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Plant protein diet suppressed immune function by inhibiting spiral valve intestinal mucosal barrier integrity, anti-oxidation, apoptosis, autophagy and proliferation responses in amur sturgeon (*Acipenser schrenckii*)

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ABSTRACT

An 8-week growth trial was conducted to investigate the effects of replacing dietary fishmeal with a plant protein blend on the growth performance, mucosal barrier integrity and the related regulation mechanism in Amur Sturgeon (*Acipenser schrenckii*) with initial weight of 87.48 g. Three isonitrogenous and isoenergetic diets were prepared. A basal diet containing 540 g/kg fishmeal (P0), whereas the other two diets were formulated by replacing 50% and 100% of FM with plant protein blend (soybean protein concentrate and cottonseed protein concentrate), and named as P50 and P100, respectively. Although essential amino acids, fatty acids, and available phosphorus had been balanced according to the nutrient requirement of sturgeon, compared with the fish of P0 and P50, the full plant protein diet (P100) significantly reduced growth performance and survival, and accompanied with serious spiral valve intestinal (SVI) damage. The increased tissue necrosis and failed responses in anti-oxidation, programming apoptosis, autophagy and cell proliferation system were regulated by inhibiting ERK1 phosphorylation, which indicated that SVI hypoimmunity and functional degradation were the main reasons for the high mortality and low utilization ability of plant protein in Amur sturgeon.

1. Introduction

Integrated nutrient plays a vital role in the aquaculture industry, and the protein is not only one of the most important nutrient, but also the most expensive stuff in the cost of fish feed [1]. High demands and limited resources have led to high price for fishmeal (FM), which led to an inevitable requirement to find alternative proteins for sustainable aquaculture [2]. The necessary factors influenced the well utilization of an alternative protein include the availability, palatability, digestibility, antinutritional factors (ANFs) and price [3,4]. The plant protein (PP), such as by-product meals from oil-seeds, including soy, cotton and rape seeds, have been widely researched and used in feed of omnivorous and herbivorous fish species [5], and even in some of carnivorous species, such as European seabass and Japanese seabass [6,7]. Generally, carnivorous fish showed a much lower ability to accept high PP diets even with balanced essential nutrients [8,9].

All sturgeon are identified as endangered animals by CITES (Convention on International Trade in Endangered Species), and Amur sturgeon is one of the most primitive Actinopterygii species distributed in the Amur River [10]. In recent years, Amur sturgeon aquaculture has received much attention in some countries and regions for its considerable value in meat and also in caviar [11]. Although sturgeons have various feeding habits, all sturgeons share the same feed mainly based on the nutrient requirement of white sturgeon and Siberian sturgeon, those had been relatively well studied [12–15]. Accordingly, they showed different responses to the diets with high level of PP [12–16], in which, Siberian sturgeon (*Acipenser baerii*) can well digest and utilize a full PP diet with essential amino acids (EAA) and phosphorus balance [13], but significantly negative effects on growth and digestive enzyme activity in Amur sturgeon (*Acipenser schrenckii*) were observed when half of FM was replaced by soy protein isolate [16]. Previous study in our laboratory also found that Amur sturgeon is with

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more carnivorous habit than that of Siberian sturgeon when they were fed high FM or PP diets (unpublished data).

The immune dysfunction problem attributed to the oxidation stress fed PP diet had been widely studied in many species [17–19]. The intestinal health of fish relies on the integrated functions on the mechanical, chemical, biological and immune barriers of the intestine [20,21]. Compared with FM, the negative factors in PP can induce intestinal barrier damage, chronic inflammation, apoptosis and immunity inhibition and even death in many fish species, such as Atlantic salmon [22], gilthead sea bream [23], large yellow croaker [24]. Chiou et al. (2007) [25] has found that extracellular regulated protein kinase (ERK) plays essential roles in growth and development on zebrafish. ERK is a subfamily of the mitogen-activated protein kinase (MAPK) superfamily, and the MAPK/ERK cascade is reported to be associated with diverse biological functions, activation of ERK can affect immunity via regulating cellular antioxidant capacity, apoptosis, and autophagy [26–28]. Moreover, both ERK and autophagy are involved in the regulation of cell proliferation, which is essential for histopathological repair [29,30].

A corkscrew-shaped spiral valve exits in the lower portion of the intestine of Acipenseriformes (sturgeon and paddlefish), sharks, rays, skates, bichirs and lungfishes [31]. The spiral valve intestine (SVI) with the thick mucosa, the spiral valve and the pyloric caecum to effectively increase the absorptive surface area. SVI in sturgeon acts as the key organ for nutrients absorption and immune response due to these unique structural features [32,33]. Until now, there is no report on the effect of PP diet on the Amur sturgeon valvular intestine yet. The objectives of the present study were to investigate the growth performance, SVI health and the underlying regulatory mechanism related to the anti-oxidation, apoptosis, autophagy and proliferation responses and the regulation of ERK1 activation of Amur sturgeon when dietary FM were partially or fully replaced by PP.

2. Materials and methods

During the feeding period, the experimental fish were maintained in compliance with the Laboratory Animal Welfare Guidelines of China (Decree No. 2 of Ministry of Science and Technology, issued in 1988).

2.1. Experimental diets

Three experimental diets were formulated to be isonitrogenous and isoenergetic. A basal diet was used as the control contain 54% low-temperature steam-dried FM (named as P0), whereas 50% and 100% of FM were substituted with a PP blend, which was composed by soybean protein concentrate (SPC) and cottonseed protein concentrate (CPC), and named as P50 and P100, respectively. Adding the crystallized amino acids (CAAs, including *L*-Lys-HCl, *DL*-Met, and *L*-Thr), fish oil and monocalcium phosphate to the PP inclusion groups to balance essential amino acid (EAA), essential fatty acids (EFA) and available phosphorus (AP), respectively according to the EAA requirement of white sturgeon [14]. The diets were made into dry sinking pellets (diameter: 3 mm) under the extrusion condition as: feeding section (90 °C/5 s), compression section (130 °C/3 s) and metering section (60 °C/4 s) using a twin-screwed extruder (EXT50A, Yang Gong Machine, Beijing, China), then air-dried in natural conditions and kept in freezer at −20 °C. The diet formulation and analyzed chemical compositions were showed in Table 1. The amino acid composition of experimental diets was showed in Table 2.

2.2. Experimental fish, feeding and sampling

Amur Sturgeon were obtained from the chinese academy of fishery sciences sturgeon breeding technology engineering center (Fangshan, Beijing, China). All fish were acclimated in laboratory conditions and fed the control experimental diet for 4 weeks before the commencement of the trial. Fish (initial body weight = 87.49 ± 0.02 g) were selected and distributed into 256 L tanks after 24 h of starvation with 20 fish per tank and four tanks per treatment. The water temperature was maintained at 20 ± 2 °C, pH = 7.5–8.5, dissolved oxygen (DO) > 7.0 mg/L and total ammonia nitrogen levels < 0.5 mg/L. Aeration was supplied to each tank 24 h per day, fluorescent light was separately designed above the tanks and kept on from 8:00 to 22:00 for photoperiod of 13L: 11D. Fish were fed to apparent satiation three times a day at 9:00, 15:00 and 21:00 for 56 days. Feed intake was measured daily.

The fish from each tank were batch weighed at the end of the growth trial, randomly collected one fish per tank and kept in the freezer at −80 °C for whole-body composition analysis, including moisture, ash, crude protein and crude lipid. At the end of the growth trial, twelve fish were anesthetized with chlorobutanol (300 mg/mL) at random in each treatment (3 fish from each tank) after 24 h starvation. The body weight, body length, liver and viscera weight of the fish were recorded individually to calculate condition factor (CF), hepatosomatic index (HSI), and viscerosomatic index (VSI), respectively. Blood samples were drawn from the caudal part of the fish. Blood samples were centrifuged at 1000 rpm for 10 min at 4 °C to obtain serum. Two parts of SVI samples were collected, one fixed in 4% paraformaldehyde solution for histology and the other one was fast frozen in liquid nitrogen for RNA isolation. All samples (Except for histological samples) were stored at −80 °C until analysis.

