ball (100 mm in diameter punctured with three 50-mm holes and manufactured for rodents) was set in cages for environmental enrichment and relief of stress that could occur due to the animals being raised individually [2]. After a 3-day acclimation period, the rats were divided into four groups of eight: the No-stress control (control-N) group, the Stress control (control-S) group and the No-stress OLS (OLS-N) and Stress OLS (OLS-S) groups. OLS was mixed and administered in feed. The rats in the two OLS groups were given 0.9% OLS mixed in feed for 28 days. The feed was prepared according to AIN93 and given *ad libitum*. The feed composition was calculated to give the

Table 1. Lipid composition of tested diets

Lipids		Control	OLS
Corn oil	(%(w/w))	–	1.20
Rapeseed oil	(%(w/w))	3.00	–
Sunflower oil	(%(w/w))	2.00	2.90
Ovary lipid from skipjack tuna	(%(w/w))	–	0.90
n-3 fatty acid	(energy%)	0.79	0.81
n-6 fatty acid	(energy%)	1.62	1.67
n-6/n-3		2.1	2.1

OLS: Ovary lipid extracted from skipjack tuna.

same energy percentages as n-6 and n-3 fatty acids and to maintain the n-6/n-3 ratio (Table 1). Drinking water was given *ad libitum*. The animals in the control-S and OLS-S groups were exposed to the WIRS stressor (27°C, 2 hr) every day.

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

Sacrifice and collection of serum and organs: Rats were sacrificed by decapitation immediately after relief from stress on the final day of the experiment. Rats that were not subjected to the stressor were sacrificed in the same way as the rats that were subjected to WIRS. Serum was separated from collected blood and preserved at –80°C until component determination. The brain was dissected into the hypothalamus, prefrontal area (cortex), hippocampus, and locus coeruleus, keeping the right and left hemispheres separate. Each area was weighed and preserved at –80°C until monoamine determination. The adrenal gland, spleen, and epididymal fat pads were removed and weighed.

Determination of serum components: Serum total cholesterol (Cho), triglyceride (TG), HDL-cholesterol (HDL), phospholipids (PL), and non-esterified fatty acid (NEFA) were measured using the 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 [12,13,19]. CORT was extracted with dichloromethane and was determined by high performance liquid chromatography (HPLC), according to the method of Scott *et al.* [17].

Determination of monoamines: To remove protein, 0.2 M perchloric acid was added to each brain area according to its weight and was homogenized using ultrasound. The homogenate was centrifuged to obtain and 1 M sodium acetate was added to the supernatant, which was then adjusted to a pH level of about 3 and measured by HPLC to determine monoamines [18, 23].

Statistical analysis: Data were expressed as mean ± standard error (mean ± SE). Data were analyzed for significant difference using two-factor factorial ANOVA (at a significance level of 5%). When no interaction was observed and a significant difference was observed both between the presence and absence of stress application (stress factor) and between the presence and absence of OLS ingestion (OLS factor), a post-hoc test was performed by the Tukey-Kramer method.

RESULTS

Growth parameters and organ weight: Growth parameters and organ weights are shown in Table 2. Final body weight, food intake, adrenal gland weight per 100 g body weight, and epididymal fat pad weight per 100 g body weight were significantly different between the presence and absence of the stress factor. The spleen weight per 100 g body weight was the highest in the control-N group, being significantly different even from the OLS-N group. The spleen weight was significantly lower in the two groups subjected to WIRS than in the groups that were not subjected to WIRS. No significant difference was observed between the two groups.

