International Food Research Journal 29(4): 776 - 785 (August 2022)
Journal homepage: *http://www.ifrj.upm.edu.my*

Acute and subacute oral toxicity evaluation of Antarctic krill protein in Kunming mice

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Article history

Received:
2 November 2020
Received in revised form:
30 October 2021
Accepted:
26 November 2021

Keywords

Antarctic krill,
protein,
acute oral toxicity,
subacute oral toxicity,
safety assessment

Abstract

Antarctic krill (*Euphausia superba*) protein is widely acknowledged as a potential animal protein source due to its large biomass with excellent nutritional and utilisation properties. However, safety assessments of Antarctic krill protein (AKP) are highly warranted before its use as human food. The present work thus assessed the safety of AKP in a Kunming mice model through acute toxicity and a 28-day feeding study, where the Kunming mice were fed with AKP or control diets. In the acute toxicity study, a single oral dose of 10 g/kg bodyweight (BW) AKP caused no death or abnormal effects in male and female mice, and the bodyweight gain remained within the normal range. In the repeated dose 28-day oral toxicity study, AKP was orally administered to Kunming mice at the doses of 2.5, 5.0, and 10.0 g/kg BW/day for 28 days. The absolute and relative liver weight gained was only observed in the mice administered with high-dose of AKP. However, this increase was incidental as no weight gain or histopathological alterations were observed in the main groups. These findings were consistent with the normal background lesions in the clinically normal mice used in the present work, which were considered spontaneous and/or incidental in nature and unrelated to the treatment. These results demonstrated that AKP did not exert significant acute and subacute toxicity upon oral administration to Kunming mice.

DOI
https://doi.org/10.47836/ifrj.29.4.05

Introduction

Increasing population growth consequently increases the demand for high-quality protein, thus necessitating the search for novel protein source. Antarctic krill, a shrimp-like crustacean, mostly harvested in the Southern Ocean, is one of the largest animal protein sources worldwide (Wang *et al.*, 2011). It is estimated that Antarctic krill biomass is 400 - 1,550 million tons, with a sustainable annual harvest of about 70 - 200 million tons (De Pitta *et al.*, 2008). Antarctic krill consists of 60 - 80% protein, 7 - 26% lipid, and 12 - 17% ash on a dry mass basis (Grantham, 1977). Accumulating studies have demonstrated that the amino acid and protein compositions of Antarctic krill could meet the amino acid requirements of humans and infants as prescribed by FAO/WHO/UNU (Suzuki and Shibata, 1990). Antarctic krill has the advantages of huge biomass and superior quality. However, attention should also be paid to the high value-added utilisation of AKP in the food industries to alleviate food deficiency and meet the high-quality protein demand of the population.

AKP can be produced by any recovery technology (Chen *et al.*, 2009). Recently, AKP has been extensively used for human dietary supplementation (Yoshitomi and Nagano, 2012). Gigliotti *et al.* (2011) reported that AKP could prevent early renal injury leading to nephrocalcinosis and potential bone loss. The hydrolysates prepared by Antarctic krill exert high amino acid nitrogen level and high recovery of total nitrogen, which could be used for high quality krill sauce preparation (Wang *et al.*, 2015).

Irrespective of these advantages, AKP has been tested in very few pre-clinical studies in mammals so far (Chen *et al.*, 2009). This lack of biological safety evaluation and toxicological risk assessment has obscured the toxicological information of AKP. Therefore, more toxicological risk assessments are essential to evaluate the safety of AKP. The present

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work thus provided insights into the safety assessment of AKP through acute toxicity and a 28-day subacute toxicity study in Kunming mice.

Materials and methods

AKP preparation

Antarctic krill was supplied by Shanghai Kai Chuang International Marine Resources Co., Ltd. (Shanghai, China) from the South Shetland Islands. AKP was extracted from the whole Antarctic krill using isoelectric solubilisation/precipitation. Briefly, Antarctic krill was diluted with alkali, and the insoluble substances were removed by centrifugation and acid leaching liquid by adjusting pH to the isoelectric point of AKP. Following centrifugation, AKP was washed, neutralised, and lyophilised (Gao *et al.,* 2016). The amino acid composition of AKP was analysed and profiled using the standard methods. Bovine serum protein (BSA) was purchased from Sinopharm Chemical Regent Co., Ltd (Shanghai, China), and used as a control. AKP and BSA were stored at -80°C until further use.

Animals

The present work was carried out at Fudan University Laboratory Animal Centre (Shanghai, China) in accordance with the laboratory animal administration rules of the Ministry of Science and Technology of the People's Republic of China. The procedures for care and use of animals were approved by the Ethics Committee of the Department of Laboratory Animal Science (202107007Z) of Fudan University, and all applicable institutional and governmental regulations concerning the ethical use of animals were followed. The healthy Kunming strain mice (30 males and 30 females) of 7-week old and weighing 18 - 22 g were procured from Fudan University Laboratory Animal Centre (Shanghai, China). The mice were randomised to cages on racks, separated by treatment group and sex, and housed in animal rooms located within an SPF rodent barrier facility. Animal rooms were maintained at a temperature between 20 and 22°C with a relative humidity of 60 ± 5%, air ventilation at 18 times per hour, and 12 h light/dark cycle. Mice were kept individually in stainless steel wire mesh cages; five mice per cage. They were acclimatised for 1 w with food and water available *ad libitum* before the experiment. All mice were fed with sterilised tap water and radiation-sterilised diets *ad libitum*. Food consumption and bodyweights of mice were recorded twice weekly during the study period. All treated mice were monitored daily for detailed neurotoxicological observations.

