原　著

Effect of Pufferfish liver on the ability of mice in learning and memorizing

マウスにおけるフグ肝の記憶学習能力効果

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〈原著論文〉

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Abstract：It is clearly proved now that nontoxic pufferfish can be produced if they are cultivated in a net at sea or an aquarium in the land and fed a TTX-free diet. It was found that they contain functional IPA and DHA, especially in their liver. DHA is related to learning and memorizing. From the effect of the pufferfish liver on the ability in learning and memorizing by the passive avoidance test using the mice, improvement in their ability was shown.

Mice were divided into two groups, "Control"（n=16）whose food contained no-IPA and no-DHA and "the Pufferfish Liver"（n=16）whose food contained a lot of IPA and DHA. After electric stimulation, the mice in the "pufferfish liver group" seemed to be watching the dark compartment but those in the "control group" moved to the dark compartment immediately（$p < 0.05$）. Moreover, after electric stimulation, the LT of "pufferfish liver group" tended to be longer than that of the "control group" and showed a significantly different behavior（$p < 0.05$）.

Therefore, it might be proven from the result of the passive avoidance test in the mice that "pufferfish liver" including a lot of functional components, IPA and DHA showed an effective improvement in the learning and memorizing ability of the mice. It could be a promising functional food for the near future.

Keywords：pufferfish liver, functional component, DHA（docosahexaenoic acid）, passive avoidance test, learning and memory ability

キーワード：フグ肝、機能性成分、ドコサヘキサエン酸、受動回避試験、記憶学習能力

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Introduction

The Japanese Ministry of Health and Welfare prohibited the serving of all kinds of pufferfish liver as a food in 1983[1)]. Since then, such dishes have not been traditional food. But it has been already proved that nontoxic pufferfish can be produced if they are cultivated in a net at sea or an aquarium in the land and fed a TTX-free diet[2-3)]. Physiochemical properties of the non-toxic pufferfish liver[4-6)] have been investigated to revive it as a traditional dish and further make it a valuable processed dish. Consequently, it was found that it contains rich and functional IPA（icosapentaenoic acid）and DHA（docosahexaenoic acid）.

These components are involved in the effective prevention of cardiovascular disease[7)] and improvements in visual function[8)], dementia[9)], mental stabilization[10)], the development, learning and memory of the brain[11-12)] and others[13-14)].

There are a lot of animal tests carried out to evaluate their functionality as follows: passive

avoidance, active avoidance, maze tests (water maze, redial maze, Y-maze) [15].

Improvements in learning and memorizing from the effect of pufferfish liver were clearly shown by the passive avoidance test. This passive avoidance test was found to be a practically available tool for the evaluation of functionality in the pufferfish liver.

Material and Methods

1. Material

The livers dissected from the non-toxic pufferfish reared in a circular aquarium with an open system in Yobuko, Saga Prefecture, Japan in 2009 were submitted as material.

Their toxicity was examined by an intraperitoneal injection using male mice of ddY strain (18－20 g) according to the Japanese official method[16] for determination of TTX. Their proximate composition, fatty acid (IPA and DHA) and vitamins were analyzed according to the standard table of food composition[17].

2. Methods

2.1 Animals

For the passive avoidance test, five-weeks-old male mice of the ddY strain, weighing 26－30 g were used. They were housed in individual cages in an air-conditioned room with a controlled temperature (23 ± 2℃) and humidity of 50～60% under 12 hours light-dark cycle. Water and food *ad libitum* until used.

2.2 Mouse food

The mice were divided into two groups, of "Control" (n=16) whose food contained no-IPA and no-DHA and "Pufferfish Liver group" (n=16) whose food contained a lot of IPA and DHA. The food of the mice in each group was adjusted according to the feed mixture of AIN (American Institute of Nutrition) -93G[18].

The vitamin A content in the pufferfish liver analyzed was 9910 μg%. The tolerable upper intake level (UL) of vitamin A for the human by Dietary Reference Intakes for Japanese, 2010 is 2700 μgRE/day (above 18 years old) [19]. Taking account of the above, AIN-93G was feasibly modified for the material used and its content as follows.

Although for the lipid of mice food in AIN-93G is the soybean oil used, it is not suitable for evaluating the effect of the functional components of the pufferfish liver because it includes a lot of n-3 polyunsaturated fatty acid (linoleic acid (50 g%))[17]. Therefore, lard (SHIGMA-ALDRICH) was used instead of the soybean oil in this study.

