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Original Article

Genotoxicity and acute oral toxicity of peptides ferrous chelates of Hairtail protein

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Abstract: Objective: To investigate the genotoxicity and acute oral toxicity of peptides ferrous chelates of Hairtail protein (Fe (II)-FPH) and to provide scientific and rational experimental basis for its application. Method: In this study, acute oral toxicity test, Ames test, mouse bone marrow micronucleus test, mice sperm abnormality test, and Chinese hamster ovary cell culture experiments were performed to detect the toxicological characteristics of peptides ferrous chelates of Hairtail protein. Results: The acute oral LD50 value of Fe (II)-FPH in mice was 7454.8 mg/kg, with the 95% confidence interval of 6258.9-9022 mg/kg. Two genotoxicity tests showed positive. In addition, octopus Fe (II)-FPH can affect the growth of normal CHO cells. Conclusions: Octopus Fe (II)-FPH had mild toxicity and had some genotoxicity and cytotoxicity. Direct use as food additives needs to be carefully considered.

Keywords: Fe (II)-FPH, genotoxicity, acute oral toxicity

Introduction

Hairtail, also known as saury, strip-shaped, is one of the most important marine commercial fish; in recent years its catches is at around 1.1 million tons, accounting for about 70%-80% of the catches of the same kind fish in the world; the annual output ranks first in marine economic fish [1]. Octopus in the processing process will produce a large number of protein-containing head, offal and other wastes, which accounts for about 40%-50% of raw fish [2]. According to previous reports [2-4], octopus scraps can be subjected to digestion, chelation, purification, lyophilization and other steps to become peptides ferrous chelates of Hairtail protein, labeled as Fe (II)-FPH. Studies have pointed out that Fe (II)-FPH was rich in amino acids [5], and had antioxidant [6] and antimicrobial effect [7]; It also can improve iron deficiency anemia [8] and had anti-hyperlipidemia [9] effect, with a wide application prospect in food fields; so it gets much attention in recent years. However, the toxicity characteristics of Fe (II)-FPH have still not yet been reported. In order to study the toxicological profile of Fe (II)-FPH and verify its safety and efficacy in food filed, according to GB 15193.1-2003 "food safety toxicology evaluation procedures and methods", acute oral toxicity and genetic toxicity experiments were performed in this study. In addition, this experiment also explored the cell toxicity of Fe (II)-FPH. The results were reported as follows.

Material and methods

Material

Peptides ferrous chelates of Hairtail protein (Fe (II)-FPH): Fe (II)-FPH was voluntarily prepared by the laboratory; Concrete steps were as previously described [3]. In the experiment, the required amount of the test drug was measured exactly; the solution of required concentrations was formulated with distilled water.

Animals and experimental environment: SPF male BALB/c mice, 18-22 g, were purchased from Beijing HuaFukang Biological Technology Co., Ltd.; the experiment started after adaptive feeding for 3-7 days; all animals were fed with the standard pellet feed provided by Tengxin Bio Co., Ltd. During the experiment, ambient temperature of animal room was 22°C-24°C, and humidity was 52%-58%.

Table 1. The acute oral toxicity results of octopus Fe (II)-FPH in Mice

Daily death/survival	Dose group				
	80×	64×	51×	41×	33×
D1	3/2	1/4	1/4	0/5	0/5
D2	5/0	2/3	1/4	0/5	0/5
D3	5/0	2/3	1/4	0/5	0/5
D4	5/0	2/3	1/4	0/5	0/5
D5	5/0	2/3	1/4	0/5	0/5
D6	5/0	2/3	1/4	0/5	0/5
D7	5/0	2/3	1/4	0/5	0/5
D8	5/0	2/3	1/4	0/5	0/5
D9	5/0	2/3	1/4	0/5	0/5
D10	5/0	2/3	1/4	0/5	0/5
D11	5/0	2/3	1/4	0/5	0/5
D12	5/0	2/3	1/4	0/5	0/5
D13	5/0	2/3	1/4	0/5	0/5
D14	5/0	2/3	1/4	0/5	0/5

Experimental strains and liver microsomal enzyme (S9) vitro metabolic activation system: TA97, TA98, TA100 and TA102, a group of standard Salmonella typhimurium histidine auxotrophic strains, were used as test strains, which were identified before the experiment to ensure that all the characteristics were qualified; identification method referred to the literature [10]; S9 was made by the laboratory.

Experimental mammalian cell lines: CHO cells (Chinese hamster ovary cells), were purchased from Shanghai Academy of Sciences.

