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Perspective

Stress responses of testicular development, inflammatory and apoptotic activities in male zebrafish (*Danio rerio*) under starvation

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ABSTRACT

Food deprivation is a severe stress across multiple fields and challenged to organismal development and immune system. Here, adult male zebrafish were used to investigate the starvation stress on organismal development, spermatogenesis, testicular inflammation and apoptosis. Results showed that the biological indexes, blood parameters, and RNA/DNA ratio in testis dramatically decreased after 1–3 weeks of starvation. The testicular architecture was impaired and the spermatogenesis was retarded with increased proportions of spermatogonia and spermatocytes, and decreased proportion of spermatozoa in the starved fish. The mRNA expressions of *amh* and *sycp3* were downregulated, the retinoic acid content increased at later stage of starvation through the transcriptional regulation of *aldh1a2* and *cyp26a1*. Besides, the immune response was elevated with upregulated mRNA and protein expressions of TNF-α, IL-6, and IL-1β, which indicated the inflammation of opportunistic risk in testis. The apoptotic activity was stimulated, accompanied by differentially upregulated expressions of *baxa*, *casp9*, *casp3*, *casp2*, and decreased ratio of Bcl-2/Bax in the attenuate testis. Taken together, our findings revealed that the stress responses of testicular development, inflammatory and apoptotic activities in male zebrafish under starvation and pointed out the susceptibility of fish gonad to food fluctuation.

1. Introduction

Food fluctuation is a common and severe stress in aquaculture due to the fluctuations in natural activities of fish reproduction, migration, as well as rearing habits of feeding rhythmicity and nutrient intake (McCue, 2010; Secor and Carey, 2016). Besides, some fish species also cope with the feeding adaptations of short-/long-term fasting when submitted to the environmental challenges (Volkoff et al., 2009). When the circumstance deviates from the optimum to starvation, fish respond with physiological and behavioral modulations to ameliorate the adverse effects (Fan et al., 2019a). And the individual fitness would be inevitably weaken if the stress response is costly or the organismal compensation is imperfect (Schulte, 2014). The interindividual and interspecific variabilities and susceptibility to starvation would help to elaborate the framework for environmental health research. In contrast, there is limited knowledge about fish gonadal responses to starvation stress, and the underlying mechanisms need further investigations.

Throughout the adult life of male fish, germ cells in the testis produce highly differentiated sperm that transmit genetic information to offspring. Spermatogenesis as a set of dynamic process, in which the undifferentiated spermatogonia self-renew or differentiate through meiosis into spermatocytes, and then metamorphosis to spermatids and fertile spermatozoa (Brinster, 2002; Xu et al., 2010). The sex-determining genes such as *amh* (anti-Müllerian hormone, AMH), *dmrt1* (doublesex and mab-3 related transcription factor 1, DMRT1), and *sox9* (SRY-box 9, SOX9), are involved in activating downstream factors and essential for testicular development and spermatogenesis (Lin et al., 2017). Another key signaling molecule is the retinoic acid (RA), a member of the oxidized and derivative form of retinol (vitamin A) participating in the meiosis initiation in fish testis (Crespo et al., 2019; Feng et al., 2015). The balance between RA-synthesizing enzymes (aldehyde dehydrogenase 1A, Aldh1a) and RA-degrading enzymes (cytochrome P450, family 26, Cyp26) precisely regulate the spatial-temporal distribution and level of RA in gonad for meiosis (Reijntjes et al., 2005; Rodriguez-Mari et al., 2013). Nevertheless, the lack or excess of RA would cause developmental defects in fish (Dobbs-McAuliffe et al., 2004).

The immunity of teleost fish gonad modulates the reproductive

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process that guarantees germ cell development and permits qualitatively inflammatory responses to fight off the infections. The germ cells and somatic cells in testis could produce immune molecules that are essential for the orchestration of gametogenesis and the maintenance of gonadal homeostasis (Chaves-Pozo et al., 2008). Once acidophilic granulocytes infiltrate into the gonad, they could trigger the production of interleukins that stimulate pathological inflammatory process, accompanied by the testicular degenerative process with high level of apoptosis and necrosis (Chaves-Pozo et al., 2005, 2008; Liarte et al., 2007). Besides, it is well accepted that the macrophages are not only important in immune system but also pivotal in development and homeostasis, in which the macrophages efficiently respond to environmental changes and modify their phenotype with engulfing and digesting the microbes or cell debris (Mosser and Edwards, 2008; Nguyen-Chi et al., 2015). And some pro-inflammatory immune molecules such as tumor necrosis factor alpha (TNFα), interleukin-6 (IL-6), and interleukin-1 (IL-1) are central cytokines in immune system, while their imbalance could lead to the critical inflammation that irreversibly induce multiple organ failure and organismal disorder as well (Novoa et al., 2009).

Zebrafish (*Danio rerio*) is a prominent animal model that has been extensively used in toxicological and ecological researches (Lieschke and Trede, 2009; Novoa and Figueras, 2012). In this study, adult male zebrafish were used to investigate the testicular responses to starvation stress. The histological and molecular biological examinations related to the testicular development, inflammation, and apoptosis were conducted to elaborate the mechanism research of pathological or immune changes and underline the susceptibility of fish gonad under starved condition.