Table 1
Formulation and compositions of experimental diets (g/kg).

Ingredients	P0	P50	P100
Fish meal[a]	540	270	0
Wheat gluten meal	180	200	180
Cottonseed protein concentrate[a]	0	180	408
Soy protein concentrate[a]	0	140	230
Fish oil[a]	30	45	55
Soybean oil[a]	20	30	40
Wheat middling	181	67	0
$Ca(H_2PO_4)_2$	5	14	22
L-Lysine monohydrochloride[b]	0	5	11
DL-Met[b]	0	3	6
L-Thr[b]	0	2	4
Soylecithin	20	20	20
Yeast extract	10	10	10
Vitamin and mineral premix[c]	14	14	14
Total	1000	1000	1000
Analyzed nutrients compositions (g/kg, in dry matter basis) and feed hardness			
Moisture	68.9	68.8	68.6
Crude ash	89.9	87.4	86.7
Crude protein	448	445	445
Crude lipid	114	118	120
Gross energy (MJ/kg)	20.2	20.1	20.2
Feed hardness (N)	10.3 ± 0.43[a]	13.9 ± 0.47[ab]	21.8 ± 0.77[b]

Mineral premix (mg/kg diet): $CuSO_4{\cdot}5H_2O$ 10; $FeSO_4{\cdot}H_2O$ 300; $ZnSO_4{\cdot}H_2O$ 200; $MnSO_4{\cdot}H_2O$ 80; KI (1%) 1.5; Na_2SeO_3 (10% Se) 10; $CoCl_2{\cdot}6H_2O$ (10% Co) 20; Zeolite 378.5.

[a] Fishmeal (crude protein content was 68.8%) and fish oil were supplied by Triple Nine Fish Product Co., Esbjerg, Denmark; CPC (crude protein content was 61.5%, low free gossypol and raffinose) was supplied by Sino-Leader Biotech Co. Ltd. Beijing, China; SPC (crude protein content was 65.2%) and soy oil were supplied by Yihai Kerry Investment Co. Ltd. Shandong, China.

[b] The addition levels of CAA (L-Lys-HCl, DL-Met and L-Thr) were calculated by the ideal AA model of white sturgeon [24].

[c] Vitamin premix (mg/kg diet): Vitamin A 20; Vitamin B_1 12; Vitamin B_2 10; Vitamin B_6 15; Vitamin B_{12} (1%) 8; Niacinamide 100; Ascorbyl calcium phosphate (35%) 1000; Calcium pantothenate 40; Biotin (2%) 2; Folic acid 10; Vitamin E (50%) 400; Vitamin K_3 20; Vitamin D_3 10; Inositol 200; Choline chloride (50%) 4000; Corn protein powder 150.

Table 2
Amino acid composition of experimental diets (g/kg crude protein).

Amino acid	P0	P50	P100	EAA requirement[a] [14]
Essential amino acid, EAA				
Arginine	59.4	77.1	97.1	47.7
Histidine	23.0	24.5	26.1	22.5
Isoleucine	43.1	40.2	35.5	29.9
Lysine	72.1	67.9	63.1	53.6
Leucine	72.5	68.1	61.3	42.7
Methionine	27.9	26.7	25.6	20.3
Phenylalanine	41.7	46.5	50.6	29.8
Threonine	40.4	40.4	40.2	32.8
Tryptophan	11.6	11.9	12.1	2.71
Valine	48.2	45.2	41.6	32.8
ΣEAA[b]	440	449	454	
Conditionally Essential Amino Acid, CEAA				
Cystine	10.3	12.4	14.4	
Glycine	53.6	46.1	38.7	
Proline	42.4	42.5	41.6	
ΣCEAA[c]	106	101	94.6	
Nonessential amino acid, NEAA				
Alanine	56.9	47.2	37.5	
Aspartic acid	90.2	92.4	93.0	
Glutamic acid	145	165	186	
Serine	40.8	42.7	44.0	
ΣNEAA[d]	333	348	360	

[a] The ideal dietary indispensable amino acid pattern for growth of white sturgeon.
[b] ΣEAA: sum of essential amino acids.
[c] ΣCEAA: sum of conditionally essential amino acid.
[d] ΣNEAA: sum of non-essential amino acids.

2.3. Chemical analysis

All chemical analyses were carried out in duplicate according to AOAC (2006). The dry matter was analyzed by drying the samples to constant weight at 105 °C. Crude protein was determined with a Kjeltec™ 2300 Unit (FOSS, Denmark) using the Kjeldahl method. Crude lipid was analyzed by acid hydrolysis with a Soxtex System HT 1047 Hydrolyzing Unit (Foss, Hillerød, Denmark), followed by Soxhlet extraction using a Soxtex System 1043 (Foss, Hillerød, Denmark). Ash was analyzed by combustion in a CWF 1100 muffle furnace (Carbolite, UK) at 550 °C for 6 h. The hardness of feed was determined by the Texture Analyzer (TA-XY2i, Stable Micro, England). The amino acid contents in the diets were analyzed in the lab of Evonik Degussa (Beijing, China) Co., Ltd.

2.4. Antioxidant parameters and immunoglobulin in serum and SVI

Serum total antioxidative capability (T-AOC), total superoxide dismutase (T-SOD), Malondialdehyde (MDA), Catalase (CAT), Anti-superoxide anion radical (Anti-$O_2^{\cdot-}$), Immunoglobulin M (IgM) and Immunoglobulin G (IgG) were determined by assay kits (Nanjing jiancheng Co., Nanjing, China) following the protocols given by the supplier. SVI homogenate parameter of reactive oxygen species (ROS) was determined by assay kit (Beyotime Biotechnology, Shanghai, China) following the protocols given by the supplier.

2.5. RNA isolation, reverse transcription and mRNA levels analysis

Total RNA was isolated from SVI using RNAiso Plus reagent (Takara, Japan), spectrophotometrically quantified using a NanoDrop 2000 (Thermo, USA) and electrophoresed on a 1% denaturing agarose gel to test the integrity. For each reverse transcription reaction, 1.0 μg of total RNA was first treated with gDNA Eraser to remove genomic DNA contaminants and then subjected to cDNA synthesis by reverse transcription in a 20 μL volume using the PrimeScript RT reagent Kit (Takara, Japan).

The core fragments of all the genes were obtained from the database of RNA-Seq. β-actin (GenBank accession no. AY649619), a housekeeping gene whose expression was found to be unaffected by the treatment in the present experiment, was used as an endogenous reference to normalize the template amount. The gene-specific primers used for mRNA quantification by RT-qPCR were showed in Table 3. Serial dilutions of cDNA generated from SVI tissues were used to make a standard curve to determine the amplification efficiency (E-values) of reference and target genes. The RT-qPCR analysis was performed using a CFX96TM Real-Time System (Bio-Rad, USA) in a 20 μL reaction volume containing iTaqTM Universal SYBR® Green Supermix (Bio-Rad, USA).

The RT-PCR temperature profile for all genes was 95 °C for 30 s followed by 40 cycles of 10 s at 95 °C, 30 s at Tm (Table 3) and 40 s at 72 °C. After the final cycle of PCR, the melting curves were systematically monitored (65 °C temperature gradient at 0.05 °C/10 s from 65 °C to 95 °C). During the detection, each sample was run in triplicate. PCR-grade water in place of the template served as the negative control. The $2^{-\Delta\Delta Ct}$ method was used to analyses RT-qPCR data [34]. The mRNA levels of target genes were shown as the n-fold difference relative to the calibrator.

2.6. Histopathological examination of the SVI

After 24 h of fixation, all SVI samples were dehydrated by the standard procedures, and the samples were embedded in paraffin and cut to 6 μm sections. Intestinal sections were stained following the protocols of hematoxylin and eosin (H & E) and Alcian blue staining for mucus and goblet cell mucin. The height of mucosal folds (HMF), the thickness of circular muscle (TCM) and the thickness of mucus (TM) were observed and quantified by LAS V4.0 of light microscopy (Leica DM2500, Leica, Germany). The terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL) assay was performed for tissue necrosis detection, the fluorescent signal was captured using a confocal microscope (Zeiss LSM700, Germany).

