Journal of Fish Biology (2005) **67,** 1446–1459
doi:10.1111/j.1095-8649.2005.00854.x, available online at http://www.blackwell-synergy.com

Ovarian regression and apoptosis in the South American teleost *Leporinus taeniatus* Lütken (Characiformes, Anostomidae) from the São Francisco Basin

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(Received 23 December 2004, Accepted 17 May 2005)

Involution and resorption of both postovulatory and atretic follicles were analysed in piau-jejo *Leporinus taeniatus* (Characiformes, Anostomidae) in order to evaluate the role of apoptosis during ovarian regression. Histological and ultrastructural analyses showed hallmarks of apoptosis in the granulosa: aggregation of compacted chromatin against the nuclear envelope, cell shrinkage, surface blebbing, loss of cell adhesion and cell fragmentation into apoptotic bodies. Protein synthesis activity preceded the onset of the cell death. The breakdown of the basement membrane led to the detachment of the granulosa cells into the follicular lumen. TUNEL-positive reactions were detected in *in situ* DNA fragmentation of granulosa of both postovulatory and atretic follicles. Apoptosis increased in a time-dependent manner contributing to reduction of the follicular areas. The apoptotic index (per cent of apoptotic cells) of the granulosa increased in postovulatory follicles soon after spawning, then these follicles degenerated and only remnants were observed at 7 days. In contrast, the granulosa cells reabsorbed the yolk during follicular atresia and the apoptotic index increased only in the late stage of regression. The results indicated apoptosis as the major mechanism to rapidly eliminate postovulatory follicles and being an essential process in the ovarian regression after spawning.

Key words: apoptosis; follicular atresia; granulosa; *L. taeniatus*; postovulatory follicle; TUNEL.

INTRODUCTION

Postovulatory follicles are morphological indicators of spawning in fishes that are quickly reabsorbed during postspawning ovarian regression (Lang, 1981*a*; Saidapur, 1982; Isaac-Nahum *et al.*, 1988; Selman & Wallace, 1989). Studies on degeneration and resorption of these follicles provide information in the analysis

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of the time and frequency of spawning during the reproductive cycle (Stequert *et al.*, 2003). Follicular atresia is a common degenerative process in ovaries of vertebrates that can be induced by several factors such as hypophysectomy, irradiation, anti-gonadotrophic agents, fasting, stress and confinement (Saidapur, 1978; Lang, 1981*b*; Bromley *et al.*, 2000; Wood & Van Der Kraak, 2002). In teleosts, atresia is more frequently found in vitellogenic oocytes and, as a consequence, it may reduce the reproductive potential of the species (Rizzo & Bazzoli, 1995; Miranda *et al.*, 1999).

The programmed cell death or apoptosis is an energy-dependent process, conserved during evolution and essential to maintain the tissue homeostasis in all multicellular organisms (Kerr *et al.*, 1972; Wyllie *et al.*, 1980). It can be triggered by physiological or pathological stimuli, involving the participation of caspase-type proteases that activate Ca^{2+}- and Mg^{2+}-dependent endonucleases, which cleave DNA into multiples of 180–200 base pairs (Thompson, 1995; Huettenbrenner *et al.*, 2003). The process affects single cells that detach from neighbouring ones and basement membrane; cell integrity is maintained during apoptosis, avoiding an inflammatory reaction (Robertson & Orrenius, 2000). The apoptotic cell exhibits typical morphological features such as aggregation of compacted chromatin in a crescent pattern underlying the nuclear envelope, cell shrinkage, surface blebbing and cell fragmentation into apoptotic bodies (Wyllie *et al.*, 1980). Externalization of phosphatidylserine on the plasma membrane plays a signalling role for phagocytes and neighbouring cells in the withdrawal of the apoptotic bodies (Tyurina *et al.*, 2000). In mammals, at least two distinct signalling pathways have been described: an extrinsic pathway initiated by extracellular ligands of the tumor necrosis factor (TNF) superfamily and an intrinsic pathway including anti-apoptotic and pro-apoptotic members of the Bcl-2 family (Huettenbrenner *et al.*, 2003). The inhibition of the anti-apoptotic Bcl-2 pathway induces apoptosis of the granulosa in the follicular atresia of mammals and birds (Hsueh *et al.*, 1994; Johnson *et al.*, 1996; Johnson, 2003).