Acute toxicity

The acute toxicity study of AKP in mice was performed using the maximum tolerated dose (MTD) method following the procedures of food safety toxicological assessment (Zhou and Han, 2006). Briefly, the mice were allowed to fast for 12 h, and then AKP was administered by oral gavage, thrice daily, at a dose of 10 g/kg body weight (BW), by dissolving in 0.9% normal saline (5 g/20 mL). BSA was used as the control diet (5 g/kg body weight (BW)). The mice were continuously observed for behavioural changes, and also signs of toxicity and mortality for 1 h after treatment, twice daily. All mice were monitored for 14 d after the treatment. The body weights of the mice were measured on day 7 and 14 prior to dosage. All observations were recorded. On day 15, the mice were anesthetised with ether, and killed by exsanguination to collect blood samples for biochemical and haematological analyses, and to conduct gross and histopathological examinations.

Subacute toxicity

The subacute toxicity study of AKP in mice was performed using the maximum-tolerated doses (MTD) method following the procedures of food safety toxicological assessment, China's Ministry of Health (GB15193.22-2014). AKP was orally administered at doses of 2.5, 5.0, and 10 g/kg BW for 28 d, respectively. BSA was used as the control diet (5 g/kg BW) (Papineni *et al*., 2017). The mice were observed for general behavioural changes, signs of toxicity, and mortality for 1 h after treatment, twice daily. The bodyweights of mice were measured on days 0, 7, 14, 21, and 28 prior to dosage. On day 29, mice were anesthetised with ether, and killed by exsanguination to collect blood samples for biochemical and haematological analyses, and to conduct gross and histopathological examinations.

Haematology

Blood samples were collected from the retro-bulbar venous plexus from all non-fasted animals anesthetised with isoflurane (Isoba®, Essex GmbH, Munich, Germany). The following haematology parameters were evaluated using a haematology instrument (Advia 120; Bayer, Munich, Germany):

red blood corpuscles (RBC) count, haematocrit (HCT), haemoglobin (HGB), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC), red blood cell distribution width-CV (RDW-CV), red blood cell distribution width (RDW), reticulocyte (RET), platelet count (PLT), platelet distribution width (PDW), mean platelet volume (MPV), platelet large cell ration (P-LCR), plateletocrit (PCT), white blood count (WBC), neutrophil (NEUT), lymphatic (LY), monocyte (MON), eosinophil (EO), and basophil (BAS).

Serum chemistry parameters were assessed with an automatic analyser (Cobasc501; Roche, Mannheim, Germany) including alkaline phosphatase (ALP), alanine aminotransferase (ALT), aspartate aminotransferase (AST), lactate dehydrogenase (LDH), total bilirubin in serum (TBIL), cholesterol (CHOL), triglyceride (TG), total protein (TP), albumin (ALB), globulin (GLO), glucose, phosphorus, and calcium. Haematology and serum chemistry parameters were measured according to the methods reported by Berge *et al.* (2015).

Histopathology

All mice were subjected to a detailed necropsy examination, and the gross pathological changes in the vital organs (liver, kidney, spleen, heart, lung, thymus gland, adrenal gland, ovary, and testis) were observed according to the methods reported by Berge *et al.* (2015). The collected organs were embedded in paraffin wax, sectioned, stained with haematoxylin, and examined under a light microscope.

Statistical analysis

All parameters are presented as means ± SD, and the significant differences between the parameters were assessed through an independent *t*-test and One-way ANOVA using SPSS 22.0 (IBM, USA). $p < 0.05$ was considered statistically significant.

Results

Acute toxicity

Mortality, observations, and body weight

The mice that survived in the 14-d observation period were fed with a single oral gavage dose of 10 g/kg BW AKP. When compared with the control group, the groups of mice (male and female) fed with AKP had normal appearance (eyes, skin, and fur), movement, and behaviour, except for increasing locomotor activity at day 7 of the experimental period. However, no significant differences were observed in hardness and size of mice faeces between the AKP-treated and control groups. Nevertheless, pale and yellow-coloured faeces were observed for the mice fed with AKP.

When compared with the control group, AKP did not affect body weight gain in the male and female mice after 7 and 14 d of feeding. The average bodyweight of the male mice was significantly higher than the female at day 0, 7, and 14 in the control and treated groups. Overall, the mice in control and treated groups showed continuous weight gain from the beginning to the end of the study.