2. Materials and methods

2.1. Fish rearing and experiment design

Adult male zebrafish of wild type (AB strain, five months old) were bred and reared in our laboratory according to the zebrafish book (Westerfield, 2000). The fish were living in the glass tanks with a density about 1.0 g fish/L dechlorinated tap water (pH 8.11 ± 0.26, dissolved oxygen 7.33 ± 0.21 mg/L, total hardness 188.53 ± 15.03 mg/L, ammonia nitrogen 0.44 ± 0.05 mg/L, total nitrogen 0.56 ± 0.04 mg/L, total phosphorus 2.21 ± 0.08 mg/L) and fed with commercial dry bait (AQUAFIN, Malaysia; ~4% of fish body weight) twice daily at 28 ± 1 °C on a light-dark cycle of 14 h: 10 h. The culture water was renewed daily and the tanks were periodically cleaned using siphon. For the starvation treatment, randomly selected fish were deprived of feed for 1–3 weeks after 2 weeks acclimation. There were no fish died during the starvation period. The control group and starved group (named CON and STA) contained three repeat tanks of 30 fish, respectively.

The fish were anesthetized with MS-222 (500 mg/L, Sigma Chemicals Inc., USA) before dissection. The body mass and body length were measured to calculate the condition factor [(body weight/body length^3) × 100]. The testis was sampled to measure the gonadosomatic index (gonad weight/body weight × 100). Besides, six testes per group were fixed in paraformaldehyde and other tissue samples were snap-frozen in liquid nitrogen and stored at −80 °C for further use. All procedures during the experiment were permitted by the regulations on experimental animals of Management Methods of Laboratory Animals in Shaanxi Province, China (No. 150, 2011) and the Animal Ethics Committee of Northwest A&F University, Yangling, Shaanxi, China.

2.2. Measurements of the blood parameters and the RNA/DNA ratio in fish testis

Total of 30 blood samples (~5 μL/fish) per group were collected from caudal artery after anesthetization. Blood glucose was directly detected by the glucometer (LifeScan, USA). Blood triglyceride and blood total cholesterol were quantified using Triglyceride Quantification Kit (Sigma Chemicals Inc., USA) and Cholesterol Quantitation Kit (Sigma Chemicals Inc., USA) according to the manufacturer's instructions. The serum was collected by centrifugal separation (1000 r/min, 4 °C, 10 min) and mixed with the reaction solutions in 96-well plate. After incubation at 37 °C for 10 min, each well was measured the optical density at 570 nm using a microplate reader (Bio Tek, USA). The concentrations of triglyceride and cholesterol were determined by the standard curve.

Nucleic acids were extracted from the testis by homogenizing in a glass homogenizer with Tris-HCL buffer (0.05 M Tris, 0.1 M NaCl, 0.01 M EDTA, pH = 8) at 4 °C. The RNA/DNA ratio was analyzed based on the UV-based method according to the publication (Kuropat et al., 2002). After series of washing with cold perchloric acid ($HClO_4$) to remove free nucleotides, the RNA content was determined by hydrolyzing with potassium hydroxide solution. Then, the hydrolysate was acidified with cold $HClO_4$ to remove the RNA, and the rest of DNA was hydrolyzed and separated by adding hot $HClO_4$. Finally, RNA and DNA were estimated from the absorbance of the hydrolysate at 260 nm by a spectrophotometer (Thermo Electron Corporation, USA) using the extinction coefficient: A_{260} of 1 μg/mL solution of hydrolyzed RNA or DNA is 0.3.

2.3. Histological observation of testis

After dissection of fish, the testes were immediately fixed in 4% paraformaldehyde solution overnight and dehydrated through the graduated concentrations of ethanol (50, 70, 80, 90, and 100%) and xylene for 10 min. Then, the samples were embedded in paraffin and cut in 6 μm thick sections with rotary microtome (Leica RM2235, USA) for haematoxylin-eosin (HE) staining. Finally, tissue sections were observed using an optical microscope (Nikon, Japan). The relative ratio of different phase cells in testis was analyzed by ImageJ 1.44 software (National Institutes of Health, USA). More than six individual sections were calculated per group in triplicate.

2.4. Quantitative real-time PCR (qRT-PCR) assay

Testis samples were homogenized in TRIZOL regent (Takara, China) using a tissue grinder. Total RNAs were extracted and treated with DNase to remove genomic DNA contamination. The quality and concentration of RNAs were checked by a nanodrop spectrophotometer (Thermo Electron Corporation, USA). Total of 3 μg RNA with M-MLV reverse transcriptase (Invitrogen, USA) and oligo $(dT)_{18}$ primer were used to synthesize cDNAs in 20 μL reaction volume.

The qRT-PCR was performed by Real-Time PCR System Thermocycler (Bio-Rad CFX96, USA) with SYBR Green ExTaq II Kit (Takara, China). Following the manufacturer's instructions, the reactions were carried out in final volume of 25 μL, with 1 × SYBR Premix ExTaq™, 0.4 μM of each primer, and 2.5 μL RT reaction solution. Each sample was examined in triplicate following the protocol: initial denaturation 95 °C for 30 s, following denaturation 95 °C for 5 s and annealing 60 °C for 30 s of 40 cycles. The fluorescent density of SYBR Green and threshold cycle (Ct) value were determined by CFX manager program (Bio-Rad, USA). The specific primers used are listed in Table 1. The gene expression was analyzed using the $2^{-\triangle\triangle Ct}$ method. *β-actin* and *ef1a* were used as the reference genes, their geometric mean was used to normalize those target genes expression (Vandesompele et al., 2002).

2.5. Western blot analysis

The testis tissues were lysed in pre-cooling RIPA lysis buffer (CWBIO, China) to extract proteins. The proteins were quantified using BCA Protein Assay Kit (Solarbio, China). Total of 100 μg extract proteins were separated using 10% sodium dodecyl sulfate polyacrylamide gradient gel, and electro-blotted onto the polyvinylidene fluoride membrane (Millipore, USA). Then, the membrane was blocked with 5% BSA (Bio-Rad, USA), incubated with primary anti-β-Tubulin (1:1000, Gxybio,

Table 1
Primer sequences of qRT-PCR experiment in the present study. F and R represent forward primer and reverse primer, respectively.