Table 1. Proximate composition of feed for a mouse

	Composition (g/100 g)	
	Control	Pufferfish liver
Corn Starch	52.9486	52.9286
Casein	20.0	19.816
Sucrose	10.0	10.0
Cellulose	5.0	5.0
Lard	7.0	4.168 ＊1
Pufferfish liver	—	4.0 ＊1
AIN-93 mineral mixture	3.5	3.5
AIN-93 vitamin mixture	1.0	1.0
L-Cystine	0.3	0.3
Choline Hydrogen Tartrate	0.25	0.25
tert-Butyl Hydroquinone	0.0014	0.0014
Total	100	100.964＊2

＊1: Crude fat components lard (4.168 g) and pufferfish liver (4 g). Net fat in pufferfish liver is 2.832 g/4 g.

＊2: Moisture in total is 0.964 g/100 g.

Table 2. Fatty acid compositions of feed for a mouse

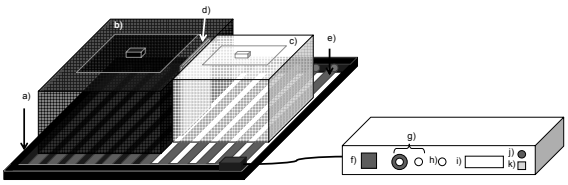
			Composition (%)	
			Control	Pufferfish liver
Saturated fatty acids			27.7	23.7
Unsaturated fatty acids			70.8	70.6
	Monoenoic acid		47.9	38.4
	Dinoeic acid		21.4	2.1
	Trienoic acid		1.3	1.0
	Tetraenoic acid		0.2	3.2
	Pentaenoic acid		0.0	13.6
	Hexaenoic acid		0.0	12.3
Saturated fatty acids	14:0		0.6	2.9
	15:0		0.0	0.3
	16:0		21.7	16.1
	17:0		0.0	0.5
	18:0		5.4	3.7
	20:0		0.0	0.2
Monounsaturated fatty acids	14:1		0.2	0.0
	16:1		6.4	8.6
	17:1		0.0	0.4
	18:1		41.0	20.3
	20:1		0.3	5.4
	22:1		0.0	3.0
	24:1		0.0	0.7
Polyunsaturated fatty acids	16:2		0.0	0.5
	16:3		0.0	0.3
	16:4		0.0	0.3
	18:2	n-6	21.2	1.3
	18:3	n-3	1.3	0.7
	18:4	n-3	0.0	1.5
	20:2	n-6	0.2	0.3
	20:4	n-3	0.0	0.6
	20:4	n-6	0.2	0.6
	20:5	n-3	0.0	7.4
	21:5	n-3	0.0	0.5
	22:4	n-6	0.0	0.2
	22:5	n-3	0.0	5.4
	22:5	n-6	0.0	0.3
	22:6	n-3	0.0	12.3

Finally, the feed for the mice was fixed as shown in Table 1-2, and the pufferfish liver content was adjusted to 4 g%.

2.3 System of passive-avoidance (system of step-through test)

Mice learning and memory ability was evaluated using a step-through passive avoidance test.

A step-through apparatus (Muromachi Kikai) was used for the passive avoidance test and the system consists of a shock generator (SGS-003DX) for the electrical stimuli trial and a step-through cage (STC-001M) for the acquisition trial (Fig. 1).



Left :	Right :
Mouse cage (STC-001M)	Shock generator (SGS-003DX)
a) Excreta tray	f) Power
b) Dark compartment	g) Shock intensity (mA)
c) Lighted compartment	h) Timer Remote
d) Guillotine door	i) Timer (min)
e) Grid part	j) Indicator (Shock rump)
	k) Manual operate button

Fig.1. A step – through test system for a mouse.

In addition, the cage consists of two compartments, a lighted compartment (W90 × D115 × H150 mm) and a dark compartment (W145 × D175 × H150 mm). They are separated by a guillotine door (W3 × H3.5 cm). The dark compartment has a series of stainless-steel rods through which a constant electrical current is delivered.

3. Step-through passive avoidance test (Fig. 2)

3.1 The acquisition trial

To learn and memorize the dark compartment as being safe for mice, the following trials were carried out.