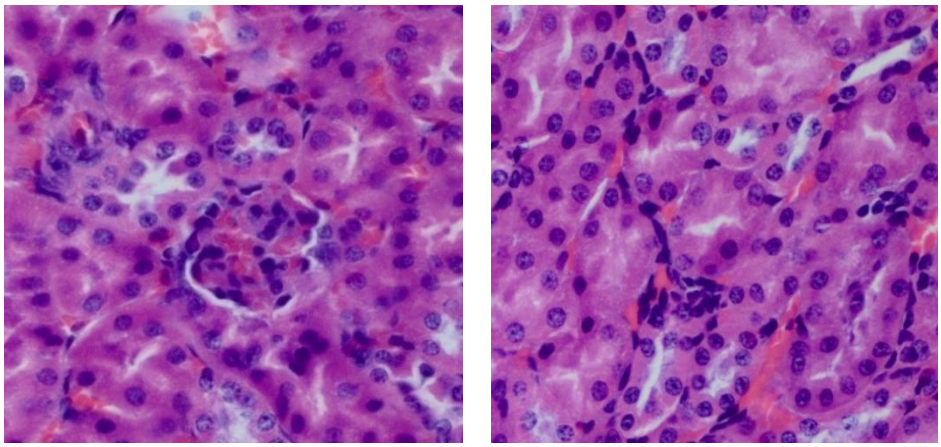
Histopathology

The organs were evaluated for AKP toxicity (Bridges *et al.,* 2010). Gross necropsy showed no abnormality in the heart, liver, spleen, lung, kidney, thymus, and testis/ovary of the mice in the AKP-treated and control groups (male and female). Furthermore, the AKP-treated groups (male and female) showed no significant negative effect on either absolute or relative organ weights as compared to the heart, liver, spleen, lung, thymus, and testis/ovary of the control group. Besides, AKP caused absolute kidney weight gain in the male mice group. However, after normalised to their bodyweight, AKP did not increase the relative kidney weight.

The histopathological results revealed that the mice fed with AKP diet had normal kidney contour without exudate (Figure 1). The glomerulus and renal capsule structures were clear, and the cell number and blood vessel walls were normal. There were no exudate and cast in the renal capsule and lumen tubule, and no oedema in the epithelium of renal convoluted tubules. Kidneys did not show significant differences except for slight congestion, which might be attributed to the stress produced in the mice during handling and blood collection (Mcclure, 1999). These results showed no statistically significant difference. Additionally, there were no significant histopathological changes in other tissues in either mice groups (control and treated groups) or sex.

Haematology and serum biochemistry

The development of blood conventional index during oral acute toxicity study is listed in Table 1.



Control group **AKP group**

Figure 1. Histopathological changes in kidneys of male mice (40 × 10 times).

Table 1. Haematological values for each group in acute toxicity study of AKP.

Group	Male		Female	
	Control	AKP	Control	AKP
RBC (M/uL)	10.1 ± 0.9	10.0 ± 0.3	10.8 ± 1.0	9.9 ± 0.4
HGB (g/dL)	15.6 ± 1.1	15.1 ± 0.5	16.2 ± 0.9	14.8 ± 0.8
HCT (%)	51.6 ± 2.9	49.6 ± 1.6	51.9 ± 4.0	47.7 ± 2.0
MCV (fL)	51.3 ± 1.8	49.4 ± 0.8	48.3 ± 1.1	48.2 ± 0.3
MCH (pg)	15.5 ± 0.3	15.1 ± 0.1	15.1 ± 0.6	15.0 ± 0.3
MCHC (g/dL)	30.2 ± 0.5	30.5 ± 0.4	31.2 ± 0.6	31.1 ± 0.5
RDW-SD (fL)	28.6 ± 0.7	29.9 ± 1.2	29.7 ± 0.4	29.9 ± 0.5
RDW-CV (%)	23.7 ± 1.2	24.7 ± 0.7	26.1 ± 1.9	24.9 ± 0.4
RET# (K/uL)	427.1 ± 201.3	385.1 ± 41.6	437.7 ± 133.7	358.6 ± 54.1
RET% (%)	4.4 ± 2.5	3.8 ± 0.5	4.1 ± 1.2	3.6 ± 0.5
PLT (K/uL)	946.7 ± 134.0	1021.7 ± 128.3	930.7 ± 98.9	847.3 ± 134.4
PDW (fL)	8.6 ± 1.0	7.7 ± 0.5	8.3 ± 0.1	8.2 ± 0.8
MPV (fL)	6.8 ± 0.8	6.4 ± 0.4	6.2 ± 0.2	6.3 ± 0.5
P-LCR (%)	7.0 ± 4.4	6.7 ± 2.2	4.4 ± 1.8	5.5 ± 1.9
PCT (%)	0.6 ± 0.2	0.7 ± 0.1	0.6 ± 0.0	0.5 ± 0.1
WBC (K/uL)	3.3 ± 1.7	4.2 ± 2.8	2.7 ± 1.5	3.9 ± 0.9
NEUT# (K/uL)	0.6 ± 0.3	1.2 ± 1.3	0.2 ± 0.1	0.6 ± 0.3
NEUT (%)	17.3 ± 1.5	24.8 ± 13.0	13.3 ± 10.2	17.1 ± 9.5
LY# (K/uL)	2.6 ± 1.3	2.9 ± 1.6	1.9 ± 1.4	3.2 ± 0.9
LY (%)	80.0 ± 1.5	72.8 ± 13.1	84.4 ± 11.0	80.2 ± 10.7
MON# (K/uL)	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.1 ± 0.1
MON (%)	1.1 ± 0.7	0.9 ± 0.2	1.4 ± 1.0	1.6 ± 0.9
EO# (K/uL)	0.1 ± 0.1	0.1 ± 0.0	0.0 ± 0.0	0.1 ± 0.1
EO (%)	1.6 ± 0.6	1.5 ± 1.5	0.7 ± 0.2	1.0 ± 0.5
BAS# (K/uL)	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
BAS% (%)	0.0 ± 0.0	0.0 ± 0.0	0.3 ± 0.5	0.1 ± 0.1

Control group: saline water irrigation stomach. Values are mean ± SD, n = 5 animals/group. Statistically significant differences were analysed for males or females as compared to control by an independent t-test, respectively.