Gene	Primer sequence(5′ to 3′)	Accession number
β-actin	F: CCTGTATGCCAACACAGTGC R: GAAGCACTTCCTGTGAACGA	NM_131031.1
ef1a	F: GGAAATTCGAGACCAGCAAATAC R: GATACCAGCCTCAAACTCACC	NM_131263.1
dmrt1	F: ACTGCACATCTGACCTAATGG R: ACACGTTATGGCTGGACAG	NM_205628.2
amh	F: CAACAGTCAATCCATCCATC R: AGGCTCATCAAGGTCAGA	NM_001007779.1
sox9a	F: GCTCGGAGACTTCAAGAA R: CCACCTCGTTCAGTAATCT	NM_131643.1
sycp3	F: CATAGAGGAGATGGAGAAAAGCC R: ATGGACTGAAGAGACTTGCG	NM_001040350.1
aldh1a2	F: AGAGACAGTGCTTACCTTGC R: TTGGAATTGTAGACCCGTGG	NM_131850.1
cyp26a1	F: CTCTGACACCCTCTCCAATG R: ATTCCTGTATGGCGCTCTTC	NM_131146.2
tnfa	F: GGTGTTTGGGATCATTTTGGC R: GCCTTGGAAGTGAAATTGCC	NM_212859.2
il6	F: GGTGAGAGACGGAGAGATGGAT R: CACGCTGGAGAAGTTGAACAG	NM_001261449.1
il1b	F: GTTCAGATCCGCTTGCAATG R: TGCTTCATTCTGTTCAGGGC	NM_212844.2
bcl2a	F: TGGATGACTGACTACCTGAAC R: GTATGAAAACGGGTGGAAC	NM_001030253.2
baxa	F: TCTGATGGCAAGTTCAACTGG R: ATGACGTGCTCCTGAATGTAG	NM_131562.2
casp9	F: GTCTTCACTCAGGACATGATCG R: ACGCAGGGAATCAAGAAAGG	NM_001007404.2
casp8	F: CTACAGACGCAGAAACTCCAT R: TGTCCATATCAGTGCCTGTTC	NM_131510.2
casp3	F: TCAATCCCATGCCTTCAGATAC R: ACATTTCCTGCATCTACGTCAG	NM_131877.3
casp2	F: CAACATCACCTCTGCAAACTG R: TCAGCATCCAAACACATCTCC	NM_001042695.1

USA), anti-TNF-α (1:500, Abcam, UK), anti-IL-6 (1:500, Cell Signaling Technology, USA), anti-IL-1β (1:500, Cell Signaling Technology, USA), anti-Bcl-2 (1:500, Cell Signaling Technology, USA), anti-Bax (1:500, Cell Signaling Technology, USA) at 4 °C overnight and secondary antibodies (1:2000, CWBIO, China) at room temperature for 2 h. After washing with Tris-buffered saline for three times, the immunoreactive bands were visualized using the enhanced chemiluminescence regents with imaging system (Bio-Rad, USA) and analyzed their density using ImageJ 1.44 software (National Institutes of Health, USA). Results of protein expressions were normalized to the expression of β-Tubulin.

2.6. Enzyme-linked immunosorbent assay (ELISA) for RA content

The content of RA was determined using Fish Retinoic Acid ELISA Kit (Meimian Biotech, China) according to the manufacturer's protocols. In detail, tissue samples were homogenized in ice-cold phosphate-buffered saline buffer using grinder. Respective supernatant was collected after centrifugation and added to the tested wells, then mixed with horseradish peroxidase conjugated reagent and incubated at 37 °C for 60 min. After washing with the buffer for five times, the chromogen solutions were added to each well and preserved at 37 °C for 15 min in dark. Finally, each well absorbance at 450 nm was measured using Microplate reader (Bio Tek, USA) within 15 min after adding the stop solution. The amount of RA was determined by the equation of standard curve and normalized to the protein concentration.

2.7. Statistical analysis

All data are shown as mean ± standard error (SEM). Comparisons between CON and STA groups were analyzed with multiple *t*-test using SPSS 20.0 software (SPSS, Chicago, USA). Data were tested for normality of distribution (Shapiro–Wilk test) and homogeneity of variance (Levene's test) prior to analysis. Data that did not meet assumptions of normality and homoscedasticity were transformed (log). Statistical analyses of more than two groups were by one-way analysis of variance (ANOVA) with Tukey's test. *P* value of <0.05 was considered statistically significant.

3. Results

3.1. Starvation stress reduced the biological indexes, blood parameters, and RNA/DNA ratio in zebrafish

Under starvation stress, the body mass of zebrafish gradually decreased and the condition factor (CF) showed significant decreases after 2 and 3 weeks of starvation ($P < 0.05$, Fig. 1A). The gonadosomatic index (GSI) significantly decreased after 1–3 weeks of starvation ($P < 0.05$, Fig. 1B). The blood glucose (BG), blood triglyceride (BTG), and blood total cholesterol (BTC) of starved zebrafish significantly declined compared to normal feeding groups ($P < 0.05$, Fig. 1C–E). Besides, the RNA/DNA ratio in testis distinctly decreased from 1 to 3 weeks ($P < 0.05$, Fig. 1F), reflecting the restrained protein synthesized potential under starved condition.

3.2. Histological changes in testis of starved zebrafish

According to the histological observation (Fig. 2A–F), A-C imply representative sections in control groups and D-F imply starved groups from 1 to 3 weeks respectively, there were impaired architecture and suppressed spermatogenesis in testes after starvation stress. The proportion of spermatogonia (SG) was increased after 2 and 3 weeks of starvation and showed significant differences at 3 weeks ($P < 0.05$, Fig. 2G). The proportion of spermatocytes (SC) was significantly increased after 2 and 3 weeks of starvation ($P < 0.05$). Besides, there was a distinctly decreased tendency in the percentage of spermatozoa (SZ) from 1 to 3 weeks in the starved testis compared to control ($P < 0.05$). While the percentage of spermatids (ST) was not statistically different during the starvation period ($P > 0.05$). The farraginous cell in different stages and the collapsed architecture in starved testis (obviously shown in Fig. 2E and F) suggested the abnormal testicular development and potentially pathologic response.