The main reagents: PBS buffer was prepared by the PBS powder purchased from Zhongshan Company; DMEM medium and FBS were purchased from Gibco Company; cyclophosphamide and picric acid were configurable; Nanjing Jiancheng Gemsa staining solution kits.

Methods and procedures

Acute oral toxicity test: 25 mice weighing 18~22 g were weighed, numbered, and divided into five groups randomly (5/group). Reference dose 120 mg/kg was used for preliminary experiment. According to the results of preliminary experiments, in formal study, 80X of reference dose 120 mg/kg was the highest dose; the rest doses were set in a geometric ratio of 1:0.8. So five dose groups were 80× (9.60 g/kg) group, 64× (7.68 g/kg) group, 51× (6.12 g/kg) group, 41× (4.92 g/kg) group, and 33× (3.96 g/kg) group. Intragastric administration was performed; experimental animals should be fasted before the experiment (12 h), without restrictions on drinking water. Then each group was exposed by once oral gavage, 1 ml once; after exposure, fasting was continued for 3~4 h. The administered volume of the test substance for each dose group was 10 ml/kg. After administration, exposure process and the poisoning and death of animals should be observed and recorded in detail in observation period.

AMES test: Four dose gradients were set, respectively: 0.05, 0.1, 0.5 and 2 μg/dish. In the four groups of test substance, three parallel plates were set for each dose were. In addition, at the same time the blank control, solvent control, the positive mutagen control and sterile control were set. 2.0 mL top agar containing 0.5 mmol/L-histidine -0.5 mmol/L biotin was dispensed in test tubes, placed in 45°C water bath for insulation; And then test strain broth 0.1 mL, the test solution 0.1 mL and S9 mixture 0.5 mL (when metabolic activation was required) were added into each tube in order; after full mixing, the mixture was quickly poured into the bottom of the agar plate, and the plate was turned to make it evenly distributed. After condensation and curing, samples were inverted and incubated in a 37°C incubator for 48 h. Count the number of revertant colonies per dish.

Mouse bone marrow micronucleus test: 25 SPF male BALB/c mice were selected, 18-22 g; three dose groups, 1/2 LD50 (3727.4 mg/kg), 1/4 LD50 (1863.7 mg/kg) and 1/8 LD50 (931.85 mg/kg), and normal control group and cyclophosphamide control group were set. Administration was performed by multiple exposure method, one exposure daily, for four days; samples were collected at 24 h after the last administration; the negative control group was treated with the equal volume of solvent; and cyclophosphamide group was injected with cyclophosphamide by 40 mg/kg intraperitoneally. Mice were sacrificed, and then sternum bone marrow was collected, fixed with methanol, and stained by Gemsa. Under an optical microscope, 1000 polychromatic erythrocytes (PCE) were counted for each mouse; the incidence of micronuclei was PCEs containing micronuclei ‰, which was analyzed by chi-square test.

Table 2. The revertant results of Ames test

Group	Dose (μg/dish)	TA97		TA98		TA100		TA102	
		- (S9)	+ (S9)	- (S9)	+ (S9)	- (S9)	+ (S9)	- (S9)	+ (S9)
Test substance	0.05	124	231	50	186	153	253	252	496
	0.1	120	4569	47	7950	124	950	245	9560
	0.5	125	many	48	many	152	many	248	many
	2	149	many	120	many	159	many	259	many
Spontaneous revertants	no	115		45		124		248	
Solvent control	H_2O	98		42		116		240	
Positive control	0.5	448		407		2253		399	

Note: "many" represents a lot, unable to count.

Table 3. The mouse bone marrow micronucleus test results of Octopus Fe (II)-FPH

Group	Mice number	Number of observed cells (about)	Micronuclei (total)	Micronucleus rate (‰)
Normal control	5	5000	10	2
1/2 LD50	5	5000	12	2.4
1/4 LD50	5	5000	16	3.2
1/8 LD50	5	5000	21	4.2
Cyclophosphamide control	5	5000	40*	8*

Note: *Compared with normal control group, Chi-square test $P < 0.01$, with significant difference.

Table 4. The mice sperm abnormality test results of Fe (II)-FPH

Group	Mice number	Sperm number	Distortions	Distortion rate
Normal control	5	1000	78	7.8%
1/2 LD50	5	1000	182*	18.2%*
1/4 LD50	5	1000	160*	16.0%*
1/8 LD50	5	1000	121	12.1%
Cyclophosphamide control	5	1000	254*	25.4%*

Note: *Compared with normal control group, Chi-square test $P < 0.01$, with significant difference.