There was no statistically significant difference ($p > 0.05$) between the control and treated groups, but these data were considered to be of no biological or toxicological significance.

Although many Antarctic krill products are available on the market, only a few acute and subchronic toxicological assessments of AKP in animals have been published. Our results confirmed that oral administration of 10 g/kg BW AKP exerted no observed adverse effect level (NOAEL) in male and female mice.

Subacute toxicity

Mortality, observations, and body weight

There was no mortality attributed to any effect of AKP. One male fed with 10 g/kg/day does of AKP died within the first three weeks of the study due to mouse's tearing. However, histological analysis did not show any abnormal changes. The appearance, movement, and behaviour of mice fed with AKP showed no statistically significant differences during the experimental period, except for the skin and fur colours. Similarly, abnormal pale-coloured faeces were observed in the treated mice due to the colour of AKP formulations. Therefore, it was not regarded as an adverse effect.

The bodyweights of three female mice groups fed with AKP were lower than the control group, while the treated groups of male mice with all doses were higher than the control group by the fourth week. As a result, the mean body weight of the female mice AKP group administered with a dose of 10 g/kg/day was the lowest among all groups. Moreover, the bodyweights of male mice in the control group significantly decreased, which might be attributed to the aggressive behaviours of these mice.

Histopathology

No treatment-related changes were observed in any organs of the control or treated groups. Similarly, no sporadic histopathological changes were observed. Absolute and relative organ weights of the male and female mice are presented in Table 2. In the female mice, there was a decrease in the absolute heart weight, but no significant changes in the relative heart weights were observed. Moreover, only the relative liver weights of the male mice administered with 10 g/kg BW AKP significantly increased, but the increase was regarded incidental as no dose-related weight changes or histopathological lesions were observed in either group (Berge *et al.*, 2015). The increase might have been due to malnutrition (loss of appetence resulted from intragastric overfeeding in mice), which was insufficient to meet the normal mice nutrition requirements.

Haematology and serum biochemistry

Haematology results are summarised in Table 3. As compared to the control group, significant decrease in MCV and MCH levels were observed in the female mice administered with 10 g/kg BW AKP. However, the P-LCR level significantly increased in the female mice administered with 10 g/kg BW AKP as compared to the control group. RDW-CV level significantly increased in the female mice administered with 5 g/kg BW AKP as compared to the BSA control. Moreover, the increased MPV and P-LCR levels in the female mice administered with 10 g/kg BW AKP achieved statistical significance when compared with the BSA control group. HCT and MCV levels also statistically decreased in the male mice administered with10 g/kg BW when compared with the control. MPV and P-LCR levels also statistically decreased in the male mice administered with 10 g/kg BW when compared with the BSA control. However, the RET levels remarkably increased in the 5 g/kg BW AKP-treated male mice. The RDW-CV levels in the male mice in the 2.5, 5.0, 10 g/kg BW AKP-treated group achieved statistical significance when compared with the BSA control and the control group. These results indicated that the biochemical parameters of the male mice used in this study to assess the hepatocyte integrity (AST, ALT), bile duct alterations (ALP), and liver function (TBIL, ALB) were normal in this breed and age (Begona *et al.*, 2003).

Serum biochemistry parameters at the termination of the 28-day oral subacute toxicity study are presented in Table 4. ALP levels significantly decreased, ranging from 2.5 to 5 g/kg BW AKP-treated male mice, however, ALP levels increased in 10 g/kg BW AKP-treated male mice. The levels of AST, ALT, and LDH remarkably increased, ranging from 2.5 to 5 g/kg BW AKP-treated male mice. The ALP levels of the 2.5 g/kg BW AKP-treated female mice significantly increased, whereas, the LDH levels of 5.0 g/kg BW AKP-treated female mice decreased when compared with the BSA group. Furthermore, the hepatocyte integrity, bile duct alterations, and liver functions were normal in the female mice. This

Table 2. Absolute and relative organ weights for each group in repeated dose 28-d oral toxicity study of AKP.

