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Oogenesis, vitellogenin-mediated ovarian degeneration and immune response in the annual fish *Nothobranchius guentheri*

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ABSTRACT

Annual fishes of the genus *Nothobranchius* show expression of age-related biomarkers at behavioral and histological levels. They therefore represent an excellent animal model for aging studies. However, oocyte development, histological and biochemical degeneration and immune response of ovary in the annual fishes remain unclear. Here, using one of these short-lived fishes, *Nothobranchius guentheri*, we reported that oogenesis process was divided into four stages (oogonium, primary growth stage, cortical alveolus stage and vitellogenesis stage), and old ovaries showed histological degeneration (with decreased mature oocytes and increased atretic oocytes) accompaning with high levels of senescence-associated beta-galactosidase and lipofuscin by down-regulation of vitellogenin (the precursor of yolk proteins). Moreover, poly(I:C) induced inflammation with overexpression of NF-κB and IL-8, and up-regulated vitellogenin expression. It was a first analysis for vitellogenin to participate in ovarian degeneration and immune response in ovary of fish, indicating that vitellogenin fulfilled a critical role in ovary development and innate immune system.

1. Introduction

Staging series of oocyte (follicle) in zebrafish, swamp eel and catfish have shown that oocytes develop to maturation after oogonium, primary growth stage, cortical alveolus stage and vitellogenesis stage [1–3]. Vitellogenin, as the precursor of yolk proteins, involves in synthese of cortical alveoli and yolk [4,5]. On the other hand, vitellogenin purified from carp (*Cyprinus carpio*) and rosy barb (*Puntius conchonius*) possesses antimicrobial activity and its expression significantly up-regulates as response to inflammation induced by *Citrobacter freundii* in the skin of zebrafish [6–8]. However, if vitellogenin mediates ovarian degeneration and immune responses in ovary of fish remains largely unknown.

Annual fishes of the genus *Nothobranchius* are a group of annual teleosts broadly distributed in South-Eastern Africa with relatively short lifespan and accelerated growth. They are excellent vertebrate models to elucidate aging progress with expression of age-related biomarkers at behavioral, histological and molecular levels. *N. furzeri*, *N. rachovii* and *N. guentheri*, whose mean lifespan are 3–6.5, 8.5 and 12 months, are usually used to study the process of aging. For example, levels of senescence-associated beta-galactosidase (SA-β-Gal), lipofuscin, neurofibrillary, tubule dilation, malignant tumor and reactive oxygen species increase and cognitive performances decrease as aging in the annual fish [9–14]. Ovary shows degeneration with vitellum coartation, and testis shows degeneration with interstitial fibrosis in aged annual fish *N. furzeri* [15]. But oogenesis, vitellogenin mediated ovarian degeneration and immune responses in the annual fish need to be further clarified.

The annual fish *N. guentheri* are bred by mass-mating in our laboratory, and their embryos are collected and incubated at 24–26 °C. Then fry are hatched and fed with Brine Shrimp during the first 8 weeks [9,16]. Using this fish, our previous results demonstrate that oxidative damage increases with age [17]. During experimental process, we noticed that hatchability of embryos was lower in aged fish compared with young fish. Accordingly, the present study aimed at highlighting oocyte development, and if vitellogenin involved in ovarian degeneration and immune response in *N. guentheri*.

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2. Materials and methods

The protocol in this study was approved by the Ethics Committee on Animal Experiments of Medical School of Shandong University (Permit Number: ECAESDUSM 1420123009). The fish *N. guentheri* was bred and reared in our own laboratory [16,17]. The fish is similar to zebrafish in size. Each fish has two slender ovaries or testes, which are bilaterally located between the swim bladder and abdominal wall. The average age for the 'young' fish group was 6 months, whereas that for the 'old' group was 12 months. Totally, 100 fish were used to explore development of oocyte, degeneration of ovary, expression of vitellogenin and immune response in the annual fish. The fish at 6- and 12-month-old were intraperitoneally injected with poly(I:C) (polyinosinic-polycytidilic acid, Sigma) dissolved in PBS at 40 μg/g body weight for 12 h, while the control fish received same amount of PBS according to previously reported protocols in zebrafish and *N. guentheri* [18,19].

2.1. Frozen sections and HE staining

The fish were killed as rapidly as possible to minimize suffering. The gonads were freshly dissected, fixed in 10% formaldehyde, embedded in optical cutting temperature compound at −20 °C and sectioned at thickness of 10 μm on a microtome (MEV, Slee, Germany). For old fish, gonad atrophy was examined by naked eyes and the percentage of ovarian atrophy was calculated before fixative.

The sections were stained with hematoxylin and eosin and the histological structure of gonads was observed under a microscope (Olympus BX61, Japan). The numbers of oocytes at different developmental stages were counted and their percentages were calculated in ovary using 20 young females and 20 old females. Ten young males and 10 old males were used for histological structure of testis. At least 6 fish in poly(I:C) challenged group and control group at 6- and 12-month-old were used for histological changes.