Serum components: Results for the determination of serum components in rats are shown in Table 3. For CORT, an interaction was observed between the stress factor and OLS factor. For the Cho concentration, no significant dif-

Table 2. Growth parameters and organ weight of rats

Growth parameter and organ			Control		OLS	
			No-stress	WIRS	No-stress	WIRS
Growth parameters						
Body weight						
Initial		(g)	55.4 ± 1.7	55.8 ± 1.7	55.8 ± 1.8	55.4 ± 1.6
Final		(g) **	196.3 ± 3.0	137.9 ± 2.1	208.2 ± 6.8	142.5 ± 4.0
Food intake		(g/day) **	13.6 ± 0.2	10.9 ± 0.2	14.2 ± 0.5	10.8 ± 0.2
Organs						
Adrenal gland	(mg/100 g body weight)	**	6.4 ± 0.2	10.1 ± 0.3	6.6 ± 0.2	9.9 ± 0.2
Spleen	(mg/100 g body weight)		291 ± 5^{c}	210 ± 3^{a}	266 ± 6^{b}	207 ± 5^{a}
Epididymal fat pads	(g/100 g body weight)	**	1.49 ± 0.10	1.05 ± 0.05	1.67 ± 0.14	1.07 ± 0.03

OLS: ovary lipid extracted from skipjack tuna. WIRS: Water immersion restraint stress. Data are expressed as mean ± SE. ** = Parameters and organs in which the two-factor factorial ANOVA showed a significant difference (1%) between the presence and absence of stress only. The different letters indicate significant differences among the four groups ($P<0.05$) (However, the two-factor factorial ANOVA showed no interaction and showed a significant difference between the presence and absence of each of stress and OLS).

Table 3. Serum components of rats

Components		Control		OLS	
		No-stress	WIRS	No-stress	WIRS
Corticosterone	(ng/mL)	14.8 ± 3.5	645.7 ± 80.7	14.9 ± 3.5	343.1 ± 71.4
Total cholesterol	(mg/dL)	104.3 ± 8.3	90.6 ± 2.8	86.5 ± 3.1	87.5 ± 6.7
HDL-cholesterol	(mg/dL)##	60.9 ± 3.6	66.2 ± 7.2	45.6 ± 3.5	50.3 ± 3.3
Triglyceride	(mg/dL)**	189 ± 17	101 ± 5	203 ± 23	146 ± 11
Phospholipid	(mg/dL)	218 ± 7[a]	192 ± 5[ab]	201 ± 6[a]	171 ± 5[b]
Non esterified fatty acid	(mEq/L)**	0.62 ± 0.05	1.11 ± 0.08	0.45 ± 0.05	1.11 ± 0.06

OLS: Ovary lipid extracted from skipjack tuna. WIRS: Water immersion restraint stress. Data are expressed as mean ± SE. ** : Components in which the two-factor factorial ANOVA showed a significant difference (1%) between the presence and absence of stress only. ## : A component in which the two-factor factorial ANOVA showed a significant difference (1%) between the presence and absence of OLS only. The different letters indicate significant differences among the four groups ($P < 0.05$) (However, the two-factor factorial ANOVA showed no interaction and showed a significant difference between the presence and absence of each of stress and OLS).

ference was observed between the presence and absence of the stress factor or OLS factor. The HDL concentration was significantly different between the presence and absence of the OLS factor only. TG and NEFA were significantly different between the presence and absence of the stress factor only. The PL concentration was significantly higher in the two groups that were not subjected to WIRS compared with the OLS-S group; the control-S group had an intermediate value.

Monoamines in the prefrontal area (cortex): Results for the determination of brain monoamines are shown in Table 4. The NE concentration in the prefrontal area (cortex) increased after exposure to the WIRS stressor in the control and OLS groups. No significant difference was observed between the control-N and OLS-N groups or between the control-S and OLS-S groups. However, the OLS groups tended to have higher concentrations of NE. The dopamine (DA) and 3,4-dihydroxyphenylacetic acid (DOPAC) concentrations were not significantly different between the presence and absence of the stress factor or OLS factor. For the concentrations of homovanillic acid (HVA) and 5-HT, a significant difference was observed between the presence and absence of the OLS factor but not in terms of the stress factor. The total concentration of DA and the DA metabolites NE, DOPAC, and HVA was not significantly different between the presence and absence of the stress factor or OLS factor. For the 5-HIAA concentration, an interaction was observed between the stress factor and OLS factor. The total concentration of 5-HT and 5-HIAA was significantly higher in the control-S group than in the other three groups. The 5-HIAA/5-HT ratio was significantly lower in the control-N group than in the other three groups.