Mice sperm abnormality test: 25 SPF male BALB/c mice were selected, 18-22 g; three dose groups, 1/2 LD50 (3727.4 mg/kg), 1/4 LD50 (1863.7 mg/kg) and 1/8 LD50 (931.85 mg/kg), and normal control group and cyclophosphamide control group were set. Administration was performed by once intragastric exposure. Samples were collected two weeks later; the negative control group was treated with the equal volume of solvent; and cyclophosphamide group was injected with cyclophosphamide by 40 mg/kg intraperitoneally. In microscopic examination, 200 sperms were counted for each mouse; the number of abnormal sperm was counted; the chi-square test was used for statistic processing.

CHO cell culture and drug treatment: Specific operations were as previous [11]. Subculture of CHO cells used DMEM medium containing 10% fetal calf serum, double-antibody (final concentration of penicillin and streptomycin was 100 u/mL) as growth medium. In the drug culture experiments, CHO cells were transferred to 6-well plates. When the monolayer cells covered 70-80% of the bottom, the following growth medium containing seven different concentrations of drug was added to stimulate cells. Drug final concentrations: 100 μg/mL, 200 μg/mL, 400 μg/mL, 800 μg/mL, 1 mg/mL, 2 mg/mL, 4 mg/mL. A negative control (growth medium) was also set; two parallel wells were set for each drug concentration and negative control respectively. After 24 h, cell morphology was observed. According to the following degrees of cell shrinkage and morphological changes, record the results:

"++++" 80% of the cells showed shrinkage, morphological changes or cell shedding; "+++" 60% of the cells showed shrinkage, morphological changes or cell shedding; "++" 40% of the cells showed shrinkage, morphological changes or cell shedding; "+" 20% of the cells showed shrinkage, morphological changes or cell shedding; "-" ≤ 10% of the cells showed shrinkage, morphological changes or cell shedding.