Group	Control		BSA Control		2.5 g/kg BW		5.0 g/kg BW		10.0 g/kg BW	
	Absolute	Relative	Absolute	Relative	Absolute	Relative	Absolute	Relative	Absolute	Relative
Male										
Heart	0.31 ± 0.04	0.77 ± 0.11	0.29 ± 0.03	0.67 ± 0.04	0.33 ± 0.01	0.78 ± 0.03	0.31 ± 0.02	0.74 ± 0.01	0.28 ± 0.05	0.69 ± 0.11
Liver	2.12 ± 0.16	5.36 ± 0.36^{ab}	2.09 ± 0.27	5.04 ± 0.39 ^{ab}	1.96 ± 0.03	4.65 ± 0.03^{b}	2.02 ± 0.15	4.90 ± 0.47^{b}	2.40 ± 0.20	5.91 ± 0.32^{a}
Spleen	0.10 ± 0.00	0.25 ± 0.01	0.15 ± 0.06	0.22 ± 038	0.12 ± 0.03	0.29 ± 0.08	0.15 ± 0.05	0.37 ± 0.13	0.14 ± 0.03	0.35 ± 0.06
Lung	0.29 ± 0.03	0.73 ± 0.09	0.28 ± 0.02	0.69 ± 0.03	0.28 ± 0.01	0.67 ± 0.03	0.31 ± 0.03	0.75 ± 0.05	0.30 ± 0.02	0.73 ± 0.02
Kidney	0.76 ± 0.06	1.92 ± 0.23	0.71 ± 0.14	1.70 ± 0.26	0.71 ± 0.01	1.69 ± 0.02	0.75 ± 0.05	1.81 ± 0.09	0.77 ± 0.14	1.88 ± 0.32
Thymus	0.15 ± 0.05	0.39 ± 0.13	0.14 ± 0.04	0.34 ± 0.08	0.17 ± 0.01	0.40 ± 0.02	0.18 ± 0.02	0.43 ± 0.03	0.14 ± 0.01	0.35 ± 0.04
Testis/ovary	0.32 ± 0.03	0.81 ± 0.10	0.24 ± 0.13	0.58 ± 0.29	0.34 ± 0.06	0.82 ± 0.14	0.30 ± 0.03	0.73 ± 0.04	0.32 ± 0.02	0.78 ± 0.03
Female										
Heart	0.28 ± 0.03^{a}	0.78 ± 0.08	0.26 ± 0.02^{ab}	0.76 ± 0.04	0.25 ± 0.02^{ab}	0.74 ± 0.07	0.25 ± 0.03^{ab}	0.73 ± 0.11	0.21 ± 0.03^{b}	0.62 ± 0.05
Liver	1.87 ± 0.13	5.19 ± 0.25	1.81 ± 0.13	5.23 ± 0.40	1.67 ± 0.07	4.91 ± 0.24	1.80 ± 0.57	5.21 ± 1.38	1.77 ± 0.32	5.25 ± 0.55
Spleen	0.14 ± 0.01	0.40 ± 0.03	0.13 ± 0.02	0.38 ± 0.06	0.11 ± 0.02	0.33 ± 0.06	0.13 ± 0.05	0.37 ± 0.12	0.12 ± 0.04	0.34 ± 0.08
Lung	0.27 ± 0.03	0.75 ± 0.39	0.28 ± 0.05	0.81 ± 0.17	0.26 ± 0.03	0.77 ± 0.08	0.29 ± 0.03	0.85 ± 0.09	0.28 ± 0.04	0.82 ± 0.05
Kidney	0.52 ± 0.02	1.44 ± 0.02	0.49 ± 0.05	1.41 ± 0.18	0.48 ± 0.09	1.40 ± 0.25	0.51 ± 0.05	1.50 ± 0.21	0.46 ± 0.06	1.37 ± 0.14
Thymus	0.27 ± 0.13	0.75 ± 0.39	0.21 ± 0.07	0.61 ± 0.17	0.17 ± 0.03	0.49 ± 0.09	0.17 ± 0.02	0.51 ± 0.06	0.20 ± 0.01	0.60 ± 0.07
Testis/ovary	0.04 ± 0.02	0.11 ± 0.05	0.05 ± 0.01	0.14 ± 0.02	0.04 ± 0.00	0.12 ± 0.00	0.04 ± 0.02	0.12 ± 0.04	0.05 ± 0.02	0.15 ± 0.04

Control group: saline water irrigation stomach. Bovine serum albumin (BSA) group: BSA irrigation stomach. Values are mean ± SD, n = 5 animals/group. Statistically significant differences were analysed for males or females by One-way ANOVA. Means followed by different lowercase superscripts in the same column indicate significant difference.

Table 3. Repeated dose 28-d oral toxicity study of AKP in mouse-haematology values (mean ± SD).