2.7. Western blot

SVI tissues were homogenized in RIPA buffer (Beyotime, China) with an added phosphatase inhibitor cocktail (Thermo Scientific™, USA). The protein concentration was measured using a BCA Protein Quantification Kit (Bio-Rad, USA). Protein extracts were run on TGX Stain-Free polyacrylamide gels (Bio-Rad, USA) and blotted onto polyvinylidene fluoride (PVDF) membranes (Millipore, USA). After blocking for 1 h at room temperature, immunoblots were incubated overnight at 4 °C in primary antibodies, including a loading control antibody, β-actin (Gene Tex Technology, USA), and target proteins including Microtubule Associated Protein 1 Light Chain 3 Beta (LC3B, Cell Signaling Technology, USA), Extracellular Signal-Regulated Kinase1 (ERK1, Gene Tex Technology, USA) and Phospho-ERK 1(p-ERK1, Cell Signaling Technology, USA). β-actin was incubated for 1 h in goat anti-mouse IgG-HRP secondary antibody (Santa Cruz Biotechnology, USA), others were incubated for 1 h in goat anti-rabbit IgG-HRP secondary antibody (Santa Cruz Biotechnology, USA). Proteins were detected using Clarity™ ECL Western Blotting Substrate (Bio-Rad, USA). Quantification was performed by Image J software (Rawak Software Inc. Germany).

2.8. Statistical analysis

All data were subjected to one-way analysis of variance (one-way ANOVA). Homogeneity of variance was confirmed by Levene's test before ANOVA and differences among the means were analyzed by Duncan's multiple-range test using SPSS Statistics 22.0 (SPSS Inc. USA). The differences of data from different groups were regarded as significant when $P < 0.05$, data were reported as mean value ± standard error of mean (S.E.M). The graphics were drawn using GraphPad Prism 6.0 (GraphPad Software Inc. USA).

Table 3
Primer sequences for real-time PCR.

Gene	Primers	Sequence 5′–3′	Target size (bp)	E-Values (%)	TM (°C)
β-actin	F*	CGGTTTCGCTGGAGATGATG	135	99.6	60.0
	R*	TCAGTGAGCAGGACGGGGTG			
caspase2	F	GGTGGGTTCCTTCAGTCAGA	152	90.3	58
	R	CACTGCTCTCTCTTGCGATG			
caspase3	F	GACATGCAGGTGGATGCCAA	80	90.2	60.0
	R	ATACACTGGCCGATGCTTGGA			
caspase6	F	AGCGTCGAGGTATCGCTTTA	206	91.7	57
	R	GTCTGCATGATTGGATGTGG			
caspase8	F	AAGAGTGCGTCCTTGGAACAGATG	120	93.2	60.0
	R	ACGCCTGAATGAAGAAGACCTTGG			
caspase9	F	CGGAAACGCCTCTTCCAAGT	178	97.8	60.0
	R	GCCCTTCCAGGCACTCAATAC			
caspase10	F	GCCAAACGTGTACAGATGGA	155	91.9	57.0
	R	CACCTGGGGCAATACAACTC			
TNF-α	F	AAAATGTCCAGCCATCAAAGCA	132	91.5	60.0
	R	GTGAGGAATGATCCCGTTGGT			
IL1-β	F	TGGTGGAGAAGAGAACGCAAGATG	114	91.3	60.0
	R	GCGTCACTGCCTGTCTCATGG			
TGF-β1	F	TGCTCCTGTGCTCGGCTCTC	80	90.6	60.0
	R	CAGCTCCATGTCCAGAGTCTTGC			
IL10-	F	GCAAAGCTGAGGGAGCTGAG	194	92.1	60.0
	R	CCCAGATTGACAGCTGGAGG			
Occludin	F	TCCGATCAGTGGAGACCAGCAG	120	95.6	60.0
	R	TCCAGCCGAGACAGGTCCTTG			
ZO1	F	AAGCACGGCCTCGTTATGAACC	182	90.2	60.0
	R	TGGAGGTGGTGGCAGAGCAG			
ZO2	F	TCTCACTCCAGCAGCCAGAGC	189	91.5	62.0
	R	CACTGCCGGATCATGGACACTG			
ZO3	F	GGAGGAGGATCTCGACGCTCTG	100	94.2	62.0
	R	CGCCTCTCCGTCCGTGTCC			
Claudin2	F	TGTCTGCCTTGCTCTCCTTT	165	91.6	57
	R	CTTTGCTCTGGGGATGGTTA			
Claudin3	F	TAGGCAGCATTGTGGCTTGCAC	161	92.0	60.0
	R	AGCCAGCATGGAGTCGTAGACC			
Claudin4	F	GGCAGCAACATTGTGACATC	228	90.3	57
	R	GGATTCGTCCTCCACACAGT			
Claudin12	F	GCTTGCCGCAACATACATGCAG	183	95.1	60.0
	R	GTCCAGTCGAACGCACTTCACC			
Claudin15	F	TGGTCGCCATCTCCTGGTACG	154	90.9	60.0
	R	CAGGAGCAGCACAGACAGCAC			
Keap1	F	CCAGACCGGATAACGAGCTGAATC	101	91.3	60.0
	R	GCCTGGCTTGTGAGACTCTGTATC			
Nrf2	F	TGGAAGTGTCCAGCCTGAGT	138	94.2	60.0
	R	GTCGGCAGTCCCTTCTTTGG			
MafG	F	ACAGGCGCCAGGTCTTAGTA	155	90.8	58.0
	R	GAACTCAACCACTGCCACAA			
MafK	F	AAATCAGCCGACCACAAGAC	161	93.0	58.0
	R	ACTGGATGGACTGGCAAGAG			
CuZn/SOD	F	ACTCCTGGAGAGCATGGCTT	83	98.5	60.0
	R	GGGTTGAAGTGAGGACCAGC			
CAT	F	TGGACACCGACACATGAATGGC	190	97.9	60.0
	R	GGCGATGGCGTTGTACAGGTC			
GST1	F	ACACCGTGGCCTACCTGCTG	105	96.3	60.0
	R	TGGTGCTCAGGAGCCGATACC			
GST2	F	GCTACCAGTCCTGCTTGCTG	95	91.0	62.0
	R	CGCTGTGCTTCATCCTAGACA			
GST3	F	TGGCTACGTGGTGCTGACTGG	163	92.0	60.0
	R	CTGGTGCGCTCGCTGAATGC			
PCNA	F	ATGGTCAGCCAGGAAACAAG	212	91.4	60.0
	R	CTGGCCTGCAATCAACTTTT			
CyclinB1	F	GAAATTCCAGCTGCTTCAGG	203	91.6	58.0
	R	TCTGCAGTAGGGGTTCCATC			
CyclinD1	F	CATTGCTTAAAGCGGAGGAG	159	90.3	57.0
	R	TCATAGCCAATGGGAAAACC			
CyclinE	F	TAAAGCCTTCCCCACTACCA	158	90.0	57.0
	R	GAGCCAGTCGAGAAGAATCG			
ATG5	F	TTTCTGCTGCGGTAACTCCT	150	96.7	60.0
	R	GCATGGGAATGACACTGATG			
ATG7	F	GACCGTACTTTGGACCAGCA	112	95.0	58
	R	AACCTCCCTCTGAATGCTGC			
ATG16	F	ACCCTGAAGGACGAGTACGA	164	93.9	58
	R	CACCTGCAGTCCTTCTCGTT			

F: forward primer; R: reverse primer.