The role of apoptosis in the reproductive dynamics of teleosts has been poorly investigated. It has been related, however, to the elimination of needless cells during gonadal differentiation (Uchida *et al.*, 2002), development of ovarian follicles (Janz & Van Der Kraak, 1997), postovulatory follicles regression (Drummond *et al.*, 2000) and follicular atresia (Wood & Van Der Kraak, 2001). Both, *in vitro* and *in vivo* studies have shown that gonadotropins, 17-ß oestradiol and the epidermal growth factor act as apoptosis suppressers in preovulatory follicles of the rainbow trout *Oncorhynchus mykiss* (Walbaum) (Janz & Van Der Kraak, 1997; Wood & Van Der Kraak, 2002). Recent studies indicate apoptosis as a biomarker of environmental impact, since fishes exposed to xenobiotics have shown a decrease in the gonado-somatic index associated with an increase in the apoptosis rate (Janz *et al.*, 1997, 2001; Piechotta *et al.*, 1999; Weber & Janz, 2001; Weber *et al.*, 2002).

The teleost *Leporinus taeniatus* Lütken (Characiformes: Anostomidae) is a medium-sized species native to the São Francisco Basin. This species reproduces during the rainy season from December to February, exhibiting the following reproductive strategies: total spawning, group synchronous oocyte development, non-adhesive eggs, quick embryonic development and absence of parental care (Rizzo *et al.*, 2002; Sato *et al.*, 2003*a*). Like other anostomids, *L. taeniatus* does

not reproduce spontaneously in captivity; hormonal induction is required to complete its reproductive cycle in hatcheries.

Considering the importance of apoptosis in the ovarian function, the present investigation analysed the resorption dynamics of both postovulatory and atretic follicles using *L. taeniatus* as an experimental model to evaluate apoptosis of the granulosa during ovarian regression.

MATERIALS AND METHODS

SAMPLING

Adult females of *L. taeniatus* (19·5 ± 1·4 cm standard length, L_S, and 80·0 ± 20·8 g body mass) in advanced gonadal maturation were submitted to hypophysation at the Hydrobiology and Hatchery Station of Três Marias, Minas Gerais, Brazil (18°12′ S; 45°15′ W), as described by Sato *et al.* (2003*b*). Oocytes extrusion was performed through coelomic wall massage at 12 h after hormonal injection. For postovulatory regression analyses, 34 spawned females were kept in concrete tanks with continuous water flow at 23–24° C for 7 days. Eight females, which did not respond to the hormonal treatment, were also used to examine follicular atresia. Ovarian regression during postspawning was assessed through the gonado-somatic index (I_G, $I_G = 100 M_G\ M_B^{-1}$, where M_G is gonadal mass and M_B is body mass).

LIGHT MICROSCOPY

Samples of ovaries at different time intervals, varying from recently spawned ovaries to 7 days after spawning were fixed in Bouin's fluid for 6–8 h or in Carnoy solution for 24 h at 4° C. The specimens were embedded in paraffin or glycol methacrylate, sectioned in 3–5 μm thickness and stained with haematoxylin-eosin, toluidine blue-sodium borate and periodic acid-Schiff (PAS) counter-stained with haematoxylin.

TRANSMISSION ELECTRON MICROSCOPY

Ovary samples at different time intervals after spawning were fixed in modified Karnovsky solution (2·5% glutaraldehyde and 2% paraformaldehyde in 0·1 M phosphate buffer at pH 7·3) for 18 to 24 h at 4° C. Post-fixation was performed in 1% osmium tetroxide with 1·5% potassium ferrocyanide for 2 h, then the tissues were embedded in Epon/Araldite plastic resin. Ultrathin sections were stained with uranyl acetate and lead citrate.