Monoamines in the hippocampus: The NE concentration and total concentration of 5-HT and 5-HIAA in the hippocampus were significantly different between the presence and absence of the OLS factor only. The 5-HIAA concentration was significantly different between the presence and absence of the stress factor only. The 5-HT concentrations for the OLS-N and OLS-S groups were significantly lower than those for the control-N and control-S groups, respectively. The 5-HIAA/5-HT ratio was significantly higher in the OLS-S group than in the two groups not subjected to WIRS; the control-S group had an intermediate value.

Monoamines in the hypothalamus: The NE and DA concentrations and the total concentration of DA, NE, and DOPAC in the hypothalamus were significantly different between the presence and absence of the OLS factor only. For the total concentration of 5-HT and 5-HIAA, an interaction was observed between the stress factor and OLS factor. The DOPAC concentrations for the control-S and OLS-S groups were significantly higher than those for the control-N and OLS-N groups, respectively. The 5-HT concentration for the control-N group was significantly higher than that of the other three groups. The 5-HIAA concentration was significantly higher in the OLS-N group than in the control-N group; the OLS-S group had a higher value than the OLS-N group. The 5-HIAA concentration for the control-S group was between that of the control-N and OLS-N groups. The 5-HIAA/5-HT ratio was significantly higher in the OLS-N group than in the control-N group and the OLS-S group had a higher value than the OLS-N group. The value for the 5-HIAA/5-HI ratio in the control-S group was intermediate between that of the control-N and OLS-N groups.

Monoamines in the Locus coeruleus: One sample of locus coeruleus from the OLS-N group was mixed with one sample from the OLS-S group and these were not distinguished. Both samples were excluded; the number in each group was seven. The NE concentration and total concentration of 5-HT and 5-HIAA in the locus coeruleus were not significantly different between the presence and absence of the stress factor or OLS factor. The 5-HT concentration significantly differed between the presence and absence of the OLS factor only. The 5-HIAA concentration significantly differed between the presence and absence of the stress factor only. The OLS-S and OLS-N groups had a significantly higher value for the 5-HIAA/5-HT ratio than the control-S and control-N groups, respectively.