3.3. Spermatogenesis related gene expressions and RA content in testis

As shown in Fig. 3A, the mRNA expression of *amh* was significantly downregulated after 2 and 3 weeks of starvation ($P < 0.05$), while the transcriptions of *dmrt1* and *sox9a* showed no obvious differences ($P > 0.05$, Fig. 3A). The transcript level of *sycp3* (synaptonemal complex protein 3, SYCP3), as a meiotic maker molecule (Ozaki et al., 2011), was gradually decreased and showed significant difference after 3 weeks of starvation ($P < 0.05$, Fig. 3B). Besides, starvation stress had a pronounced effect on RA contents that appeared significant increases by 37%–118% at 2 and 3 weeks compared to control ($P < 0.05$, Fig. 3C). The mRNA expression of *aldh1a2* was dramatically increased after 2 and 3 weeks of starvation ($P < 0.05$) and the expression of *cyp26a1* was significantly decreased from 1 to 3 weeks ($P < 0.05$, Fig. 3D), showing the responsive feedback of these two genes that regulate the RA signaling pattern in testis.

3.4. Elevated inflammatory response in the starved testis

To reveal the inflammatory response in testis after starvation stress, the mRNA and protein expressions of TNF-α, IL-6, and IL-1β were analyzed. The transcriptions of *tnfa* and *il6* were significantly upregulated after 3 weeks of starvation ($P < 0.05$, Fig. 4A and B), the mRNA expression of *il1b* showed significant increases at 2 and 3 weeks ($P < 0.05$, Fig. 4C). The protein expressions of TNF-α, IL-6, and IL-1β were consistently elevated and exhibited marked differences in all starved

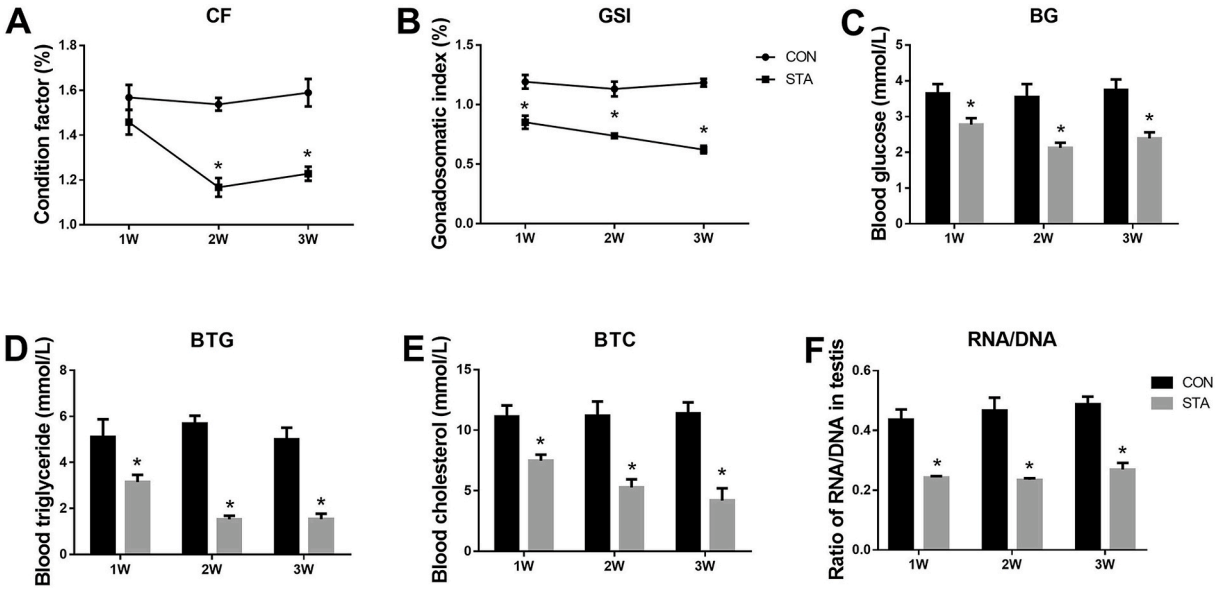


Fig. 1. Changes of developmental and biological indexes in zebrafish upon starvation stress. (A) Condition factor (CF), (B) gonadosomatic index (GSI), (C) blood glucose (BG), (D) blood triglyceride (BTG), and (E) blood cholesterol (BTC) in male zebrafish after 1–3 weeks of starvation (n = 30). (F) The ratio of RNA/DNA in zebrafish testis (n = 9). CON and STA represent the control and starved groups, respectively. The single asterisk above the columns or lines denotes statistically significant between CON and STA groups analyzed by two-tailed *t*-tests ($P < 0.05$). *$P < 0.05$. No single asterisk denotes there were no significant difference, $P > 0.05$.