TUNEL

Ovary samples were fixed in 4% paraformaldehyde solution in 0·1 M phosphate buffer solution (PBS), embedded in paraffin and submitted to the TUNEL technique by using the *in situ* Cell Death Detection kit-POD (Roche, Mannheim, Germany). Sections of 6 μm thickness were washed in PBS and treated with 20 μm ml^{-1} of proteinase K in 10 mM Tris-EDTA buffer at pH 7·5 for 15 min, permeabilized with 0·1% Triton X-100 and treated with 0·3% H_2O_2 in methanol to inactivate endogenous peroxidase. The sections were treated with terminal deoxynucleotidyl transferase (TdT) and fluorescein-conjugated deoxinucleotides for 3 h at 37° C, then with anti-fluorescein antibody conjugated with peroxidase for 1·5 h at 37° C, developed with diaminobenzidin (DAB) for 15 min and counter-stained with haematoxylin. Rat thymus sections previously treated with corticoid were used as positive control. The negative control was performed without the treatment with TdT/labelled-deoxinucleotides.

MORPHOMETRY

Areas of both postovulatory and atretic follicles were measured by using the software for digital image analyses, Kontron KS 400 (Carl Zeiss Vision GmbH, Germany; www.zeiss.de). The mean areas of the follicles were calculated using 60 postovulatory follicles at different time intervals from recently spawned ovaries to 72 h postspawning and 60 atretic follicles in early, advanced and late regression.

The apoptotic index for the granulosa (I_A, $I_A = 100C_A\ C^{-1}$, where C_A is number of apoptotic cells and C is number of whole cells) in both postovulatory and atretic follicles was determined according to Moro *et al.* (2003). At least two of the following histological alterations were considered as characteristics of apoptosis: cell shrinkage, loss of adhesion, detachment from basement membrane, chromatin condensation in a crescent pattern underlying the nuclear envelope and cellular fragmentation into apoptotic bodies. Mean I_A was obtained using 60 postovulatory follicles in each time until 72 h postspawning or 60 atretic follicles in each regression phase.

STATISTICAL ANALYSIS

An ANOVA followed by Tukey's test was used to compare the mean ± S.D. of I_G, I_A and area during regression of the postovulatory follicles and atretic follicles ($P < 0{\cdot}05$). Pearson's correlation was used to correlate time, area and I_A.

RESULTS

Immediately after spawning, the ovaries of *L. taeniatus* contained ovigerous lamellae with early and advanced perinucleolar follicles, postovulatory follicles, atretic follicles and rare residual mature vitellogenic follicles with the nucleus displaced toward the micropyle, which was occluded by micropylar cell. During ovarian regression, increased vascularization and granulocytes were observed in the connective stroma. Females that did not respond to the hormonal treatment had atretic follicles at different regression stages. The I_G decreased gradually with no significant difference up to 72 h postspawning. Normal vitellogenic follicles of *L. taeniatus* had nuclei with various nucleoli, yolk globules filling most of the ooplasm, peripheral cortical alveoli, thick zona radiata, squamous follicular cells and thin connective theca.

REGRESSION OF THE POSTOVULATORY FOLLICLES

Immediately after spawning, postovulatory follicles had a convoluted broad lumen, low granulosa cells supported by the PAS-positive continuous basement membrane, and a thin connective theca [Fig. 1(a), (b)]. The micropylar cell was attached to the follicle [Fig. 1(c)]. At 6 and 12 h postspawning, granulosa cells were hypertrophied, consisting of a single layer of high columnar cells [Fig. 1(d)]. At 24 h postspawning, the basement membrane was diffuse as visualized by PAS reaction and the theca was thickened [Fig. 1(e)]. Apoptosis figures were frequently observed at 48 and 72 h postspawning [Fig. 1(f)]. In addition, the breakdown of the basement membrane led to detachment of the granulosa cells into the partially occluded follicular lumen [Fig. 1(f)]. The micropylar cell was undamaged at 72 h after spawning. In intervals from 4 to 7 days, postovulatory follicles gradually collapsed and showed progressive occlusion of the lumen,