2.2. Transmission electron microscopy

Ovarian samples isolated from 6-month-old fish were immediately fixed in 4% glutaraldehyde and 1% osmium tetroxide. After dehydration, ovarian tissues were embedded in epoxy resin. Ultrathin sections were stained with uranyl acetate and lead citrate and observed using a transmission electron microscope.

2.3. SA-β-Gal and lipofuscin

The activity was detected by a SA-β-Gal staining kit (Cell Signaling Technology). Frozen sections were stained with X-gal staining solution overnight at 37 °C according to the manufacturer's instructions and observed under bright field using a microscope.

The frozen sections were mounted onto glass slides and observed under fluorescence microscope for lipofuscin assay.

Activity of SA-β-Gal and accumulation of lipofuscin were analyzed in ovary and testis without treatment and in ovary with poly(I:C)/PBS injection both in 6- and 12-month-old fish (n = 6 in each group).

2.4. Quantitative real-time PCR

Livers and ovaries from young and old fish were used to detect expression of vitellogenin, NF-κB and IL-8 at mRNA level as aging and poly(I:C) stimulation. Total RNA was extracted with RNA simple Total RNA Kit and rapidly reverse-transcribed to cDNA with FastQuant cDNA (TIAN GEN) according to manufacturer's instructions. Total RNA of 2 μg was used for cDNA synthesis in a 20 μl volume. PCR reactions were performed in 20 μl volume with 2 μl cDNA using Quantitect SYBR Green PCR kit (TIAN GEN). The amplification was performed on a Roche LightCycler Real-Time PCR System at 95 °C for 15 min, followed by 40 cycles of 95 °C for 10 s and 60 °C for 20 s. The gene-specific primers were presented in Table 1.

Six fish both in young and old groups were used for vitellogenin expression during aging process. Six fish both in poly(I:C) challenged group and control group for young and old fish were used to detect expression of vitellogenin, NF-κB and IL-8 in ovary and liver.

2.5. Statistical analysis

All of the assays were repeated in 6 fish independently at least. Graphpad Prism 5 and Adobe Illustrator CS6 were used to process figures. SPSS 17.0 software was used to perform statistical analysis. Changes of oocytes percentages at different stage, SA-β-Gal activity, lipofuscin accumulation and vitellogenin, NF-κB and IL-8 levels were analyzed by one-way analysis of variance (ANOVA). Values represent the mean ± SD, and a value of $P < 0.05$ was considered statistically significant.

3. Results

3.1. Oogenesis of the annual fish

Histological structure of ovary and ultrastructure of oocytes were assayed to investigate the development of oocyte in 6-month-old fish.

Oocyte development was divided into four stages in the annual fish, oogonium (stage 1), primary growth stage (stage 2), cortical alveolus stage (stage 3) and vitellogenesis stage (stage 4) histologically.

Oogonium was comparatively small, ranged in size up to approximately 20 μm in diameter. Nucleus was relatively large with a centrally located nucleolus. No follicle cells were observed around oogonium (Fig. 1A).

Based on growth of follicle cells, primary growth stage could be separated into two phases, prefollicle phase and follicle phase. Oocyte with a diameter of about 30 μm in prefollicle phase was surrounded by a few follicle cells (Fig. 2A). Nucleus grew into germinal vesicle with increase of cell volume. Several small nucleoli were clearly visible throughout nucleus (Fig. 1B). These oocytes with large nucleo-cytoplasmic ratios lay in nests and contained electron-dense nuage, a typical structure of small oocytes (Fig. 2B). The diameter of oocytes was ranging from 35 to 70 μm in follicle phase. Similarly to prior phase, the germinal vesicle comprised of several peripherally located nucleoli. An oocyte was surrounded by follicle cells arranged in a single layer, which was called granulosa layer (Fig. 1C).

A number of cortical alveolus (yolk vesicles) were observed in the periphery of the oocytes at cortical alveolus stage (Fig. 1D), and they were dispersed throughout the ooplasm progressively with increase in number and size (Fig. 1E). They were firstly appeared

Table 1
The primers of genes for quantitative real-time PCR.

Gene	Primer sequence (5′-3′)
vitellogenin	F: CGTAACTGTATGTTGTGGTGCCT
	R: GGAACTGAACATCAAGAAGACCC
NF-κB	F: CTTCAGCCACTCCACCTAGC
	R: GTCCACTCTTCAGCTCCTGG
IL-8	F: GCACAGCTATTGCCTTCTCC
	R: CAGCGTGGCAATGATCTCTA
β-actin	F: CACCTTCTACAATGAGCTCCGT
	R: GCAGGAGTGTTGAAGGTCTCAA