Table 4. Regional distribution of monoamines and metaolites in rat brain

Cerebral components		Control		OLS	
		No-stress	WIRS	No-stress	WIRS
Prefrontal area (cortex)					
NE	(pmol/mg tissue)	0.79 ± 0.05^{a}	1.05 ± 0.03^{bc}	0.94 ± 0.04^{ab}	1.16 ± 0.08^{c}
DA	(pmol/mg tissue)	0.77 ± 0.12	1.32 ± 0.42	0.75 ± 0.16	0.71 ± 0.09
DOPAC	(pmol/mg tissue)	0.82 ± 0.14	1.41 ± 0.37	0.89 ± 0.18	0.91 ± 0.16
HVA	(pmol/mg tissue)$^{\#\#}$	0.44 ± 0.03	0.56 ± 0.06	0.63 ± 0.04	0.68 ± 0.05
NE+DA+DOPAC+HVA	(pmol/mg tissue)	2.82 ± 0.28	4.33 ± 0.79	3.21 ± 0.35	3.47 ± 0.33
5-HT	(pmol/mg tissue)$^{\#\#}$	1.93 ± 0.08	1.85 ± 0.06	1.52 ± 0.09	1.45 ± 0.10
5-HIAA	(pmol/mg tissue)	1.78 ± 0.07	2.54 ± 0.06	1.85 ± 0.05	2.15 ± 0.14
5-HT+5-HIAA	(pmol/mg tissue)	3.71 ± 0.13^{a}	4.39 ± 0.10^{b}	3.37 ± 0.09^{a}	3.60 ± 0.22^{a}
5-HIAA/5-HT		0.92 ± 0.03^{a}	1.38 ± 0.04^{b}	1.25 ± 0.09^{b}	1.50 ± 0.09^{b}
Hippocampus					
NE	(pmol/mg tissue)$^{\#\#}$	0.52 ± 0.03	0.44 ± 0.03	0.38 ± 0.03	0.32 ± 0.05
5-HT	(pmol/mg tissue)	1.26 ± 0.05^{a}	1.04 ± 0.06^{ab}	0.97 ± 0.07^{bc}	0.77 ± 0.06^{c}
5-HIAA	(pmol/mg tissue)**	1.31 ± 0.02	1.66 ± 0.08	1.49 ± 0.08	1.61 ± 0.06
5-HT+5-HIAA	(pmol/mg tissue)$^{\#}$	2.56 ± 0.06	2.69 ± 0.11	2.47 ± 0.09	2.38 ± 0.07
5-HIAA/5-HT		1.05 ± 0.04^{a}	1.63 ± 0.12^{ab}	1.59 ± 0.15^{a}	2.22 ± 0.24^{b}
Hypothalamus					
NE	(pmol/mg tissue)$^{\#\#}$	2.05 ± 0.09	2.06 ± 0.08	1.73 ± 0.10	1.60 ± 0.12
DA	(pmol/mg tissue)$^{\#\#}$	0.48 ± 0.04	0.46 ± 0.03	0.41 ± 0.02	0.32 ± 0.02
DOPAC	(pmol/mg tissue)	0.34 ± 0.02^{a}	0.58 ± 0.04^{bc}	0.50 ± 0.02^{ab}	0.75 ± 0.07^{c}
NE+DA+DOPAC	(pmol/mg tissue)$^{\#}$	2.87 ± 0.12	3.10 ± 0.12	2.63 ± 0.11	2.67 ± 0.16
5-HT	(pmol/mg tissue)	0.83 ± 0.04^{a}	0.65 ± 0.04^{b}	0.56 ± 0.05^{b}	0.50 ± 0.04^{b}
5-HIAA	(pmol/mg tissue)	1.05 ± 0.03^{a}	1.16 ± 0.03^{ab}	1.30 ± 0.04^{b}	1.59 ± 0.07^{c}
5-HT+5-HIAA	(pmol/mg tissue)	1.88 ± 0.04	1.81 ± 0.06	1.86 ± 0.07	2.09 ± 0.08
5-HIAA/5-HT		1.29 ± 0.10^{a}	1.83 ± 0.13^{ab}	2.47 ± 0.24^{b}	3.34 ± 0.28^{c}
Locus coeruleus					
NE	(pmol/mg tissue)	0.54 ± 0.03	0.52 ± 0.04	0.49 ± 0.05	0.46 ± 0.04
5-HT	(pmol/mg tissue)$^{\#}$	0.57 ± 0.05	0.54 ± 0.03	0.47 ± 0.09	0.39 ± 0.01
5-HIAA	(pmol/mg tissue)*	1.12 ± 0.03	1.46 ± 0.05	1.45 ± 0.20	1.56 ± 0.09
5-HT+5-HIAA	(pmol/mg tissue)	1.68 ± 0.08	2.00 ± 0.06	1.93 ± 0.28	1.95 ± 0.10
5-HIAA/5-HT		2.05 ± 0.13^{a}	2.77 ± 0.20^{ab}	3.29 ± 0.37^{bc}	4.06 ± 0.20^{c}

OLS: ovary lipid extracted from skipjack tuna. WIRS: water immersion restraint stress. NE: Norepinephrine, DA: Dopamine, DOPAC: 3,4-Dihydroxyphenylacetic acid, HVA: Homovanillic acid, 5-HT: 5-Hydroxytryptamine, 5-HIAA: 5-Hydroxy-3-indoleacetic acid. Data expressed as mean ± SE. n is 8 for each data point except for the locus coeruleus OLS groups, where it is 7. *, **: Components in which the two-factor factorial ANOVA showed a significant difference (5%, 1%, respectively) between the presence and absence of stress only. #, ##: Components in which the two-factor factorial ANOVA showed a significant difference (5%, 1%, respectively) between the presence and absence of OLS only. The different letters indicate significant differences among the four groups ($P<0.05$) (However, the two-factor factorial ANOVA showed no interaction and showed a significant difference between the presence and absence of each of stress and OLS.).