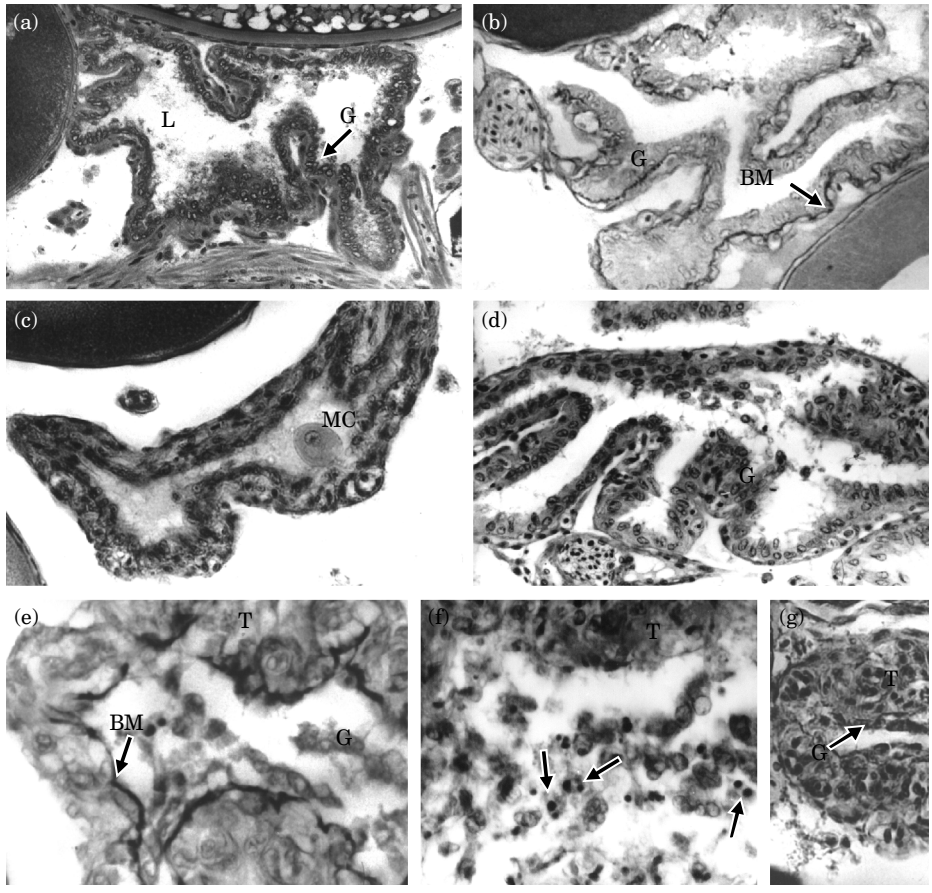

FIG. 1. Histological sections of postovulatory follicles of *Leporinus taeniatus* stained with (a) toluidine blue, (b), (e) PAS-haematoxylin and (c), (d), (f) haematoxylin-eosin. (a) Recently spawned follicle, ×239. (b) PAS-positive intact basement membrane in recently spawned follicle, ×367. (c) Micropylar cell attached to the granulosa at 6 h postspawning, ×327. (d) Columnar granulosa cells at 6 h postspawning, ×287. (e) Detached granulosa cells from the damaged basement membrane and thick theca at 48–72 h postspawning, ×638. (f) Granulosa cells in apoptosis in the follicular lumen at 72 h postspawning, ×654. (g) Squamous granulosa cells and fibrous theca at 7 days postspawning, ×375. L, lumen; G, granulosa; BM, basement membrane; MC, micropylar cell; T, connective theca; ➞, apoptotic granulosa cell.

decrease of the granulosa and thickening of the theca. Finally, granulosa remains were squamous and the theca became fibrous at 7 days postspawning [Fig. 1(g)].