2.9. Key resources table

Resource	Source	Identifier
Antibodies		
goat anti-mouse IgG-HRP secondary		
goat anti-rabbit IgG-HRP secondary		
ß-actin		
Chemical		
Alcian blue		
amino acids		
Carbolite		
chlorobutanol		
eosin		
fluoride		
hematoxylin		
L-Thr		
Malondialdehyde		
monocalcium phosphate		
phosphorus		
SOD		
ProteinPeptide		
Extracellular Signal-Regulated Kinase1(ERK1)		
Microtubule Associated Protein 1 Light Chain 3 Beta (LC3B)		
Phospho-ERK1		
ß-actin		

3. Results

3.1. Growth performance, morphometric parameters and whole-body composition

The results of growth performance and morphometric parameters were presented in Table 4. SR significantly decreased in P100 group ($P < 0.05$). FBW, CF and VSI were no significant differences among the three groups ($P > 0.05$). SGR, WGR and PPV of P0 and P50 groups significantly higher than P100 group ($P < 0.05$). FCR of P0 and P50 groups significantly lower than P100 group ($P < 0.05$). HSI of P0 group significantly higher than P50 and P100 ($P < 0.05$). FR of P0 group significantly higher than P50 group and significantly lower than P100 group ($P < 0.05$).

There were no significant differences in whole-body moisture, crude protein, crude lipid and ash among three groups (Table 5).

Table 4
Effects of dietary plant protein replace fishmeal on the growth performance and morphometric parameters in Amur Sturgeon (means ± SEM).

	P0	P50	P100
Growth performance			
SR %[b]	100 ± 0.00[b]	98.8 ± 1.25[b]	78.8 ± 5.15[a]
FBW	228 ± 5.29	223 ± 4.35	210 ± 6.67
SGR %/d[c]	1.71 ± 0.04[b]	1.67 ± 0.04[b]	1.53 ± 0.04[a]
WGR[d]	160 ± 5.98[b]	152 ± 4.22[b]	87.8 ± 7.75[a]
FR % bw/d[e]	1.52 ± 0.03[a]	1.42 ± 0.01[a]	1.78 ± 0.05[b]
FCR[f]	0.96 ± 0.02[a]	0.92 ± 0.02[a]	1.68 ± 0.15[b]
PPV (%)[g]	30.3 ± 2.12[b]	27.2 ± 1.21[b]	17.7 ± 1.85[a]
Morphometric parameters			
CF[h]	0.72 ± 0.01	0.75 ± 0.02	0.73 ± 0.02
HSI[i]	2.35 ± 0.06[b]	2.09 ± 0.02[a]	2.04 ± 0.11[a]
VSI[j]	7.47 ± 0.32	7.07 ± 0.33	7.21 ± 0.04

[a] Values (mean ± SEM) in the same row with different superscript letters are significantly differences ($P < 0.05$).
[b] SR (survival rate, %) = 100 × final fish number/initial fish number.
[c] SGR (specific growth rate, %/d) = = 100 × [Ln (FBW) - Ln (IBW)]/days. FBW is final body weight, IBW is initial body weight.
[d] WGR(weight gain rate, %) = 100 × (Wf - Wi)/Wi.
[e] FR (feeding rate, %) = 100 × feed intake/[(Wf + Wi)/2]/days. Wf is the final total weight, Wi is the initial total weight. The same below.
[f] FCR (feed conversion ratio) = feed intake/(Wf - Wi).
[g] PPV (productive protein value, %) = 100 × (final total weight × terminal fish protein content-initial total weight × initial fish protein content)/(total food intake × feed protein content).
[h] CF (condition factor) = 100 × (body weight, g)/(body length, cm)^3.
[i] HSI (hepatosomatic index, %) = 100 × liver weight/whole body weight.
[j] VSI (viscerasomatic index, %) = 100 × viscera weight/whole body weight.

Table 5
Effects of dietary plant protein replace fishmeal on whole-body composition in Amur Sturgeon (means ± SEM).

	P0	P50	P100
Moisture (%)	77.1 ± 0.70	75.7 ± 1.13	76.7 ± 0.18
Crude protein (%)	13.3 ± 0.59	12.2 ± 0.16	12.5 ± 0.49
Crude lipid (%)	6.23 ± 0.22	6.57 ± 0.18	5.98 ± 0.35
Ash (%)	3.04 ± 0.06	2.95 ± 0.11	2.91 ± 0.11

[a] Values (mean ± SEM) in the same row without different superscript letters have no significant differences ($P > 0.05$).

Table 6
Effects of dietary plant protein replace fishmeal on antioxidant parameters of Amur Sturgeon (means ± SEM).

	P0	P50	P100
MDA (nmol/ml)	5.77 ± 0.19	5.93 ± 0.56	5.13 ± 0.43
T-AOC (U/ml)	25.4 ± 5.90	21.5 ± 4.45	21.7 ± 5.87
T-SOD (U/ml)	141 ± 12.0	163 ± 9.91	163 ± 9.59
CAT (U/gprot)	77.5 ± 21.2	83.0 ± 33.3	80.1 ± 33.0
Anti-$O_2^{\cdot-}$ (U/gprot)	177 ± 12.0	187 ± 17.4	201 ± 17.9

[a] Values (mean ± SEM) in the same row with different superscript letters are significantly differences ($P < 0.05$).

3.2. ROS content and antioxidant capacity in serum and SVI

The ROS content and antioxidant response in serum and SVI were presented in Table 6 and Fig. 1. Serum MDA, T-AOC, T-SOD, CAT, Anti-O2$^{\cdot-}$ were no differences among groups (Table 6). However, with the increase of dietary PP blend, SVI ROS levels were increased, and the SVI ROS in P100 group was significantly higher than that in P0 group (Fig. 1A, $P < 0.05$). However, compared with the P0 group, the mRNA levels of Keap1, MafK, Cu/Zn-SOD, CAT, GST1 GST3 were significantly downregulated ($P < 0.05$), and Nrf2 mRNA levels were significantly up-regulated (Fig. 1B, $P < 0.05$).

3.3. SVI pathological examination-histology, tight junction

Fish SVI sections were examined after H & E staining and Alcian blue staining for mucus and goblet cell mucin. Twelve samples were observed in each group. Three typical phenotypes were shown in Fig. 2A. The quantification of the height of mucosal fold (HMF, marked with yellow double-headed arrow), the thickness of circular muscle (TCM, marked with green double-headed arrow), and thickness of mucus (TM, marked with purple double-headed arrow) for these three phenotypes were shown in Fig. 2B. Phenotype I showed well-shaped SVI with lowest TCM, which indicated the tight structure of the circular muscle, higher HMF, thicker TM and well adhesion of mucus on intestinal villi, and general integrated epithelial structure. Phenotype II defined mild lesion of SVI with slightly loose circular muscle layer (marked with black arrow), lower HMF, thinner TM and partial detachment of mucus from intestinal villi (marked with red arrow), and partially damaged epithelial tissue (marked with blue arrow). Phenotype III defined severe lesion of SVI with an increased TCM, which indicated a larger scale damaged circular muscle layer (marked with black arrow), lower HMF, thinner TM and much more detached mucus from intestinal villi (marked with red arrow), and more severely damaged epithelial tissue (marked with blue arrow). In the P0 group, ten samples showed normal morphological structure, and only two samples exhibited mild lesions. In the P50 group,