The mice were placed in the light compartment facing away from guillotine door and allowed to explore the room for 30 seconds. The door was opened, and as soon as mouse entered the dark compartment with all four paws, the latency time (LT) was immediately recorded. Thereafter the mice were kept inside for 30 seconds before being returned to their cages. The maximum observation period in this test was 300 seconds. This trial was continuously carried out for two weeks.

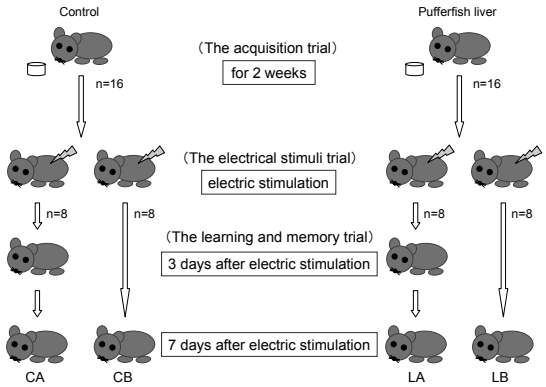


Fig. 2. Experiment schedule of step – through test for mouse.

3.2 The electrical stimuli trial

In order to learn and memorize the pain suffered in the dark compartment, the following trials were given to them.

For electrical stimuli, the mice were placed in the lighted compartment, facing away from the dark one and allowed to explore for 30 seconds. After 30 seconds, the guillotine door was opened. When the mice entered the dark one with all four paws, its door was closed, and the LT to enter was recorded. Two seconds after the door was closed, a shock (0.4 mA) was administered to their feet. Thirty seconds after the electric stimulation the mice were returned to the cage again.

3.3 The learning and memory trial

To evaluate whether the mice could learn and memorize the danger of the dark compartment or not, the following trials were given.

Three days (control group: CA, pufferfish liver group: LA) and 7 days (control group: CA/CB, pufferfish liver group: LA/LB) after electrical stimulation, the LT that the mice had moved from the lighted compartment to the dark one was measured. Further, in order to evaluate fear suffered by mice in the dark compartment, their behavior before and after the electric stimulation was observed.

4. The comparison of cholesterol content in the blood serum between "Control group" and "Pufferfish Liver" group

4.1 Preparation of blood serum

Thirty-six mice of each group were dissected to

collect their blood having fasting for 18 hours after experiment. Sample was centrifuged at 3500 rpm for 10 min at 4℃ to get the serum which was them preserved at -80℃ until use.

4.2 The total cholesterol content in each serum

Total cholesterol (TC) of each serum was measured by the enzyme method using E-TEST WAKO of clinical chemical kit (Wako Pre Chemical Industries Ltd.) according to the instructions of the manufacturer.

5. Statistical analyses

The data is presented as the mean ± SEM. Statistical analyses used SPSS(ver. 11.5)and the data was performed by Wilcoxon rank-sum test for non-parametric systems.

Result

1. Behavioral changes in the mice

The effect of the pufferfish liver in passive avoidance learning and memory was investigated through the behavioral changes in the mice(Table3).

Before the electric stimulation, each mouse (n=32) in CA and LA moved to the dark compartment immediately when both groups were put in the lighted compartment.

Three days after electric stimulation was administered to the mice that moved to the darkroom, half of the mice (n=4) in CA moved but none in LA. There was a significant difference between CA and LA in comparison to before the electric stimulation was administered ($p < 0.05$). 7 days after electric stimulation, the same result was also obtained ($p < 0.05$).

Next, the mice in LA and LB tended to watch the dark compartment after electric stimulation as follows: Some kept in the lighted compartment, and others wandered there, sometime peeping at the dark compartment and stretching their bodies, including fear. Especially, 7 days after electric stimulation, all the mice of LA showed negative-movements towards the dark compartment and the significant difference from those of CA was shown ($p<0.01$).

Table 3. Behavior modification of a mouse

	Control					Pufferfish liver				
	CA (n=8)			CB (n=8)		LA (n=8)			LB (n=8)	
〔Passage day after shock〕	0	3 days	7 days	0	7 days	0	3 days	7 days	0	7 days
It moves to the dark compartment at once.	8	4	6	8	6	8	0	0	8	3
It peeps into the dark compartment.	0	5	2	0	2	0	7	8	0	5
It is wandering in the lighted compartment.	0	2	1	0	1	0	3	1	0	0
It doesn't move at all.	0	0	0	0	0	0	2	2	0	1

2. The LT of the step-through passive avoidance test

Result of passive avoidance test by the mice was showed in Fig. 3.