Ultrastructural analyses showed that the hypertrophied granulosa cells had a rough endoplasmic reticulum, mitochondria, a developed Golgi complex [Fig. 2(a), (b)] and apical pseudopodium-like-projections toward the lumen [Fig. 2(c)]. Contacts such as tight junctions coupled adjacent cells [Fig. 2(a)]. Apoptosis characteristics such as a compacted chromatin aggregated against the nuclear envelope [Fig. 2 (b)], evident nuclear fragmentation, loss of cell adhesion [Fig. 2(c)], surface blebbing and intact organelles [Fig. 2(d)] were detected in the granulosa at different time intervals postspawning.

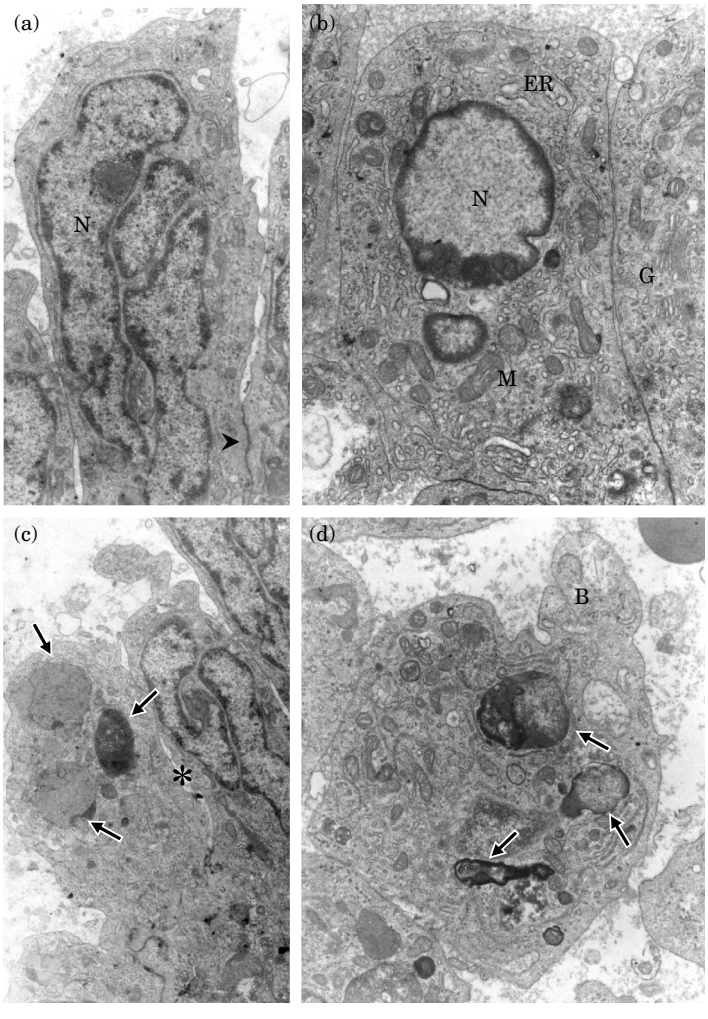

FIG. 2. Ultrastructure of the granulosa in postovulatory follicles of *Leporinus taeniatus*. (a) Hypertrophied granulosa cell and probable tight junctions in adjacent cells, ×8509. (b) Nucleus with compacted chromatin underling the nuclear envelope, indicating apoptosis, ×12 831. (c) Apical pseudopodium-like projection toward the lumen, nuclear fragmentation and loss of cellular adhesion, ×4688. (d) Blebbing on the apoptotic cell surface, ×8531. N, nucleus; ➤, tight junction; ER, endoplasmic reticulum; M, mitochondria; G, Golgi apparatus; ⟶, nuclear fragmentation; ✻, loss of cell adhesion; B, surface blebbing.