Group	Control	BSA Control	2.5 g/kg.BW	5.0 g/kg.BW	10.0 g/kg.BW
Male					
RBC (M/uL)	11.0 ± 0.4	10.3 ± 0.4	10.4 ± 0.5	10.0 ± 0.4	9.9 ± 0.3
HGB (g/dL)	16.3 ± 0.5	15.0 ± 0.2	15.3 ± 0.9	15.1 ± 0.6	14.8 ± 0.6
HCT (%)	51.3 ± 1.8^{a}	47.9 ± 2.1^{ab}	47.3 ± 2.5^{ab}	47.1 ± 1.9^{ab}	45.5 ± 2.1^{b}
MCV (fL)	46.6 ± 2.3^{a}	46.5 ± 4.0^{ab}	45.5 ± 1.3^{b}	47.0 ± 2.2^{b}	45.9 ± 0.6^{b}
MCH (pg)	14.8 ± 0.6	14.6 ± 0.8	14.7 ± 0.5	15.1 ± 0.6	15.0 ± 0.2
MCHC (g/dL)	31.7 ± 0.3	31.3 ± 1.1	32.4 ± 0.2	32.1 ± 0.3	32.6 ± 0.2
RDW-CV (%)	27.9 ± 1.7^{a}	26.9 ± 0.7^{b}	27.4 ± 2.1^{c}	30.6 ± 2.8^{c}	30.1 ± 0.9^{c}
RDW	26.1 ± 1.3	25.0 ± 2.4	26.4 ± 0.3	26.4 ± 0.8	26.6 ± 0.4
RET# (K/uL)	165.4 ± 62.6	185.0 ± 64.3	334.3 ± 128.8	490.4 ± 209.3	357.6 ± 74.6
RET% (%)	1.5 ± 0.6^{b}	1.8 ± 0.7^{ab}	3.2 ± 1.3^{ab}	4.9 ± 2.2^{a}	3.6 ± 0.7^{ab}
PLT (K/uL)	1019.0 ± 108.5^{ab}	994.7 ± 32.7^{ab}	941.7 ± 227.9^{b}	1389.0 ± 221.5^{a}	954.3 ± 75.1^{b}
PDW (fL)	7.9 ± 0.6	8.2 ± 0.8	8.2 ± 0.5	8.4 ± 0.8	7.7 ± 0.4
MPV (fL)	6.1 ± 0.5^{ab}	6.3 ± 0.6^{b}	6.4 ± 0.3^{b}	6.4 ± 0.6^{b}	6.0 ± 0.2^{a}
P-LCR (%)	4.5 ± 2.0^{b}	5.5 ± 2.8^{b}	6.1 ± 1.4^{b}	5.9 ± 2.1^{b}	4.2 ± 0.4^{a}
PCT (%)	0.6 ± 0.1	0.6 ± 0.1	0.6 ± 0.1	0.9 ± 0.1	0.6 ± 0.0
WBC (K/uL)	1.3 ± 0.5	3.0 ± 0.5	3.2 ± 1.2	2. 5 ± 1. 5	4.4 ± 2.4
NEUT# (K/uL)	0.2 ± 0.1	0.8 ± 0.6	0.6 ± 0.6	0.6 ± 0.6	0.7 ± 0.9
LY# (K/uL)	1.1 ± 0.4	2.1 ± 0.8	2.5 ± 1.0	1.7 ± 1.1	3.5 ± 1.4
MON# (K/uL)	ND	ND	ND	ND	ND
EO# (K/uL)	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.1 ± 0.1
BAS# (K/uL)	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
NEUT% (%)	15.2 ± 2.1	28.0 ± 20.7	18.2 ± 15.3	23.6 ± 15.6	13.3 ± 9.6
LY% (%)	82.9 ± 2.6	70.7 ± 20.8	79.9 ± 15.9	73.5 ± 16.2	84.4 ± 11.0
MON% (%)	1.1 ± 0.4	0.5 ± 0.3	1.0 ± 0.2	1.6 ± 0.4	1.0 ± 0.5
EO% (%)	0.4 ± 0.4	0.8 ± 0.5	0.8 ± 0.7	1.0 ± 0.4	1.2 ± 1.0
BAS% (%)	0.4 ± 0.4	ND	0.1 ± 0.2	0.2 ± 0.2	0.1 ± 0.2
Female					
RBC (M/uL)	10.1 ± 0.5	10.6 ± 0.6	10.5 ± 0.5	10.7 ± 0.6	10.1 ± 0.2
HGB (g/dL)	15.8 ± 0.3	15.5 ± 0.8	15.6 ± 0.7	15.7 ± 0.4	15.0 ± 0.6
HCT (%)	49.1 ± 2.2	49.6 ± 3.5	48.1 ± 1.7	47.8 ± 3.3	45.3 ± 1.5
MCV (fL)	48.7 ± 0.5^{a}	46.7 ± 0.5^{ab}	45. 7 ± 1.2^{b}	44.9 ± 1.3^{b}	44.7 ± 0.7^{b}
MCH (pg)	15.7 ± 0.5^{a}	14.6 ± 0.3^{ab}	14.8 ± 0.4^{ab}	14.7 ± 0.4^{ab}	14.8 ± 0.3^{b}
MCHC (g/dL)	32.2 ± 0.9	31.3 ± 0.7	32.5 ± 0.3	32.9 ± 1. 6	32.2 ± 0.2
RDW-CV (%)	30.6 ± 0.8^{ac}	29.6 ± 1.3^{c}	27.9 ± 1.8^{c}	30.5 ± 2.9^{b}	27.1 ± 0.5^{c}
RDW	25.3 ± 0.7	26.2 ± 1.0	26.0 ± 1.1	28.5 ± 0.2	26.3 ± 0.4
RET# (K/uL)	466.6 ± 200.6	228.7 ± 165.4	326.4 ± 140.6	409.2 ± 113.9	229.4 ± 60.1
RET% (%)	4.6 ± 2.0	2.2 ± 1.6	3.1 ± 1.4	3.8 ± 0.9	2.3 ± 0.6
PLT (K/uL)	853.7 ± 140.3	955.3 ± 122.6	1033.0 ± 122.6	837.3 ± 96.5	1001.3 ± 105.4
PDW (fL)	7.8 ± 0.5	7.7 ± 0.6	8.1 ± 0.5	7.6 ± 0.1	8.9 ± 0.7
MPV (fL)	6.2 ± 0.2^{ab}	5.9 ± 0.3^{b}	6.3 ± 0.2^{ab}	6.0 ± 0.1^{b}	6.8 ± 0.3^{a}
P-LCR (%)	4.8 ± 0.3^{b}	3.7 ± 0.9^{b}	5.4 ± 1.0^{b}	3.8 ± 0.1^{b}	8.0 ± 1.0^{a}
PCT (%)	0.5 ± 0.1	0.6 ± 0.1	0.7 ± 0.1	0.5 ± 0.1	0.7 ± 0.1
WBC (K/uL)	5.1 ± 1.8	3.2 ± 1.5	2.4 ± 0.8	2.9 ± 1.0	3.9 ± 1.2
NEUT# (K/uL)	1.7 ± 1.0	0.5 ± 0.4	0.4 ± 0.1	0.5 ± 0.3	1.1 ± 0.8
LY# (K/uL)	3.4 ± 0.9	2.7 ± 1.2	2.0 ± 0.9	2.3 ± 0.9	2.7 ± 1.1