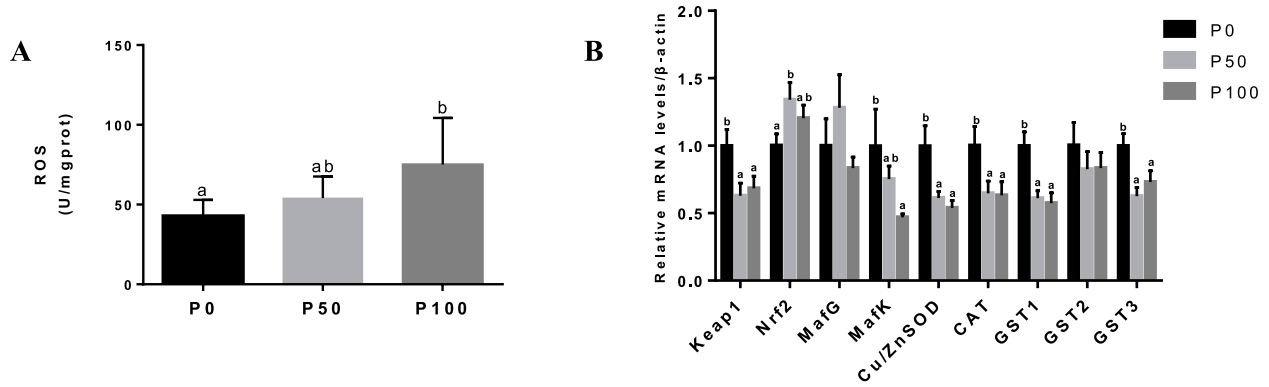
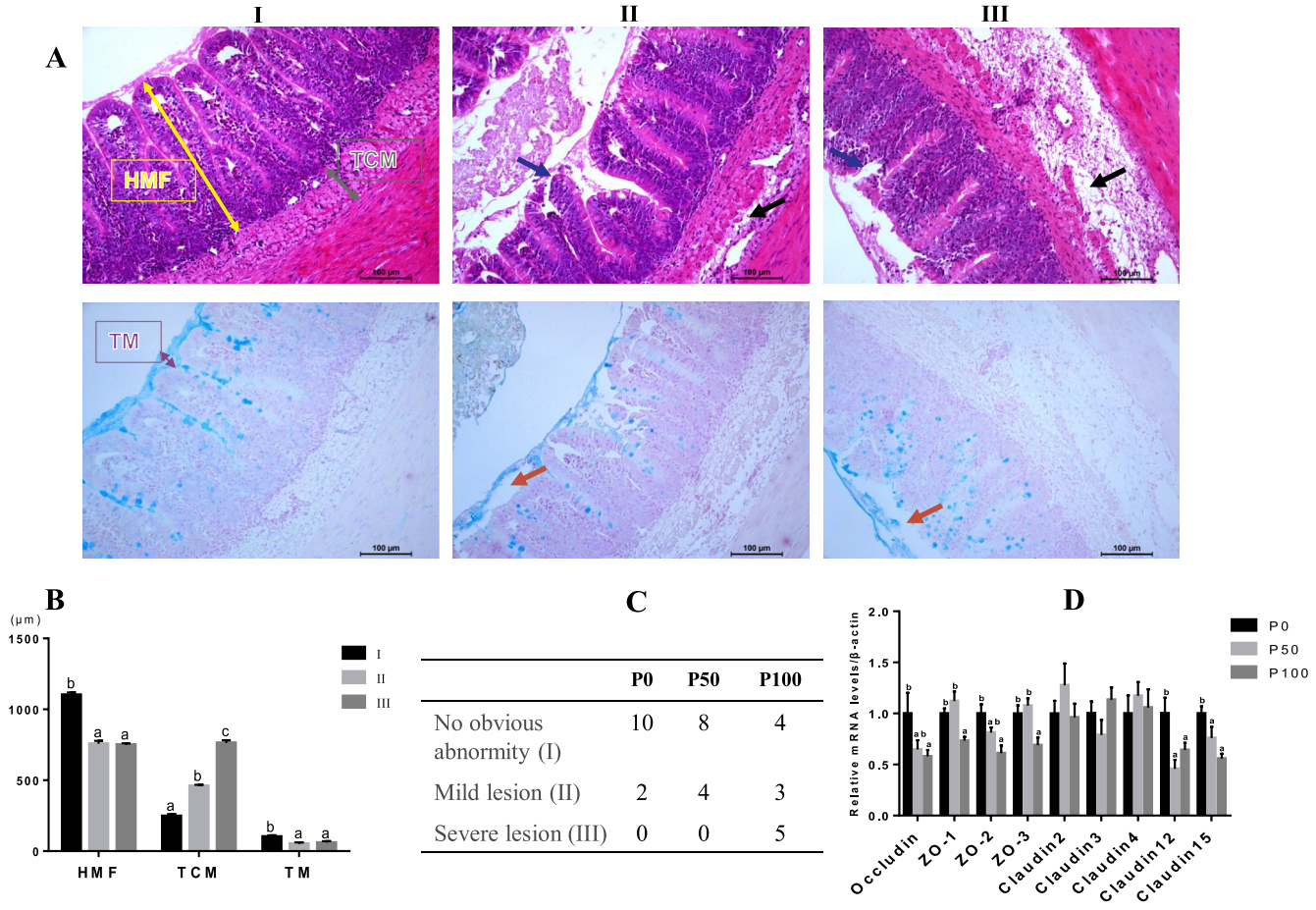


Fig. 1. Oxidation stress was shown in SVI of Amur sturgeon with symptom of increased ROS level but inhibited antioxidant response when FM was replaced by PP (Values having different letters are significantly different, $P < 0.05$, mean ± SEM, n = 8). (A): ROS levels in SVI were significantly increased in P100 group. (B): Dietary plant protein reduced expression of genes related to the antioxidant response in SVI of Amur sturgeon.

eight samples showed normal morphological structure, four samples exhibited mild lesions. In the P100, only four samples showed normal morphological structure, three samples exhibited mild lesions and five samples exhibited severe lesions (Fig. 2C).

The mRNA levels of tight junction (TJ) in SVI were presented in Fig. 2D. Occludin, ZO-2, Claudin12 and Claudin15 genes expression in both PP inclusion groups were significantly down-regulated ($P < 0.05$). ZO-1 and ZO-3 mRNA level in P100 group was significantly decreased, compared to those of P0 and P50 groups ($P < 0.05$).

The cell necrosis signal determined by TUENL assay (red color signal) of well-shaped and severe lesion SVI were shown in Fig. 3. Little TUNEL signals were shown both in villi and circular muscle layer of samples with no obvious abnormity (Fig. 3A), but highly expressed TUNEL signal (as marked by white "□") in the severe lesion samples



	P0	P50	P100
No obvious abnormity (I)	10	8	4
Mild lesion (II)	2	4	3
Severe lesion (III)	0	0	5

Fig. 2. Dietary plant protein induced SVI histological lesions and non-integrated tight junctions (Values having different letters are significantly different, $P < 0.05$). (A): H&E and Alcian blue staining of SVI sections with bar = 100 μm, in which epithelial structural integrity (marked with blue arrows), circular muscle layer (marked with black arrows) were damaged to varying degrees, and detached mucus from intestinal villi (marked with red arrow) were also observed in damaged SVI tissues (mean ± SEM, n = 12). (B): Quantification of height of mucosal fold (HMF) and thickness of circular muscle (TCM) and mucus (TM), respectively. Lower HMF, thinner TCM and TM were observed in with the more severe lesion of pathological SVI (mean ± SEM, n = 12). . (C): Phenotype of SVI histopathological examination in fish fed various diets (mean ± SEM, n = 12). (D): Dietary plant protein reduced SVI mRNA levels of tight junction genes in Amur sturgeon. (mean ± SEM, n = 8). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

