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PCNA and apoptosis during post-spawning ovarian remodeling in the teleost *Oreochromis niloticus*

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ABSTRACT

The balance between cell proliferation and apoptosis is crucial for tissue development and homeostasis. The present study investigated the contribution of proliferating cell nuclear antigen (PCNA) and apoptosis during ovarian remodeling after spawning in the Nile tilapia *Oreochromis niloticus*. Breeding females were kept in controlled conditions and ovary samples were collected weekly for TUNEL assay, immunohistochemistry for PCNA and caspase-3 and morphometric analysis. During the follicular growth, PCNA labeled mainly the nuclei of oocytes and follicular cells in a high proportion of follicles especially in primary growth, while a low occurrence of apoptosis in follicular and theca cells was detected. At 0–3 days post-spawning, post-ovulatory follicles showed no proliferative activity, however the follicular cells exhibited high rates of apoptosis. At 7–10 days, PCNA labeled the thecal cells in a low proportion of post-ovulatory follicles, which showed follicular cells with lower rates of apoptosis. PCNA labeled mainly the theca in the advanced and late stages of atretic follicles, while the follicular cells exhibited a significant increase of apoptosis along follicular atresia. We concluded that PCNA and apoptosis work cooperatively to ensuring the success of follicle development and maintaining of tissue homeostasis during follicular growth. PCNA and apoptosis are also essential mechanisms in the follicular regression during post-spawning ovarian recovery in the Nile tilapia.

1. Introduction

In all vertebrates, the propagation of the species depends on the development of reproductive organs that undergo significant morphophysiological changes to achieve reproductive success (DeFalco and Capel, 2009; Matsuda et al., 2012). Post-spawning fish ovaries are excellent experimental models for studying the mechanisms involved in the tissue remodeling, especially due to follicular growth and regression for the start of a new reproductive cycle (Santos et al., 2005; Thomé et al., 2009). During primary growth, nuclear and cytoplasmic changes occur in the oocytes as well as the formation of the zona radiata and follicular cells. The secondary growth, influenced by sex hormones, is characterized by the accumulation of yolk globules in the ooplasm leading to a large increase in the follicles' diameter (Lubzens et al., 2010; Melo et al., 2014). After spawning, fish ovaries exhibit post-ovulatory follicles comprising a basement membrane that separates the follicular cells from the theca (Santos et al., 2008a). In fish ovary, the vitellogenic follicles that are not spawned during the reproductive period become atretic follicles and are reabsorbed in a long physiological process (Miranda et al., 1999). During ovarian development, the balance between the signs of proliferation and cell death determine the fate of the ovarian follicles for ovulation or follicular atresia (Krysko et al., 2008).

The proliferating cell nuclear antigen (PCNA) is a non-histone nuclear protein, highly conserved between species, which has been used as a biomarker of cell proliferation in a variety of tissues (Tománek and Chronowska, 2006; Quagio-Grassiotto et al., 2011; Guzmán et al., 2014). This protein acts as a cofactor of DNA polymerase delta and is involved in many essential cellular processes, such as DNA replication and repair, cell cycle control, and cell survival (Essers et al., 2005; Balla et al., 2008; Stoimenov and Helleday, 2009). PCNA has been reported to participate actively in the regulation of apoptosis, either by promoting pro-apoptotic proteins or suppressing anti-apoptotic proteins (Scott et al., 2001; Xu et al., 2011).

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Programmed cell death or apoptosis is a genetically regulated process which plays a fundamental role during the development and homeostasis of multicellular organisms (Kerr et al., 1972; Jenkins et al., 2013). It is characterized by fragmentation of internucleosomal DNA and cell fragmentation forming apoptotic bodies, which are engulfed by phagocytes or neighboring cells, thus avoiding an inflammatory reaction (Bangs et al., 2000; Drummond et al., 2000). The main effectors of apoptosis are caspases that activate Ca^{2+}/Mg^{2+}-dependent endonucleases, which cleaves the DNA into fragments of 180–200 base pairs (Huettenbrenner et al., 2003). Among the caspases, caspase-3 is a key effector molecule of the apoptotic program being responsible for the proteolytic cleavage of a wide variety of cellular proteins that lead to typical morphological changes of apoptosis (Brentnall et al., 2013).

Tilapias are the second most important group of fish cultivated in aquaculture worldwide and the Nile tilapia *Oreochromis niloticus* (Linnaeus, 1758) is the main species of tilapia currently farmed (FAO, 2015). The Nile tilapia is an important experimental model for reproductive biology studies due to the spawning in different environmental conditions, adaptability to various culture systems, and resistance to diseases and infections (Little and Hulata, 2000). In captivity, females of *O. niloticus* asynchronously spawn every 3 or 4 weeks and the interspawning interval can be influenced by lineage, age and size of fish, temperature, sex ratio, stocking density, nutritional status, and photoperiod (Coward and Bromage, 2000; Lapeyre et al., 2010).

Despite recent studies on the cell proliferation and apoptosis in vertebrates ovary (Xu et al., 2011; Matsuda et al., 2012; Thomé et al., 2012), very little is known about the balance of these processes in the post-spawning ovarian regeneration. Thus, the aim of this study was to investigate the contribution of PCNA and apoptosis during ovarian remodeling in *O. niloticus* after spawning, with an emphasis on follicular growth and regression.