DISCUSSION

The rats subjected to chronic stress had a final body weight loss, decreased food intake, and the enlarged adrenal grand regardless of OLS ingestion. These findings are known to be common stress responses. The spleen weight per 100 g body weight was lower in rats given OLS but not subjected to chronic stress than in control rats. However, the difference was smaller than the difference between the OLS and control groups that were subjected to chronic stress. In the present study, the growth parameters and weights of organs other than spleen seemed to be affected by chronic stress application only regardless of OLS ingestion. The same is true of the serum components TG and NEFA. In addition, the HDL concentration was lower in the OLS groups, as reported previously [14, 15]. Although no significant difference test was performed because an interaction of the stress and OLS factors was observed, the OLS group under chronic stress, compared with the control group, tended to have a lower CORT level.

The 5-HT concentrations in various brain areas of rats given OLS were affected and lower than those in the control rats regardless of chronic stress application. On the other hand, the 5-HIAA concentrations in the hippocampus and locus coeruleus were significantly different between the presence and absence of stress application regardless of OLS ingestion. The 5-HIAA concentration in the hypothalamus was significantly different between the presence and absence of each of OLS ingestion and stress application and an interaction was observed between the stress factor and OLS factor in the prefrontal area (cortex). These may have affected the total concentration of 5-HT and 5-HIAA and 5-

HIAA/5-HT ratio. As mentioned above, stress and anxiety increase 5-HT release in the CNS [22] and 5-HIAA concentration is low in the cerebrospinal fluid of animals that display aggressive behavior [8]. Selective serotonin reuptake inhibitors (SSRIs) are used to reduce aggressive behavior and in the treatment of anxiety and/or fear. The serotonin nervous system can thus be seen to play an important role in stress and anxiety and/or fear. The ability of OLS to mitigate anxiety and/or fear demonstrated in this study may have resulted from its action on the serotonin nervous system.

OLS ingestion affected the concentrations of many brain monoamines besides 5-HT: NE in various areas other than the locus coeruleus, HVA in the prefrontal area (cortex), and DA and DOPAC in the hypothalamus. Among these, the NE concentration in the prefrontal area (cortex) and DOPAC concentration in the hypothalamus were also affected by chronic stress application. In the present experiments, the energy percentages for the essential n-6 and n-3 fatty acids and the n-6/n-3 ratio were the same for the control rats and those given OLS. Thus, the clear differences from the control group in the monoamine concentrations in various brain areas may have been due to the function of substances other than the essential fatty acids in OLS. OLS contains phosphatidylcholine, phosphatidylethanolamine, and lysophosphatidylcholine bound to DHA. These compounds may be responsible for the function.

The percentage of pet ownership in Japan is 18.8% for dogs and 15.1% for cats (according to the 11th National Investigation of Dog and Cat Ownership; Pet Food Manufacturers Association). Many families have dogs and/or cats and often raise them indoors in urban areas. Companion animals raised indoors suffer stress under stimulation from humans. Animals exposed to stress display problem behavior such as aggressive behaviors. It would be desirable if pet food or supplements that are routinely consumed could relieve stress and anxiety in companion animals to suppress aggressive behaviors.

The results of the present study showed that OLS ingestion influenced the CNS, especially the serotonin nervous system. This influence is suggestive of OLS relieving anxiety and inhibiting aggressiveness. In the future, we intend to conduct a detailed study of these potential effects.

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