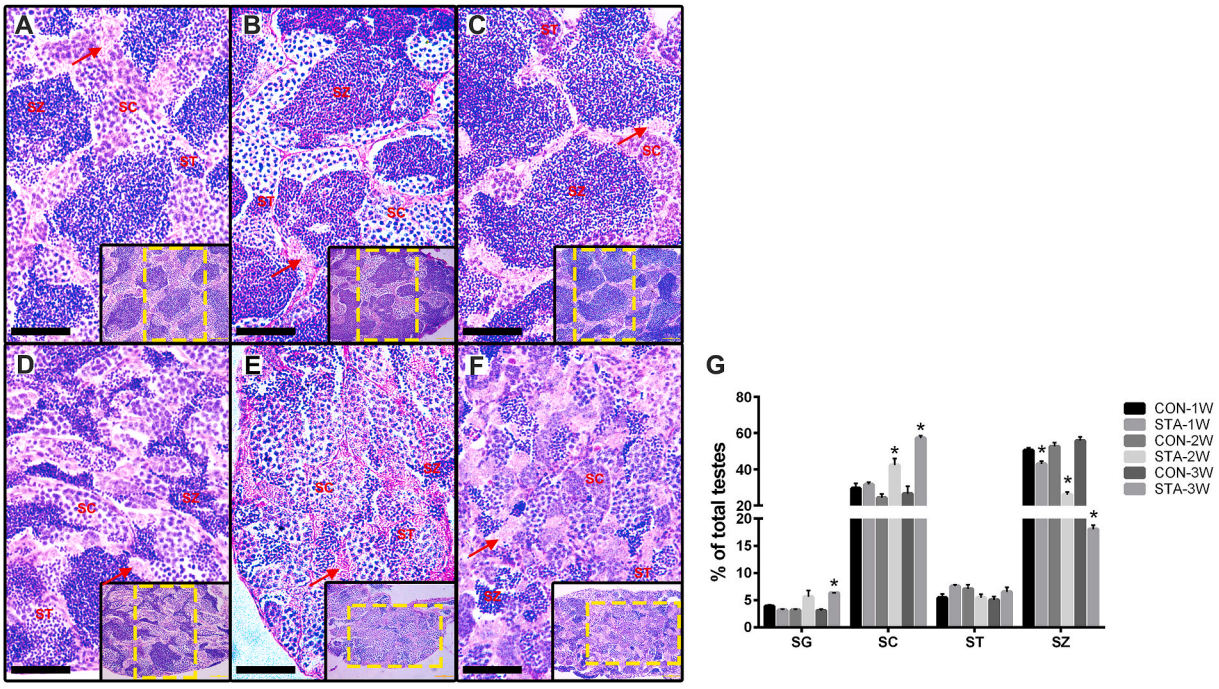


Fig. 2. Histology analysis of zebrafish testis after starvation. Histological observation of testis stained by haematoxylin-eosin (HE) in (A–C) control groups and (D–F) starved groups from 1 to 3 weeks, respectively. (G) Relative ratio of different phase cell in zebrafish testis (n = 6). SG, spermatogonia; SC, spermatocytes; ST, spermatids; SZ, spermatozoa. SG was marked by red arrow. Scale bar = 50 μm. The single asterisk above the columns or lines denotes statistically significant between CON and STA groups analyzed by two-tailed *t*-tests ($P < 0.05$). *$P < 0.05$. No single asterisk denotes there were no significant differences, $P > 0.05$. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

groups in contrast to control group ($P < 0.05$, Fig. 4D and E), indicating the continuous inflammatory response in testis.

3.5. Increased apoptosis level in testis under starvation stress

The apoptotic activity examinations of caspases (casp) and Bcl-2 family were shown in Fig. 5. The transcriptions of *baxa* and *casp9* were gradually enhanced in testis after starvation and showed statistical differences after 3 weeks of starvation ($P < 0.05$, Fig. 5B and C). Whereas, the expressions of *bcl2a* and *casp8* were of no marked changes in starved groups ($P > 0.05$, Fig. 5A and D). As to the mRNA expression of *casp3*, it had a temporary elevation after 1 week of starvation while

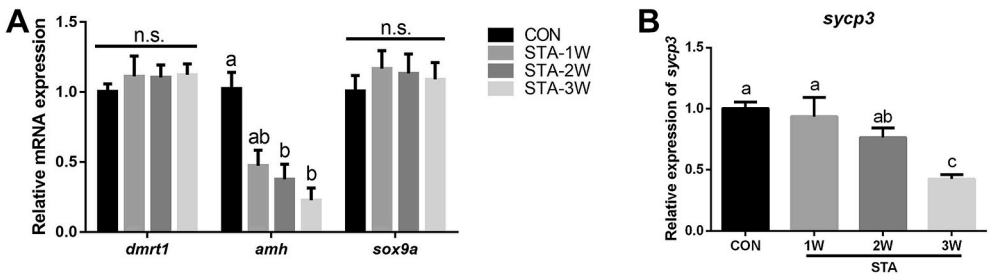
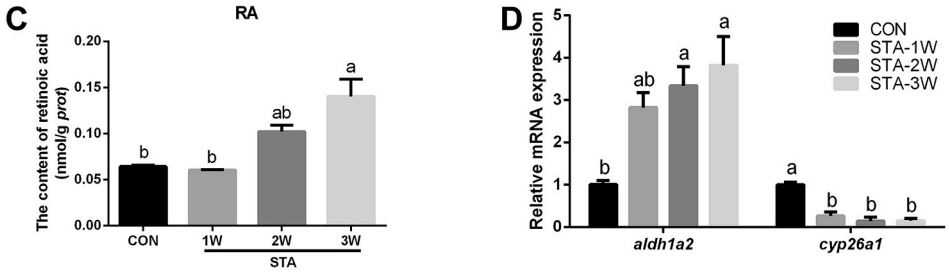
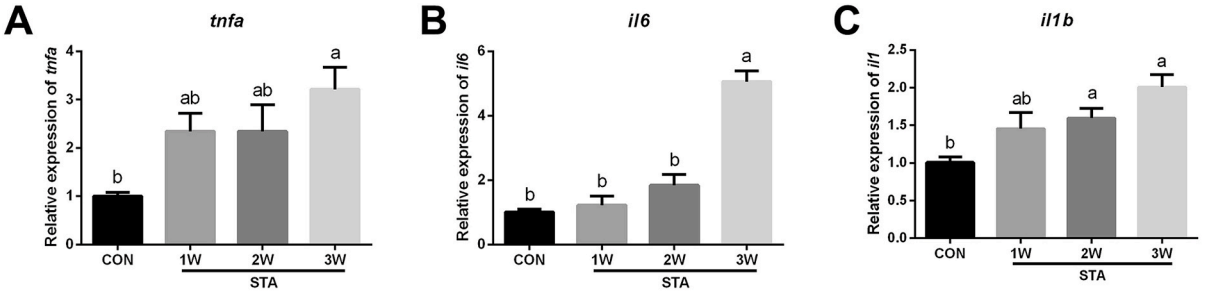