2. Materials and methods

2.1. Fish stocking and sampling

The experiment was conducted at the Aquaculture Laboratory of the Veterinary School of the Federal University of Minas Gerais (UFMG), southeastern Brazil, during the months of November and December 2010. The research was approved by the Ethics Committee of Animal Experimentation (CETEA) at UFMG. The lineage of *O. niloticus* used in this study originated from the Chitralada Nile tilapia line introduced to Brazil from Thailand in the 1990s. To induce spawning, a breeding stock (age between 12 and 18 months) of males and females, previously kept under captive conditions, were distributed in eight 1 m^3 tanks with a water temperature between 26 and 28 °C at a ratio of 2 male to 6 females. After 3 days stocked, the oral cavity of the breeding females was inspected for signs of spawning, and from those which had reproduced, the offspring were taken to prevent mouth-brooding. Then, 12 spawned females were transferred and kept in a 5 m^3 tank with geomembrane liner, heaters with thermostat for maintaining the temperature, continuous supplementary aeration by an air diffuser, and a photoperiod of 12 h light:12 h dark. The fish were fed *ad libitum* twice a day with commercial feed (32% crude protein), and 30 min after feeding the remnants were collected. The water's physicochemical parameters were monitored weekly (temperature: 29.5 ± 1.5 °C, dissolved oxygen: 6.70 ± 1.46 mg l^{-1}, pH: 7.68 ± 0.31, conductivity: 0.28 ± 0.05 mS cm^{-1}, total dissolved solids: 0.16 ± 0.03 g l^{-1}, salinity: 0.12 ± 0.02 ppt, and turbidity: 1.71 ± 0.38 NTU).

In order to examine post-spawning ovarian remodeling, three females were collected weekly: 0–3 days after spawning, 7–10 days after spawning, 14–17 days after spawning and 21–24 days after spawning. Fish were killed by transversal section of the spinal cord following the ethical principles established by the Brazilian College of Animal Experimentation (COBEA). For each female, total length (TL), body weight (BW) and gonad weight (GW) were obtained, and the gonadosomatic index (GSI) was calculated: 34.89 ± 2.64 cm TL; 661.14 ± 161.83 g BW; 12.96 ± 6.34 g GW; 1.93 ± 0.76 GSI. Serial samples of the middle region of the ovaries of each specimen were collected for analyses employing a variety of techniques.

2.2. Light microscopy

For histology, samples of the ovaries were fixed in Bouin's fluid for 24 h at room temperature and then embedded in paraffin. Transverse histological sections of 5 μm thickness were mounted on glass slides and stained with hematoxylin-eosin (HE), Gomori's trichrome (GT), and periodic acid-Schiff (PAS) counterstained with hematoxylin.

2.3. In situ *TUNEL assay*

Ovarian samples were fixed in 4% paraformaldehyde solution in 0.1 M phosphate buffer for 24 h at 4 °C, embedded in paraffin, sectioned (5 μm thickness), and submitted to TUNEL (Terminal transferase-mediated dUTP Nick-End Labeling) assay, using the TdT-FragEL DNA fragmentation detection kit QIA 33 (Calbiochem, San Diego, CA, USA) following the manufacturer's protocol. Briefly, the sections were washed in TRIS buffer saline pH 7.6 (TBS), treated with 20 μg/ml proteinase K in 0.01 M Tris–HCl buffer at pH 8 for 20 min, and then treated with 3% hydrogen peroxide (H_2O_2) in TBS for 30 min to inactivate the endogenous peroxidase. The sections were then incubated with terminal deoxynucleotidyl transferase (TdT) and biotinylated deoxynucleotides for 3 h at 37 °C. Sections were incubated with peroxidase-conjugated streptavidin for 45 min at room temperature, developed with diaminobenzidin (DAB, Sigma Aldrich's Corp., St. Louis, MO, USA) in TBS for 8 min, and counterstained with hematoxylin. The negative control excluded treatment with TdT/labeled deoxynucleotides.

2.4. Immunohistochemistry

Ovarian samples were fixed in 4% paraformaldehyde solution in 0.1 M phosphate buffer for 24 h at 4 °C, embedded in paraffin, sectioned at 5 μm thickness and submitted to immunohistochemistry reaction for detection of PCNA (mouse anti-PCNA monoclonal antibody, clone PC-10, dilution 1:200, supplier sc-56, Santa Cruz Biotechnology, Santa Cruz, CA, USA) and caspase-3 (mouse anti-caspase-3 monoclonal antibody, clone E-8, dilution 1:200, supplier sc-7272, Santa Cruz Biotechnology). The antigen retrieval was performed in citrate buffer (pH 6.0) for 5 min after boiling in a microwave oven. Next, the sections were incubated with 3% H_2O_2 in methanol to inactivate endogenous peroxidase. For permeabilisation and to block unspecific staining, blocking buffer (2% bovine serum albumin + 0.05% Triton X-100 + 0.01% Tween 20) with 10% of normal horse serum was used for 45 min. Next, primary antibody was applied to the sections overnight in a humidified chamber at 4 °C. For immunoperoxidase (PCNA), the sections were incubated for 45 min with biotinylated goat anti-mouse secondary antibody (1:200, LSAB 2 System HRP, DakoCytomation Kit, Carpinteria, CA, USA), and treated with peroxidase-conjugated streptavidin for 45 min. Labeling was visualized by diaminobenzidine (DAB) counterstained with hematoxylin. For immunofluorescence (PCNA and caspase-3), the sections were incubated with a secondary antibody ALEXA 488 (goat anti-rabbit IgG, 1:200, A11034, Life Technologies, Carlsbad, CA, USA). For the negative control, the treatment with the primary antibody was omitted. Fluorescence images were