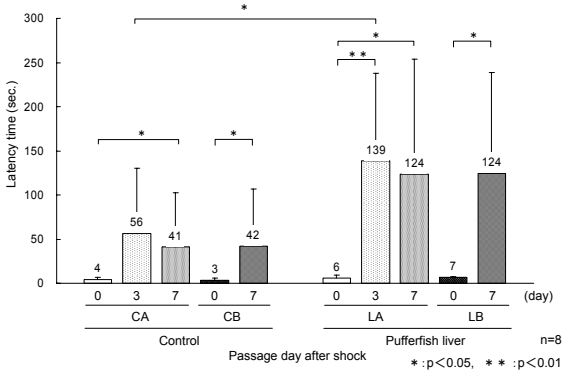


Fig. 3. Effect of pufferfish liver in the step-through passive avoidance test.

Each mean LT to enter the dark compartment in CA and CB was 4 seconds, 3 seconds respectively and in LA and LB was 6 seconds, 7 seconds. The LT to enter the dark compartment was not different among the groups. On three days after electric stimulation, the LT in CA showed a significant difference from that before electric stimulation though extended from 4 to 56 seconds. In addition, 7 days after electric stimulation, each LT of CA and CB showed a significantly different acquisition trial ($p < 0.05$), in comparison with that before electric stimulation.

On the other hand, 3 days after electric stimulation, each LT of LA extended from 6 to 139 seconds, and showed a significant difference ($p < 0.05$), in comparison with that before electric stimulation. In addition, 7 days after electric stimulation, the LT of LA and LB was the same as 124 seconds for each other and showed a significantly different acquisition trial($p < 0.05$), in comparison with that before electric stimulation.

Next, each LT of CA and LA on 3 days was 56 and 139 seconds, respectively. The LT in LA was obviously longer than and significantly different from,

that of CA resulting in effective acquisition ($p < 0.05$). On the other hand, 7 days after electric stimulation, each LT both 124 seconds of LA and LB tended to be very long in comparison with those in CA (41 seconds) and CB (42 seconds) but not significantly different from the letter ($p < 0.05$). The effective acquisition in this trial seems not continue for 7 days. Further trials will be done to confirm the time to keep effective acquisition.

3. The total cholesterol level of the mice

TC level of control and the pufferfish liver group were 148 ± 28 mg/dl and 125 ± 17 mg/dl respectively. That of the pufferfish liver group was lower than that of the control group and showed a significant difference ($p < 0.01$), in comparison with that of the control group.

Conclusion

For behavior of mice after electric stimulation, mice in LA and LB seemed to be watching the dark compartment though those in CA and CB moved to the dark compartment. It was clearly shown that mice in LA and LB did the behavioral learning and memorized fear of electric stimulation, different from the mice in CA and CB.

Since, the LT in LA and LB was much longer than that in CA and CB, it was strongly postulated that the fear of electric stimulation was maintained for one week. The effective improvement of the learning and memory ability on the aversive stimulus seems to reflect on the score of LA and LB.

Therefore, it was clearly proven from the result of the passive avoidance test in the mice that the pufferfish liver including a lot of functional components, IPA and DHA showed the effective improvement of the learning and memory ability. In addition, it was found that they were effective in reduced in cholesterol. It will be promising functional food for the near future.