MON# (K/uL)	ND	ND	ND	ND	0.1 ± 0.0
EO# (K/uL)	ND	ND	ND	ND	ND
BAS# (K/uL)	ND	ND	ND	ND	ND
NEUT% (%)	30.8 ± 8.6	13.4 ± 6.1	16.1 ± 10.3	17.2 ± 9.2	28.2 ± 19.6
LY% (%)	67.8 ± 8.4	85.4 ± 6.6	82.4 ± 10.5	80.8 ± 9.5	69.9 ± 20.5
MON% (%)	0.4 ± 0.2	0.4 ± 0.2	1.0 ± 0.4	1.6 ± 0.6	1.3 ± 0.7
EO% (%)	1.0 ± 0.4	0.6 ± 0.6	0.5 ± 0.5	0.4 ± 0.1	0.6 ± 0.2
BAS% (%)	ND	0.1 ± 0.1	ND	ND	ND

Control group: saline water irrigation stomach. Bovine serum albumin (BSA) group: BSA irrigation stomach. Values are mean ± SD, n = 5 animals/group. Statistically significant differences were analysed for males or females by One-way ANOVA. Means followed by different lowercase superscripts in the same column indicate significant difference. ND: not detected.

Table 4. Repeated dose 28-d oral toxicity study of AKP in mouse-clinical chemistry (mean ± SD).

Group	Control	BSA Control	2.5 g/kg.BW	5.0 g/kg.BW	10.0 g/kg.BW
Male					
ALP (IU/L)	443.0 ± 151.6	328.3 ± 121.9	245.3 ± 61.5	229.0 ± 35.9	282.3 ± 103.2
ALT (IU/L)	29.3 ± 4.9^{ab}	34.0 ± 9.6^{ab}	28.0 ± 2.0^{ab}	39.7 ± 6.7^{a}	22.7 ± 3.2^{b}
AST (IU/L)	74.7 ± 1.5	80.3 ± 2.1	85.7 ± 39.3	165.3 ± 70.9	76.3 ± 12.5
LDH (IU/L)	598.3 ± 140.2^{b}	886.7 ± 100.3^{ab}	690.7 ± 205.8^{ab}	1121.7 ± 78.1^{a}	986.7 ± 333.4^{ab}
Glucose (mmol/L)	4.1 ± 1.5	5.1 ± 0.4	4.0 ± 0.3	3.4 ± 0.7	5.3 ± 1.4
TBIL (mol/l)	1.2 ± 0.4	1.8 ± 0.9	1.2 ± 0.6	1.5 ± 0.7	1.0 ± 0.2
CHO (mmol/L)	2.2 ± 0.5	2.2 ± 0.5	2.1 ± 0.4	2.4 ± 0.8	1.6 ± 0.4
TG (mmol/L)	0.4 ± 0.2	0.5 ± 0.2	0.5 ± 0.1	0.8 ± 0.5	1.1 ± 0.4
TP (g/L)	54.2 ± 2.3^{ab}	55.8 ± 2.7^{a}	48.6 ± 2.3^{ab}	55.2 ± 0.8^{a}	44.5 ± 7.5^{b}
ALB (g/L)	31.9 ± 1.3^{a}	32.2 ± 0.6^{a}	28.6 ± 0.4^{ab}	31.3 ± 1.2^{ab}	26.8 ± 3.5^{b}
GLO (g/L)	22.2 ± 1.0	23.6 ± 2.3	20.0 ± 2.6	23.9 ± 1.8	17.7 ± 4.1
Phosphate (mmol/L)	2.8 ± 0.2^{a}	2.0 ± 0.4^{ab}	2.6 ± 0.1^{ab}	2.6 ± 0.3^{b}	1.5 ± 0.2^{c}
Calcium (mmol/L)	2.2 ± 0.1^{ab}	2.2 ± 0.0^{ab}	2.1 ± 0.0^{ab}	2.3 ± 0.1^{a}	1.9 ± 0.2^{b}
Female					
ALP (IU/L)	406.7 ± 113.6	384.3 ± 117.4	485.7 ± 114.0	371.3 ± 147.5	358.7 ± 64.2
ALT (IU/L)	19.7 ± 3.8	30.3 ± 11.6	28.0 ± 3.6	62.7 ± 53.4	32.0 ± 21.0
AST (IU/L)	80.7 ± 13.1	95.7 ± 27.5	81.7 ± 5.8	119.0 ± 40.1	100.3 ± 20.2
LDH (IU/L)	595.0 ± 32.2	842.0 ± 192.9	834.7 ± 83.5	703.7 ± 55.8	761.3 ± 45.7
Glucose (mmol/L)	6.5 ± 3.4	5.4 ± 1.2	4.9 ± 0.6	3.8 ± 0.6	4.6 ± 0.9
TBIL (mol/l)	0.8 ± 0.2	1.0 ± 0.1	1.6 ± 0.2	1.5 ± 0.8	0.7 ± 0.2
CHOL (mmol/L)	2.3 ± 0.9	2.0 ± 0.4	2.3 ± 0.9	1.8 ± 0.2	1.6 ± 0.0
TG (mmol/L)	0.6 ± 0.1	0.3 ± 0.0	0.4 ± 0.2	0.3 ± 0.1	0.4 ± 0.1
TP (g/L)	53.3 ± 4.1	52.3 ± 8.1	60.2 ± 5.3	49.7 ± 1.8	49.8 ± 4.3
ALB (g/L)	31.5 ± 2.3	32.1 ± 2.7	34.8 ± 2.1	31.4 ± 0.5	30.3 ± 1.9
GLO (g/L)	21.8 ± 1.8	20.2 ± 5.3	25.4 ± 3.5	18.3 ± 1.5	19.5 ± 3.3
Phosphate (mmol/L)	2.3 ± 0.4	2.3 ± 0.2	2.1 ± 0.2	2.6 ± 0.4	1.9 ± 0.4
Calcium (mmol/L)	2.2 ± 0.2	2.2 ± 0.1	2.3 ± 0.1	2.2 ± 0.1	2.1 ± 0.1