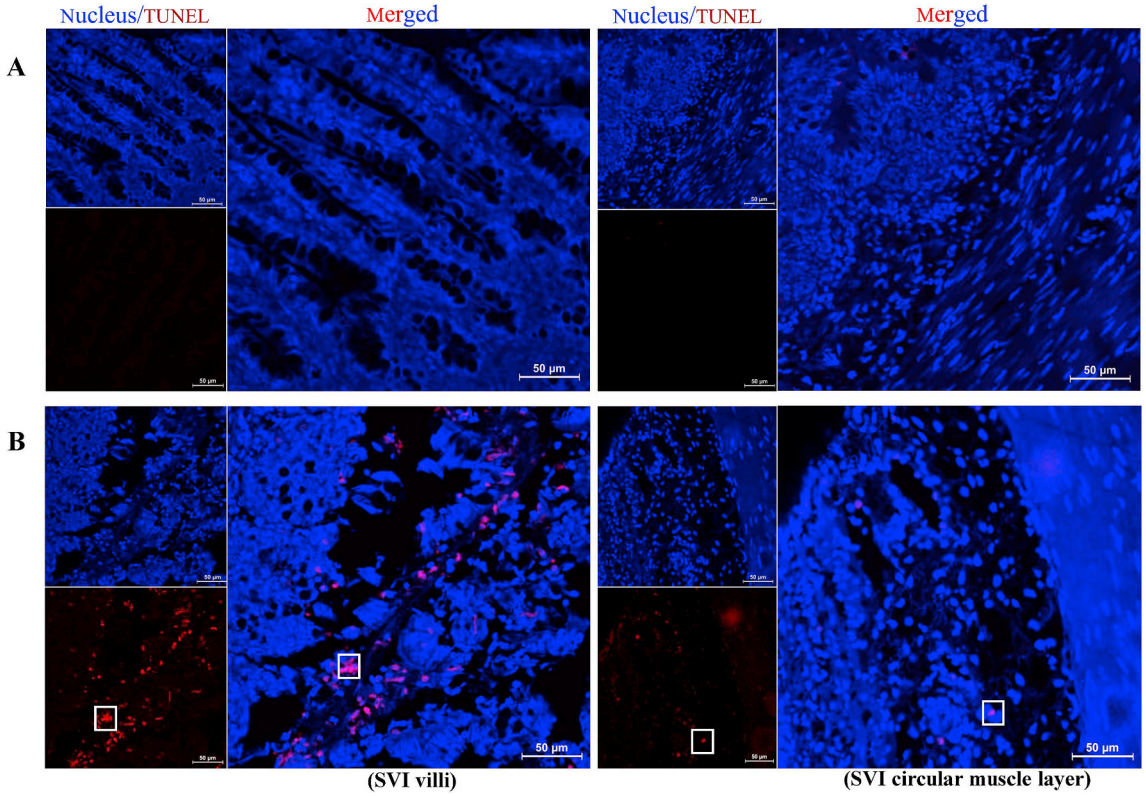


Fig. 3. Plant protein diet induced severe tissue necrosis (the TUNEL signal in red color and DAPI for nucleus) in SVI of Amur sturgeon. (A): A little TUNEL signals were shown both in villi and circular muscle layer of samples with no obvious abnormity. (B): Highly expressed TUNEL signal (as marked by white "□") in the severe lesion samples. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

were observed (Fig. 3B). The plant protein diet induced severe tissue necrosis in SVI of Amur sturgeon.

3.4. Serum immunoglobulins, inflammatory cytokines and apoptosis in SVI

Serum IgG and IgM, inflammatory cytokines and apoptosis in SVI were presented in Fig. 4. With the increase of dietary PP levels, IgG in serum were decreased, and IgG in P100 group was significantly lower than that in P0 group ($P < 0.05$), IgM have no difference among groups (Fig. 4A). Compared with the P0 group, the pro-inflammatory cytokine, IL1β and the anti-inflammatory cytokine, IL10 mRNA levels were significantly down-regulated in PP inclusion groups (Fig. 4B, $P < 0.05$). The TNFα and TGFβ genes expression were not regulated. For the apoptosis related genes, the mRNA levels of caspase1, caspase3, caspase8 and caspase9 in PP inclusion groups were also significantly down-regulated (Fig. 4C, $P < 0.05$).

3.5. Autophagy and proliferation responses in SVI

The mRNA levels of autophagic related genes in SVI were showed in Fig. 5A. ATG7 and ATG16 mRNA levels were decreased in PP inclusion groups. The protein expression of LC3B was showed in Fig. 5B. With increasing dietary PP, LC3BI had no significant difference at the protein level in each group, but LC3BII significantly lower in turn ($P < 0.05$), and accordingly LC3BII/I gradually decreased in PP inclusion groups ($P < 0.05$). The mRNA levels of proliferation related genes in SVI were showed in Fig. 5C. There was no difference on mRNA levels of PCNA and CyclinB1 among groups, but CyclinD1 and CyclinE mRNA levels in PP inclusion groups were significantly lower than those in P0 group ($P < 0.05$).

3.6. ERK1 signal activation in SVI

The ERK1 protein, phosphorylation ERK1 (p-ERK1) levels and the ratio of p-ERK1/ERK1 were presented in Fig. 6. The ERK1 protein level

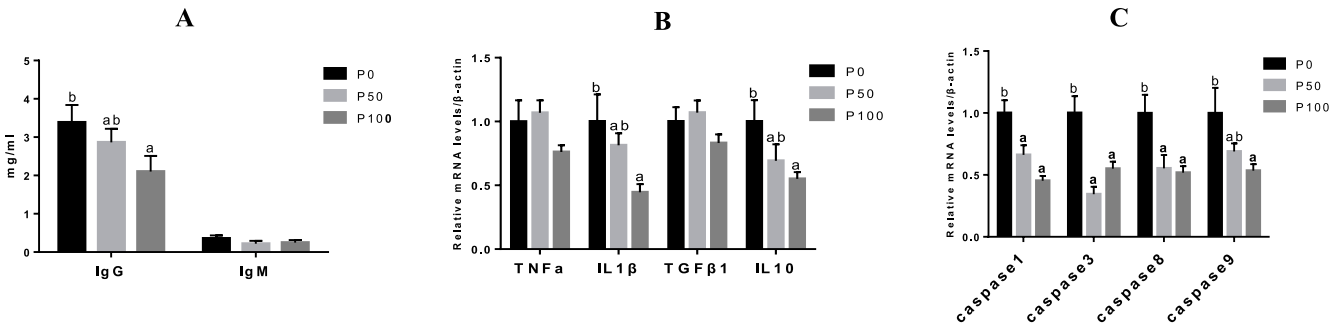


Fig. 4. Inhibited immunity, inflammatory and programming apoptosis in SVI of fish when dietary FM was partially or fully replaced by PP (Values having different letters are significantly different, $P < 0.05$, mean ± SEM, n = 8). (A): Significantly reduced serum IgG in P100 group. (B): Significantly downregulated gene expression of inflammatory cytokines, IL1β and IL10 in SVI of P100 group. (C): Downregulated mRNA levels of caspase family in P50 and P100 groups.

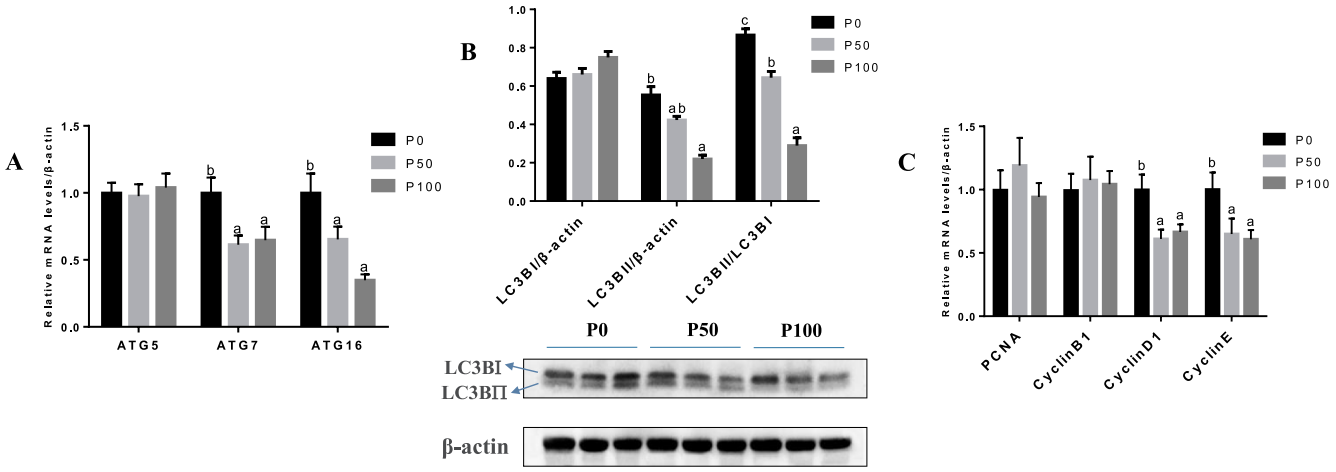


Fig. 5. Inhibited cell autophagy and proliferation in SVI of fish when dietary FM was partially or fully replaced by PP (Values having different letters are significantly different, $P < 0.05$, mean ± SEM, n = 8). (A): Dietary plant protein reduced mRNA levels of autophagy genes ATG7 and ATG16. (mean ± SEM, n = 8). (B): Gradually reduced protein expression ratio of LC3BII/I with higher PP levels in diets. (mean ± SEM, n = 3). (C): The cell proliferation genes expression were downregulated in PP groups.

of SVI has no significant difference in each group, p-ERK1 protein level and the ratio of p-ERK1/ERK1 significantly decreased in P100 group ($P < 0.05$).