Fig. 3. Transcript analysis and retinoic acid (RA) content changes in zebrafish testis. Relative mRNA expression files of (A) *dmrt1*, *amh*, *sox9a*, and (B) *sycp3* in the zebrafish testis (n = 3). (C) The content of RA and (D) relative mRNA expression files of *aldh1a2* and *cyp26a1* in the zebrafish testis (n = 3). Different small letters above the columns indicate statistically significant differences examined by one-way analysis of variance (ANOVA) with Tukey's test at the 0.05 level ($P < 0.05$). n.s. signifies no significant differences ($P > 0.05$).





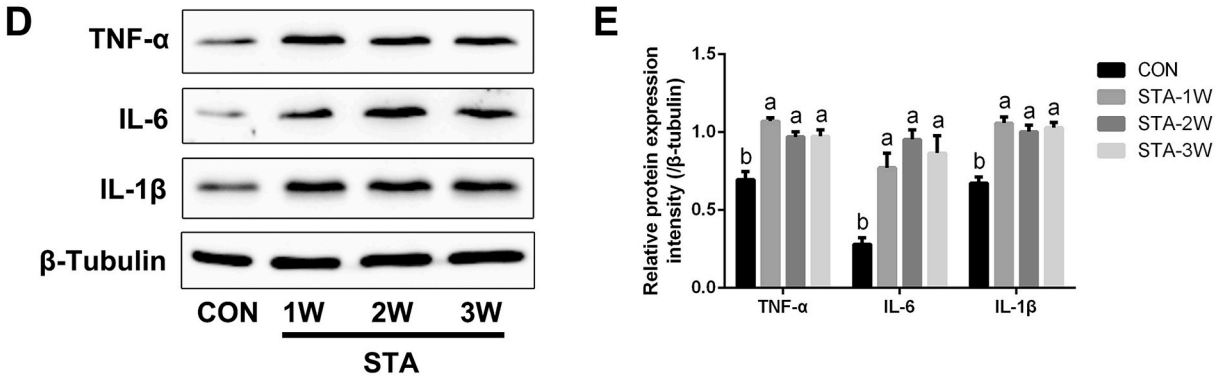


Fig. 4. Inflammatory response in zebrafish testis after starvation. Relative mRNA expression files of (A) *tnfa*, (B) *il6*, and (C) *il1b* in the fish testis (n = 3). (D, E) Relative protein expressions of TNF-α, IL-6, and IL-1β in the fish testis evaluated by Western blot (n = 3). Different small letters above the columns indicate statistically significant differences examined by one-way analysis of variance (ANOVA) with Tukey's test at the 0.05 level ($P < 0.05$).

sharply decreased afterwards ($P < 0.05$, Fig. 5E). And the expression of *casp2* were significantly raised after 2 and 3 weeks of starvation ($P < 0.05$, Fig. 5F). That temporal gene expression of caspases might be the specific regulation in the death signal cascade of apoptotic response. The protein expressions of Bcl-2 and Bax showed the similar tendency with mRNA expressions (Fig. 5G). The expression of Bcl-2 was not obviously changed in starved testis compared to control ($P > 0.05$), while the Bax significantly elevated ($P < 0.05$), and the ratio of Bcl-2/Bax exhibited distinct reduction from 1 to 3 weeks of starvation ($P < 0.05$, Fig. 5H).

4. Discussion

Nutrient deprivation stress could cause adverse effects on organism natural activities and phenotypes, and the performance characteristics are important proxies for detecting fish fitness under environmental stress (Schulte, 2014). In the present study, the body mass and testis