Control group: saline water irrigation stomach. Bovine serum albumin (BSA) group: BSA irrigation stomach. Values are mean ± SD, n = 5 animals/group. Statistically significant differences were analysed for males or females by One-way ANOVA. Means followed by different lowercase superscripts in the same column indicate significant difference.

could be attributed to an excessive dietary fat (Deng *et al.,* 2005) or loss of appetence (resulted from intragastric overfeeding in mice), which was insufficient to meet the normal mice nutrition requirements.

Discussion

Antarctic krill is gaining increasing popularity worldwide due to its large biomass and high nutritional value. AKP is widely acknowledged as a novel source of protein, with the advantages of industrial use as dietary supplement food and feed. However, each novel protein consumed as food or feed must undergo a safety assessment before use to protect human and animal health. In this regard, few researchers have conducted safety assessments of AKP on human health. Animal testing is one of the most pivotal parts of safety evaluation of new protein sources (Xu *et al.,* 2009). To directly assess the toxicity of novel proteins in mammals, acute and subacute toxicity in mice are used to test the safety of AKP.

In the present work, orally acute and subacute toxicity studies were conducted in a Kunming mice model to assess the safety of AKP. AKP was administered by gavage at a dose up to 10 g/kg/day, the maximum feasible dose-level limited by the volume and viscosity of the samples. Bodyweights were considered as indicative of AKP-induced toxicity between the control and treated groups (Berge *et al.,* 2015). The bodyweight results demonstrated that all tested animals grew normally and survived throughout the treatment period. Furthermore, no significant differences were observed in the clinical, haematological, and blood biochemistry parameters in either group. The increased locomotor activity could be attributed to the change in the environment, such as inadvertent gavage stress (Zakharova *et al.,* 2012). The faeces colours were pale and yellow for all treated groups, which might have been due to the coloured materials of the material, but not necessarily related to toxicity (Buesen *et al.,* 2015). These results showed that oral administration of 10 g/kg BW AKP did not exert acute or subacute toxicity. These results were consistent with the results of Gigliotti *et al*. (2007) who proved that AKP concentrates feeding exerted no adverse effect on the kidney function of female Sprague Dawley rats. Gigliotti *et al*. (2007) also elucidated that there was no significant toxicological difference between the treated and control group in a 13-week study with 5% krill oil (Gigliotti *et al.,* 2011). Berge *et al.* (2015) found that 9.67% krill powder was safe for a 13-week subchronic toxicity study in rats. Overall, further studies are highly warranted to obtain more comprehensive information on the safety of AKP, especially for the long-term administration of AKP.

Conclusion

The present work assessed the safety of AKP in a Kunming mice model through acute and subacute oral toxicity studies. Results showed that oral administration of 10 g/kg BW AKP did not exert acute or subacute toxicity to Kunming mice. The present work provided insights into long-term safety effects for further chronic and clinical studies.

Acknowledgement

The present work was financially supported by the National Natural Science Foundation of China (grant no.: 31471687), the Central Public-interest Scientific Institution Basal Research Fund, CAFS (grant no.: 2020XT1201), the Shanghai Municipal Natural Science Foundation (grant no.: 13ZR1449900), and the Key Project of Shanghai Agriculture Prosperity through Science and Technology [grant no.: 2015 (5-5)].

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