4. Discussion

Replacement of dietary fish meal with PP usually reduce the FR in carnivorous teleosts, such as Japanese seabass [7], turbot [35], Japanese flounder [36], In the present study, although the FR of Amur sturgeon was not affected with partial or fully PP diet, it also can not digest and utilize full PP diet well like a similar species, Siberian sturgeon [13]. Xu et al. (2012) [16] found that replacement of more than 58% FM by soy protein isolate (dietary FM content was at least 17%) would negatively affect the growth and intestinal pepsin, trypsin and amylase activity for juvenile Amur sturgeon. In this study, we reached a similar conclusion that the growth performance of the P50 group was not significantly affected, in which 27% of FM were retained. The relatively inefficient nutrient absorption and low utilization in PP compared with FM usually affect the normal growth of fish and induce various pathological reactions after high level replacing dietary FM, especially prone to intestinal diseases [37,38]. Sturgeon has a SVI that is quite different in epithelial arrangement from that of most of the teleost. A spiral valve inside the intestine could compensate for the deficiency of the short intestine of sturgeon to increase the absorbent surface and keep digestible material in the ileum for an extended nutrient absorption period [39]. The lumenal exposed epithelium is dominated by a single layer of goblet cells with a few ciliated cells, whereas the epithelium of the deeper, unexposed area consists primarily of columnar cells with only a few, scattered goblet cells [32,33]. Results from the present study showed that the growth performance of the P100 group was significantly reduced, accompanying severe SVI lesions, which further induced the poor nutrient absorption. Although CPC and SPC are high quality protein sources with relatively high digestibility and essential nutrients had been supplemented, the higher pellet hardness retarded the gastric emptying [40], the leaching loss and asynchronous absorption of CAAs in PP diet [8,41], In addition, a special consequence of the spiral valve constricting the lumen of the intestine is that sturgeon cannot pass large hard objects through their lower intestine easily. The lower slaking rate with high hardness diet could damage the integrated of SVI morphological structure [42].

Reactive oxygen species (ROS)are formed as a natural byproduct of the metabolism of oxygen and act important roles in cell signaling and homeostasis [43]. Under normal circumstances, the production and elimination of ROS maintain a dynamic balance in the SVI cells.

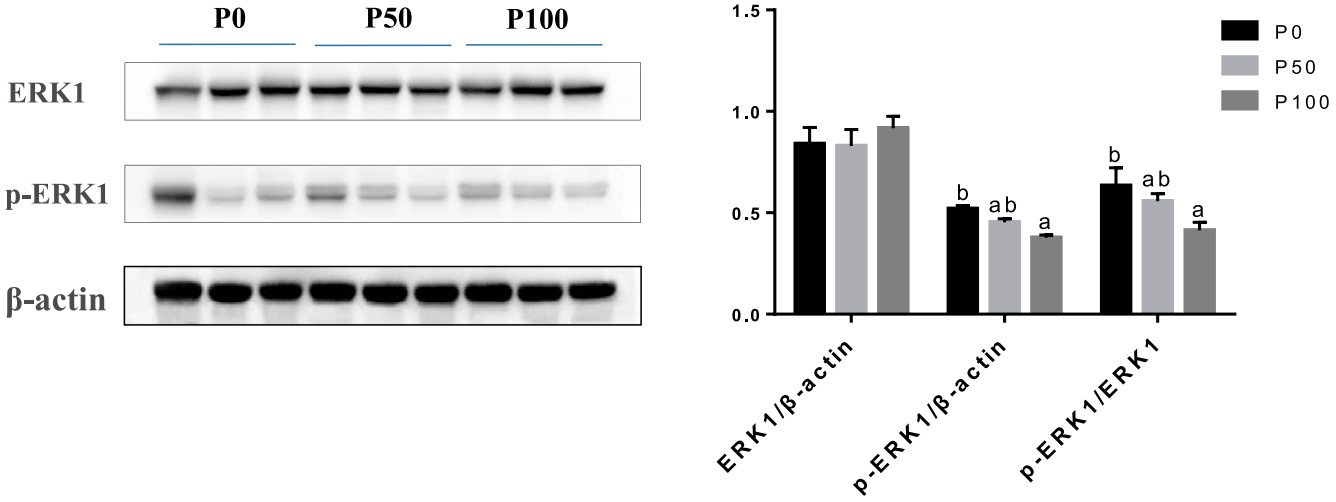


Fig. 6. ERK1 protein phosphorylation was significantly inhibited in SVI of P100 group. (Values having different letters are significantly different, $P < 0.05$, mean ± SEM, n = 3).

However, once the environmental stress occurs, ROS levels can increase dramatically and surpass the removal capacity of the antioxidant system may result in significant damage to cell structures [44]. Keap1-Nrf2-Mafs-ARE is one of the most important antioxidant signaling transduction pathway in animal [45], in which Keap1 is a repressor protein that binds to Nrf2 and promotes its degradation in cytoplasm, the key signaling protein within the pathway is Nrf2 that can translocate into the nucleus and forms a heterodimer with small Maf proteins (MafG and MafK) to bind together with the antioxidant response element (ARE) to promote downstream antioxidant genes transcription [46,47]. Whereas small Maf proteins themselves can form homodimer as a suppressor of downstream antioxidant genes transcription, and in some human cancer, Nrf2 is over-ubiquitinated by KEAP1 and NRF2 genes missense mutations into the nucleus, resulting in elevated expression of Nrf2 target genes, therefore, both Nrf2 and small Mafs family have double-edged sword effects under different situations [46–49]. In the present study, although the downregulated Keap1 and upregulated Nrf2 mRNA levels well responded to the increased ROS in PP groups, the failed positively responded MafK gene expression resulted in the decreased expression of downstream antioxidant enzyme genes, including Cu/ZnSOD, CAT and GSTs. The clear ROS stress was observed in SVI of PP100 group with high ROS level and poor antioxidant capability. Besides, these antioxidant enzyme activities are closely related to enough trace mineral efficiency [50,51]. When dietary FM was replaced by PP, the introduction of phytic acid, a strong cation-chelator could inhibit the bioavailability of minerals to animals [52], which suggests that consideration should be given to increasing the amount of trace elements or using organic chelated minerals when PP substituting FM in fish diet [53,54].

Epithelial TJ of the intestine is the most important component of physical barrier, which is multi-protein complex made up of transmembrane proteins (Occludin and Claudins), scaffolding proteins (ZOs) and regulatory molecules, etc [55]. Oxidative stress can induce TJ proteins expression changes and further negatively affect the TJ structure and function [56]. The present results of TJ related gene expression in SVI of Amur sturgeon were consistent with those of turbot and Atlantic salmon, in which high level of dietary PP can cause TJ destruction in the distal intestine [57,58]. The mRNA levels of Occludin, ZO2, Claudin12 and Claudin15 were decreased in all PP inclusion groups, and ZOs (ZO1, ZO2 and ZO3) were also significantly downregulated in SVI of P100 group, which suggested that 50%–100% of dietary PP levels disrupted the SVI TJ integrate of the Amur sturgeon. In addition to the physical barrier, mucus is one of the important mucosal barriers [59], and it is also easily damaged by oxidative stress [60]. The intestinal mucus is mainly composed of mucin and locates on top of the epithelial cells, it is the first line of defense for intestine and essential for maintaining intestinal barrier function [61]. We observed that the thickness and integrity of the mucus were negatively affected in diseased SVI. Both physical and mucosal barriers serve as indispensable defense against antigens and harmful substances, and once destroyed, further promoted oxidative stress.