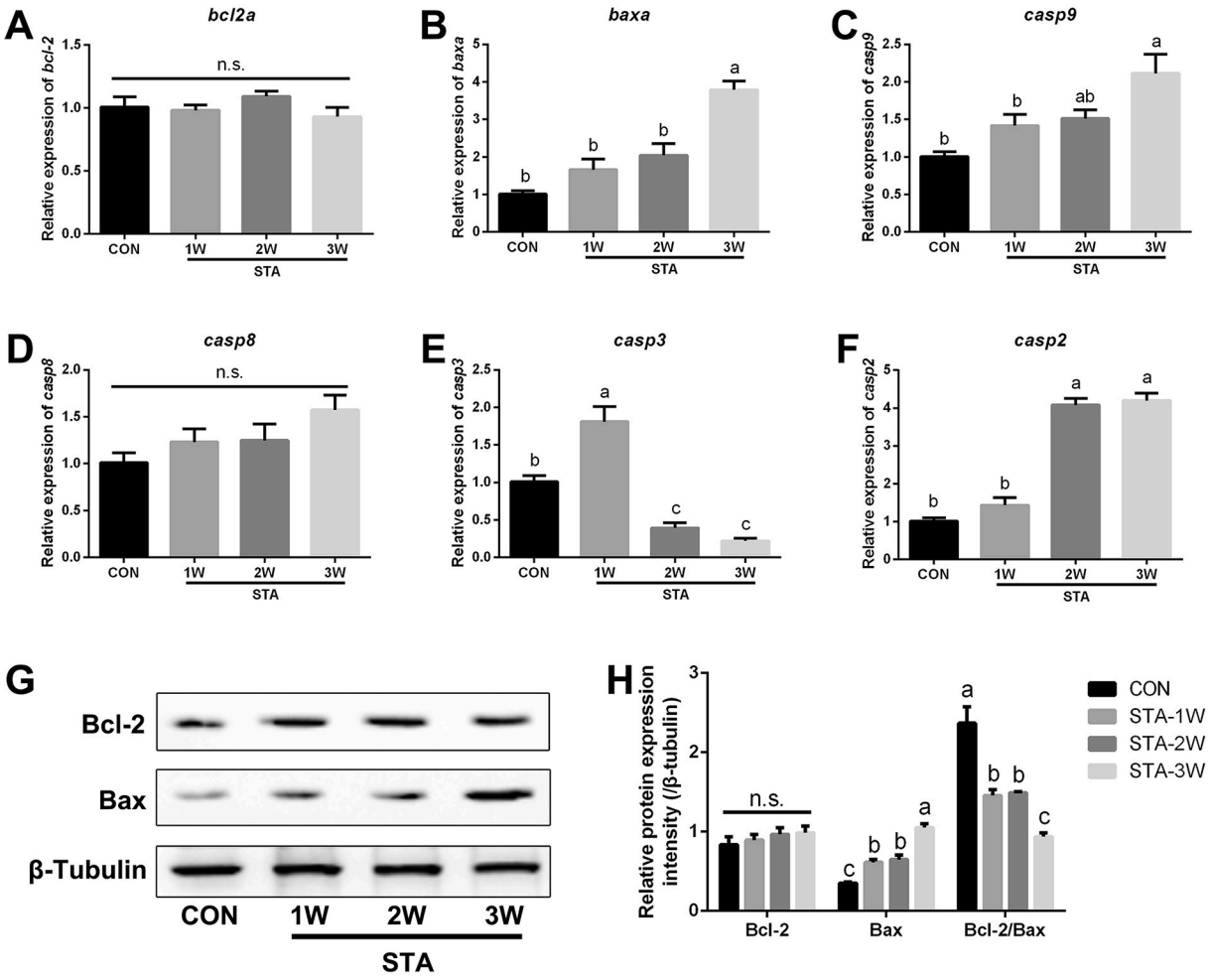


Fig. 5. Apoptotic activity in testis after starvation stress. Relative mRNA expression files of (A) *bcl2a*, (B) *baxa*, (C) *casp9*, (D) *casp8*, (E) *casp3*, (F) *casp2*, and (G, H) relative protein expressions of Bcl-2 and Bax with the ratio of Bcl-2/Bax in the zebrafish testis (n = 3). Different small letters above the columns indicate statistically significant differences examined by one-way analysis of variance (ANOVA) with Tukey's test at the 0.05 level ($P < 0.05$). n.s. signifies no significant differences ($P > 0.05$).

weight were decreased along with the 3 weeks fasting, and the blood parameters were consistently reduced, indicating the poor growth condition and disturbed organ activity. As the fasting is characterized by consistently decline of fat storage, in which the lipases within adipocytes or other cells are activated to maintain the physiological homeostasis (Huang et al., 2018; Tian et al., 2013). Underlying the capacity to survive during fasting period, the negative energy budget shifts the dependency to endogenous lipid utilization and protein catabolism thus leading to the loss of body mass and the atrophy or downregulation of organs (Caloin, 2004; Secor and Carey, 2016). The reduced blood glucose, triglyceride, and cholesterol levels were associated with the GSI reduction that may cause cell degeneration and architecture disorganization in zebrafish testis. Considerably, the rate of cell loss could exceed the rate of cell proliferation in tissue during the starvation period. Even some cellular components are recycled as energy sources for the sustainability of organ output (Fan et al., 2019a; Ma et al., 1998). As a traditional indicator, RNA/DNA ratio responds rapidly to nutrient variability and reflects recent growth and cell synthetic capacity (Diaz et al., 2018; Gwak et al., 2003). Under starvation stress, the RNA/DNA ratio in testis was significantly decreased, reconfirming their sensitive potential to the fluctuant environment.

Seasonal feeding changes coincide with the spawning migration and reproduction in fish, suggesting the energy availability is crucial for the reproductive process and gonadal function (Volkoff et al., 2009). Consequently, reproductive process is inhibited or delayed when energy deficiency (Schneider et al., 2002). In the present study, food deprivation reduced the GSI and retarded spermatogenesis with decreased proportion of mature spermatozoa detected in histological observation of zebrafish testis, which is consistent with the reduced GSI and spermatocrit in male Arctic charr (*Salvelinus alpinus*) subjected to long duration of fasting (Frantzen et al., 2004). Present study also supports the nutritional status is critical determinant of testicular function and regulates sperm maturation process (Hatef and Unniappan, 2019). *Amh*, as a member of the TGF-β superfamily of growth factors, also involved in the modulations of testicular development and spermatogenesis. It is expressed in Sertoli cells and acts as a guardian in regulating germ cells proliferation or differentiation and gamete maturation in male zebrafish (Kossack and Draper, 2019). Present results showed that the transcript of *amh* was downregulated, accompanied by the decreased expression of *sycp3*, a meiosis marker expression in spermatocytes, agreeing with previous study that the loss of Sycp3 expression in the *amh* mutant zebrafish (Lin et al., 2017), and reflecting the deficient differentiation of spermatogonium in the testis of starved fish.