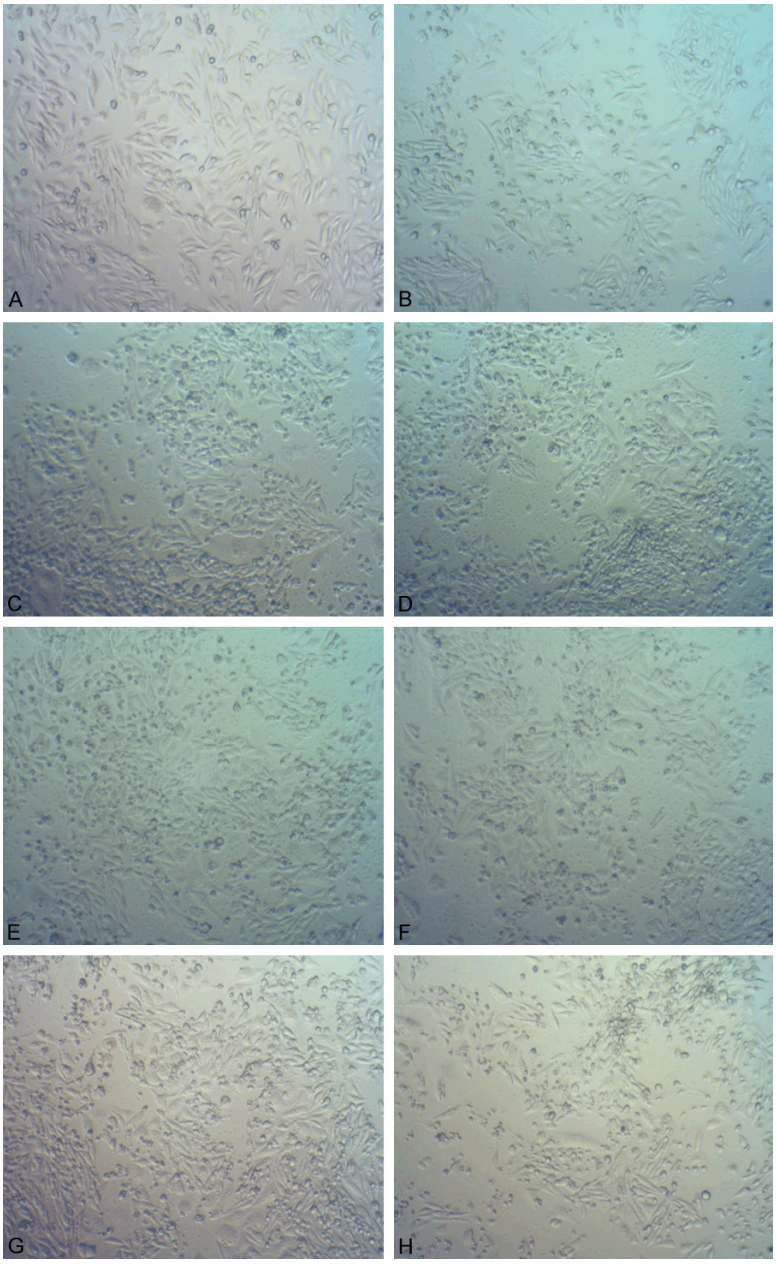

Figure 1. CHO cell morphology after drug treatment. A. Negative control; B. 100 μg/mL; C. μg/mL; D. 400 μg/mL; E. 800 μg/mL; F. 1 mg/mL; G. 2 mg/mL; H. 4 mg/mL.

Results

Acute oral toxicity test

The statistics of mice death in each group were shown in **Table 1**. According to mortality data in mice and according to the weighted probit method (Bliss's method) [12, 13], the median lethal dose was: LD50 = 7454.8 mg/kg; LD50 (Feiller correction) with 95% confidence limit = 6258.9-9022 mg/kg.

AMES test

After the test, strains TA97, TA98, TA100 and TA102 had no significant change among each dose group before adding S9 mixture; the mixture after the addition of S9, the revertant colonies of test substance were twice or more than twice the revertant colonies of solvent control and showed a dose-response trend, indicating that the test substance was positive for mutagenicity in Salmonella typhimurium (**Table 2**).

Mouse bone marrow micronucleus test

After statistical analysis, there was significant difference in the micronucleus rate between the control group and the positive control group (cyclophosphamide control group) ($P < 0.01$); there was no significant difference in micronucleus rate between each experimental group and negative control group (**Table 3**).

Mice sperm abnormality test

After statistical analysis, there were significant differences in the number of abnormal sperms between normal control group and the positive control group (cyclophosphamide control group) ($P < 0.01$); In each experimental group, in addition to 1/8 LD-50 group, there were significant differences in the number of abnormal sperms between the normal control group and other three drug concentration groups (**Table 4**).

CHO cell culture and drug treatment

The degree of Shrinkage of cells and morphological changes per well was shown in **Table 5**; **Figure 1** showed the cell morphology in some wells. The results indicated that with the in-

Table 5. CHO cell culture results after treated by Octopus Fe (II)-FPH

Well number	Negative	100 μg/mL	200 μg/mL	400 μg/mL	800 μg/mL	1 mg/mL	2 mg/mL	4 mg/mL
1	-	+	+	++	++	+++	+++	++++
2	-	-	++	++	++	++	++++	++++

crease of drug concentration, the degree of cell shrinkage and morphological changes was getting stronger.

Discussion

Acute oral toxicity test [14-17], Ames test [18], mouse bone marrow polychromatic erythrocyte micronucleus test [19] and the mouse teratosperm test [20] are the standard tests to detect toxicological properties of drugs. Chinese hamster ovary cells are the most successful host cells to express biological products [21], with a very wide range of applications. The growth status of cells after drug treatment observed by cell culture can reflect the strength of the toxicity of the drug from one aspect [22].

In this study, the acute oral toxicity test in mice confirmed that LD50 of Fe (II)-FPH was 7454.8 mg/kg, with mild toxicity. The results of three genotoxicity tests showed that, Fe (II)-FPH after metabolic activation in Salmonella typhimurium was positive for mutagenicity; it had no effect on mouse bone marrow erythrocyte micronucleus formation; when the dose was greater than or equal to 1863.7 mg/kg, it showed positive in mice sperm abnormality test. This showed that the Fe (II)-FPH was genotoxic. CHO cell culture results showed that Fe (II)-FPH also had cytotoxicity. Conclusions showed that the direct use of Fe (II)-FPH as a food additive still exists some security risks, requiring to be carefully considered.

Octopus Fe (II)-FPH is from the scraps in the processing of octopus; although it has some health risks seen from the current conclusions, we can reduce its edible risk and improve the effectiveness in the near future through a more in-depth study to improve production technology and adding other active ingredients, thus achieving more secure applications in the food industry and improving the utilization efficiency of octopus, which is one of the major ocean economic resources.

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Disclosure of conflict of interest

None.

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