Destruction of the intestinal mucosal barrier and oxidative stress usually lead to high intestinal inflammation, apoptosis and immunity response in fish [62,63], however, once pathological stimulation triggers tissue necrosis, various cellular activities will be affected [64]. Immunity includes humoral and cellular immunity. Immunoglobulins are important humoral immune antibodies [65], and for most teleosts, IgM is the major immunoglobulin in plasma, little with IgG [66,67]. Interestingly, for Amur sturgeon, serum IgG content is much higher than that of IgM, and sensitively responded to the nutrient abundance and SVI lesion in the present study. This could be related to the evolutionary status of Acipenseriformes, which has much higher identity with mammals, but not with teleosts [68,69]. Cellular immunity is largely dependent on the balance of pro-inflammatory cytokines and anti-inflammatory cytokines [58,70]. IL1β is a potent proinflammatory cytokine, which induces T-cell and B-cell activation and cytokine and antibody production [71], and can be blocked by Nrf2 binding to the pro-inflammatory genes [72], and rely on capase1 activation to split pro-IL1β into an active form [73,74]. On the contrary, as one of the most important anti-inflammatory cytokine, IL10 acts on many cells to limit excessive tissue disruption caused by inflammation [75]. Inflammatory cytokines are major roles to active cell apoptosis, which is an active programmed cell death process for the body to adapt to the environment, self-protection, and also release anti-inflammatory factors [76,77]. Caspase-3 is the most important key executioner in both extrinsic and intrinsic apoptosis processes (mitochondrial apoptosis) by activating promoter-type caspase, such as caspase 8 and caspase 9, respectively [78]. In the present study, both immunity and apoptosis responses were inhibited, but clearly increased necrosis signals and high mortality were observed in SVI tissues of P100 group. The inflammation and immune response in animal are regulated by a dynamic process, Wu et al. (2018) found that acute intestinal inflammation was found in the early stage (0–5 weeks) of feeding grass carp with 40% and 70% PP diet, and it trended to be relieved via both enhancing an immune activity and wound healing (3–5weeks) in 40% PP group. The grass carp showed upregulated pro-inflammatory cytokines (IL7 and IL34) and anti-inflammatory cytokines expression (IL4 and IL21), whereas all down-regulated during the healing stage [79]. Besides, Grenier et al. (2011) and Pierron et al. (2016) reported that the proinflammatory and anti-inflammatory factors of cells were down-regulated under the stimulation of certain oncogenic toxins [80,81]. Similarly, we found that Amur sturgeon was not tolerant to full PP diet, severe villous necrosis and clearly hypoimmunity status with failed antioxidant, inflammation and apoptosis response were observed in SVI of fish fed P100 diet, which could be more serious status than those with up-regulated inflammatory and programming apoptosis responses [82,83].

In addition to apoptosis, autophagy also is one of the mechanisms of programmed cell death [84], and also tends to be inhibited in necrotic tissue [85]. Autophagy is not only the major intracellular degradation system for resisting cell damage by removing unnecessary or dysfunctional components, but also maintains organism homeostasis by regulating nutrient sensing, xenophagy, infection, immunity and repair mechanism [86]. Furthermore, autophagy can provoke caspase-1 activation to split pro-IL1β into IL1β [87], and IL1β further mediates cyclin to regulate proliferation [88]. The hallmark feature in the process of autophagosome formation is that microtubule-associated protein 1 light chain 3(LC3-I) transform into LC3-II binding with autophagosome membrane through near the C-terminus site-specific proteolysis and lipidation. ATG5, ATG7 and ATG16 are essential proteins involved in this process [89,90]. LC3B is the most widely studied protein of the LC3 family (LC3A, LC3B, LC3C), and the protein expression ratio of LC3B-II/I is commonly used to determine the degree of autophagy [91,92]. CyclinD1 is a key protein that determines whether cells enter the mitosis, CyclinE and CyclinB1 are important promoter of G1/S and G2/M transition in the cell cycle, respectively [93]. In this research, we observed that ATG7, ATG16, CyclinD1 and CyclinE mRNAs were down-regulated, and the protein expression ratio of LC3B-II/I decreased in PP inclusion groups, it illustrated that PP diet inhibited autophagy and further negatively affected the immune and apoptosis responses and further inhibited the repairing ability in SVI.

MAPK superfamily regulates a vast array of physiological processes, p38MAPK, ERK and JNK are three cascades of MAPK, whereas their respective main functions have certain differences, p38MAPK activation primarily regulates inflammation-related factors, JNK mainly conducts signal transduction for various cellular stresses, and ERK participants the regulation on growth factors, cell proliferation and nutrient-related receptors [94]. ERK1 is a subfamily of the ERK family that can be phosphorylated and activated to transform into p-ERK1 to mediate anti-oxidation, apoptosis, autophagy, and further modulate immune response [26–28], among them, ERK and autophagy are also important for cell proliferation and tissue repair [29,30]. Moreover, p-ERK1 interacts with the TJ protein complex by binding to the C-terminal tail of Occludin [95]. Besides, ERK1 plays an extremely important role in fish growth and development [25]. Though phosphorylation activation of ERK has been documented as a double-edged signal

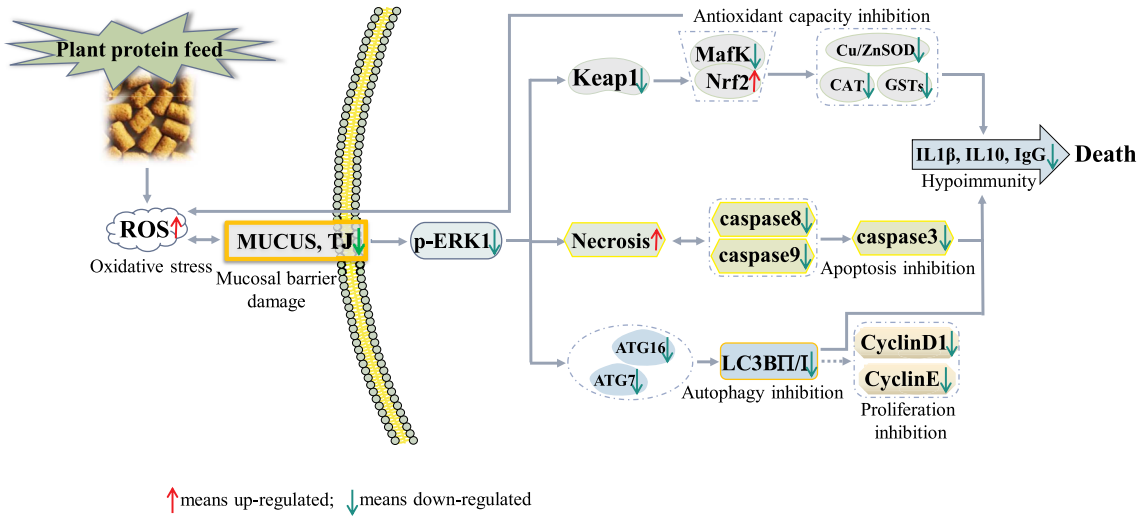


Fig. 7. The mechanism of full plant protein diet induces severe SVI damage in Amur sturgeon. Plant protein diets induced severe oxidative stress and mucosal barrier damage in SVI of Amur sturgeon. The increased tissue necrosis and failed responses in anti-oxidation, programming apoptosis, autophagy and cell proliferation system were regulated by inhibiting ERK1 phosphorylation, which indicated that SVI hypoimmunity and functional degradation are the main reasons for the high mortality and low utilization ability of plant protein in Amur sturgeon.

transducer for immunity under various contexts [96–98], in the present study, phosphorylation of ERK1 protein in SVI were significantly decreased by full PP diet, which demonstrated a pathological role of inhibition of ERK1 phosphorylation in hypoimmunity through its diverse suppression on anti-oxidation, apoptosis and autophagy. The excessive necrosis and insufficient autophagy and cell proliferation induced the high motility and atrophy phenotype in the SVI of Amur sturgeon when fed full PP diet [99].

In conclusion, the replacement of 50% FM with PP blend had no significant effect on the growth performance of Amur sturgeon. Complete replacement of dietary FM with PP blend significantly reduced the growth performance and survival of Amur sturgeon. In general, as summerirzed in Fig. 7, the plant protein diets induced severe oxidative stress and mucosal barrier damage in SVI of Amur sturgeon. The failed responses in anti-oxidation, programming apoptosis, autophagy and cell proliferation system and increased tissue necrosis indicated that SVI hypoimmunity and functional degradation are the main reasons for the high mortality and low utilization ability of plant protein in Amur sturgeon (Fig. 7).

Acknowledgments

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