The RA signaling generally underlies fecundity and plays a vital role in zebrafish spermatogenesis process (Pradhan and Olsson, 2015). Present results showed that increased RA levels with upregulated expression of *aldh1a2* and downregulated expression of *cyp26a1* in the testis of starved zebrafish. As previous study indicated that excessive RA in inappropriately spatial and temporal patterns are potently teratogenic in zebrafish embryo (Dobbs-McAuliffe et al., 2004; Shenefelt, 2010). We

speculated that the feedback mechanisms might coordinate the induction of *aldh1a2* and the repression of *cyp26a1* upon the challenge of starvation to compensate RA level for the deficient spermatogenesis in starved zebrafish. Nevertheless, excessive RA in the autocrine or paracrine manner eventually gave the potential impairments in testis.

Organism would be more vulnerable when their immune system are depressed due to the endogenous resources being diverted and allocated to life sustaining systems during the extended episodes of fasting, which partly accounts for the fitness costs of reproduction (Bourgeon et al., 2010; Bourgeon and Raclot, 2006). In the current study, the pro-inflammatory cytokine expressions of TNF-α, IL-6, and IL-1β were generally upregulated in the testis, indicating the elevated inflammatory response under starved condition. Upregulated TNF-α level is the key feature of classically activated macrophages that efficiently respond to environmental challenges and stimulate inflammation (Mosser and Edwards, 2008). Meanwhile, IL-6 and IL-1β are the best-known pro-inflammatory interleukins involved in the regulation of fish immune system (Secombes et al., 2011). Recent research on Nile tilapia (*Oreochromis niloticus*) also revealed that the mRNA expression levels of IL-6 and IL-1β increased in spleen after short-term fasting (Wang et al., 2019). Besides, the high level of cytokines might account for the disorganized testicular sections in the present study. The past research documented that short-term starvation acted as a preventive measure in red sea bream (*Pagrus major*) to prevent bacterial infection by promoting immunological function and eventually enhance the disease or bacteria resistant capacity (Mohapatra et al., 2017). As environmental studies showed that stress could affect the gene expressions of specific chemokine and cytokine, pointing out that stress is thought to be immunosuppressive and exacerbating inflammatory according to the duration of stress (Khansari et al., 2018; Tort, 2011). Therefore, the intricate influence of starvation stress and immune responses could be varied by the duration time and species or tissues. And the stimulated inflammatory responses in the starved testis might be related to the leukocyte infiltration (Chaves-Pozo et al., 2008; Liarte et al., 2007).

Continuous tissue inflammation inevitably resulted in the tissue injury, corroborated by the impaired tissue architecture in starved zebrafish. Notably, current study indicated that the upregulated transcription of apoptotic genes and decreased ratio of Bcl-2/Bax protein level, which is a rheostat appearing to determine the cell survival or death following the apoptotic stimulus (Fan et al., 2019b; Korsmeyer et al., 1993). Bcl-2 works as an apoptotic brake and primarily regulates apoptosis at the outer mitochondrial membrane, where it could block the pro-apoptotic proteins interaction (Walensky, 2006). Bax is reported as an intrinsic apoptotic activator that disturbs the outer mitochondrial permeability and results in the loss of membrane potential, the release of cytochrome *c*, and the initiation of downstream factors such as caspase9 and caspase2 with apoptosome complex (Lockshin and Zakeri, 2004; Wei et al., 2001). As TNF-α plays a major role in triggering extrinsic apoptosis changed with the activation of caspase8 and downstream effectors (Micheau and Tschopp, 2003), the increased expressions of *casp8*, *casp3*, and *casp2* reflect the elevated apoptotic activity in farraginous and inflammatory testis after starvation stress in this study. In addition, the testicular apoptosis is observed during the spermatogenesis process of teleost fish and varied in reproductive stages and living conditions (Chaves-Pozo et al., 2005; Schulz et al., 2010). Cell apoptosis occurs to regulate the germ cells stock, spermatozoa production, eliminate anomalous gametes, and contributes to the testicular homeostasis or regression (Kaptaner and Kankaya, 2013; Ribeiro et al., 2017). So that increased apoptosis could also be induced by the imbalance between germ cell proliferation and differentiation with the impaired spermatogenic cycle, which ceased sperm production and considerably decreased GSI to reduce reproductive output and maintain homeostasis under continuous starvation stress.

5. Conclusion

In conclusion, the starvation stress severely impaired the growth condition including biological indexes, blood parameters, and RNA/DNA ratio in adult male zebrafish from 1 to 3 weeks. The testicular disorganization and restrained spermatogenesis were relevant to the downregulated transcript of *amh* and the modulation of RA metabolism. Besides, the inflammation was elevated with upregulated pro-inflammatory cytokines of TNF-α, IL-6, and IL-1β, concomitant with the stimulated apoptotic activity in the testis of starved fish. Present study highlights the stress response of fish testicular development to food availability, and points out the inflammatory and apoptotic risk in testis to environmental fluctuation.

Declaration of competing interest

The authors declare no competing interests.

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Abbreviations used

Aldh1a	aldehyde dehydrogenase 1A
AMH	anti-Müllerian hormone
BG	blood glucose
BTC	blood total cholesterol
BTG	blood triglyceride
casp	caspases
CF	condition factor
CON	control group
Cyp26	cytochrome P450, family 26
DMRT1	doublesex and mab-3 related transcription factor 1
ELISA	Enzyme-linked immunosorbent assay
GSI	gonadosomatic index
HE	haematoxylin-eosin
IL-1	interleukin-1
IL-6	interleukin-6
qRT-PCR	Quantitative real-time PCR
RA	retinoic acid
SC	spermatocytes
SG	spermatogonia
SOX9	SRY-box 9
ST	spermatids
STA	starvation group
SYCP3	synaptonemal complex protein 3
SZ	spermatozoa
TNFα	tumor necrosis factor alpha

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