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References

1) Pufferfishes available in Japan -an illustrated guide to their identification -secondsond edition. Environmental Health Bureau, Ministry of Health and Welfare. Chuo-hokishuppan, Tokyo 1984; 56-79.
2) Noguchi, T., Arakawa, O., Takatani, T. Toxiciy of pufferfish *Takifugu rubripes* cultured in netcages at sea or aquaria on land. C.B.P. Part D 1 2006; 153-157.
3) Noguchi, T., Arakawa, O., Takatani, T. TTX accumulation in pufferfish. C. B. P. Part D 1 2006; 145-152.
4) Onuki K., Noguchi, T., Arakawa, O. Confirmation of non-toxicity of the pufferfish cultivated in open system of circular aquarium and functional compartments in the lipid J. J. of Food Chemistry and Safety. 2009; 16 (3) : 157-162.
5) Onuki K., Noguchi, T., Arakawa, O. Nutrition and functional components of processed pufferfish livers with safety. J. J. of Food Chemistry and Safety 2010; 17 (1) : 6-11.
6) Mineki, M., Onuki, K., Sawasaki, E., et all. The utilization, chemical composition, and fatty acid composition of non-toxic livers of pufferfish (*Takifugu rubripes*) cultured at aquaria on land. Japanese Journal of Sensory Evaluation 2009; 13: 23-30.
7) Hirai, A., Hamazaki, T., Terano, T., Nishikawa, T., et all. Eicosapentanoic acid and platelet function in Japanese. Lancet 1980; 2: 1132-1133.
8) Hoffman, DR., Birch, EE., Birch, DG., et all. Impact of early dietary intake and blood lipid composition of long-chain polyunsaturated fatty acids on later visual development. J. Pediatr. Gastroenterol Nutr. 2000; 31: 540-553.
9) Joshi, H., Kaur, N., Jyotibala, C. Evaluation of nootropic effect of argyreia speciosa in mice. J

Health Sci. 2007; 53: 382-388.
10) Hamazaki, T., Sawazaki, S., Itomura, M., et all. The effect of docosahexaenoic acid on aggression in young adults. J Clin Invest 1996; 97: 1129-1134.
11) Hashimoto, M., Tanabe Y., Fujii, Y., et all. Mechanism of improvement of spatial cognition with dietary docosahexaenoic acid. Folia pharmacologica japonica 2002; 120: 54-56.
12) Suzuki H., Lipid（oil）. Suzuki H., Wada T., Miura R., Eds. Suisansyokuhineiyougaku, Tokyo, gihodoshuppan 2004; 118-187.
13) Suzuki, H. Health effects of fish oil. Journal of Oleo Science 1999; 48（10）: 1017-1024.
14) Narayan B., Miyashita K., Hosokawa M., Physiological effects of eicosapentaenoic acid（EPA）and docosahexaenoic acid（DHA）- A review. Food Reviews International 2006; 22: 291-307.
15) Takuma, K., Nagai, T., Yamada K. Behavioral analyses for learning and memory in mice and rats. Folia pharmacologica japonica 2007; 130: 112-116.
16) Yasumoto T. Puffer toxin. Environmental Health Bureau, Ministry of Health and Welfare（ED.）, Food Hygiene Inspection Manual. Food Hygiene Association, Tokyo 1991; 232-240.
17) Report of the subdivision on resources, The council for science and technology, Ministry of Education, Culture, Sports, Science and Technology, Japan. Standard table of food composition in Japan, Fifth revised and enlarged edition -2005-. Tokyo: 2005.
18) P. G. Reevs, F. H. Nielsen G. C Fahey, Jr. AIN-93 Purified diets for laboratory rodents: Final report of the American Institute of Nutrition ad hoc writing committee on the reformulation of the AIN-76A rodent diet. J. of nutrition 1993; 123: 1939-1951.
19) Ministry Of Labor And Welfare, Vitamin. Dietary Reference Intakes for Japanese, 2010, Tokyo Daiichi-shuppan, 2009; 118-123.

要　旨：最近，フグ毒保有の餌を遮断した環境で養殖すれば無毒のフグが生産され，その肝も無毒であることが分かった。そのフグ肝には，機能性成分IPAと DHAが非常に多く含まれ，DHAに記憶学習の維持向上効果が認められている。そこで本研究によりフグ肝は食品として記憶学習能力の向上に大きな効果をもたらすことを受動回避試験によりマウスを用いて検討した。

マウスの餌には，IPAおよびDHAを多量に含むフグ肝投与群（n=16）とこれら成分を含まないコントロール群（n=16）に分けて受動回避試験により記憶学習能力への効果を調べた。電気ショック後のフグ肝投与群は，コントロール群より明室内に留まったり，徘徊したり，暗室になかば身をのばし，有意に多く暗室を警戒した行動を起こし（$p < 0.05$），さらに，電気ショック後のマウスの反応潜時（LT）は，フグ肝投与群がコントロール群より明らかに長く，有意な差が認められた（$p < 0.05$）。

これら受動回避試験の結果から，フグ肝には記憶学習能力の向上効果の可能性が認められた。将来，フグ肝は機能性を持った優れた食品になることが期待される。