FOLLICULAR ATRESIA

The atretic follicles were classified into three stages based on the morphological changes in the ooplasm and surrounding follicular layers. During the early atresia stage, yolk liquefaction, zona pellucida breakdown, yolk releasing and hypertrophy of the granulosa were observed [Fig. 3(a), (b)]. During advanced atresia, highly columnar granulosa cells exhibiting intense phagocytic activity ingested most of the yolk and zona pellucida remains [Fig. 3(c)]. During the late

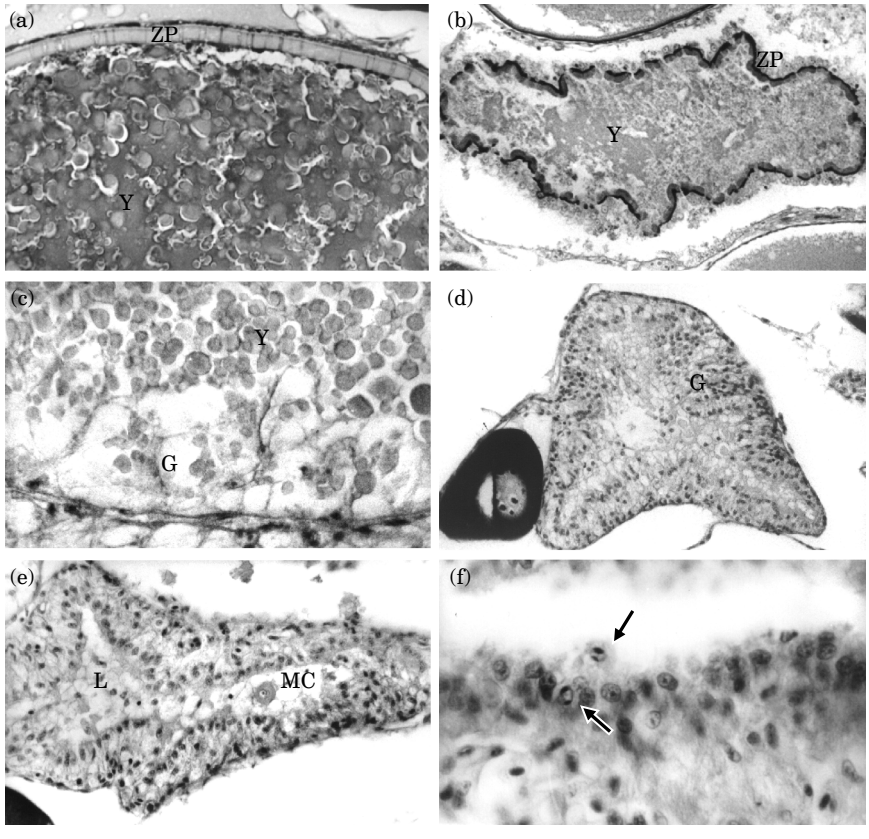

FIG. 3. Histological sections of atretic follicles of *Leporinus taeniatus* stained with haematoxylin-eosin. (a) Atretic follicle in early degeneration with delicate tears in the zona pellucida, ×410. (b) Zona pellucida breakdown and yolk liquefaction during early atresia, ×164. (c) Highly columnar granulosa engulf yolk in advanced atresia, ×566. (d) Vacuolated atretic follicle with most yolk reabsorbed in advanced atresia, ×186. (e) Micropylar cell in late atresia, ×410. (f) Apoptotic granulosa cells during late atresia, ×596. ZP, zona pellucida; Y, liquefaction of the yolk; G, hypertrophied granulosa cells; MC, micropylar cell; L, lumen; ➞, apoptotic granulosa cell.

stages, granulosa cells filled the partially occluded follicle lumen and they reabsorbed the residual yolk [Fig. 3(d)]. The theca became thicker and vascularized while granulocytes invaded the follicular lumen. The micropylar cell was unaltered in the late stage of regression [Fig. 3(e)]. Figures of apoptosis were frequent in this phase [Fig. 3(f)]. The atretic follicles progressively collapsed, the granulosa declined and the theca became fibrous. Finally, the remaining granulosa cells containing yellow-brownish pigments were associated with vascularized connective tissue.

Ultrastructural analyses revealed increased protein synthesis and endocytic activity in the granulosa [Fig. 4(a), (b)]. Digestive vacuoles with the remains of degenerated organelles and myelin figures were numerous in the cytoplasm [Fig. 4(c)]. Ingested apoptotic bodies were observed in the advanced stages of follicular atresia [Fig. 4(d)].

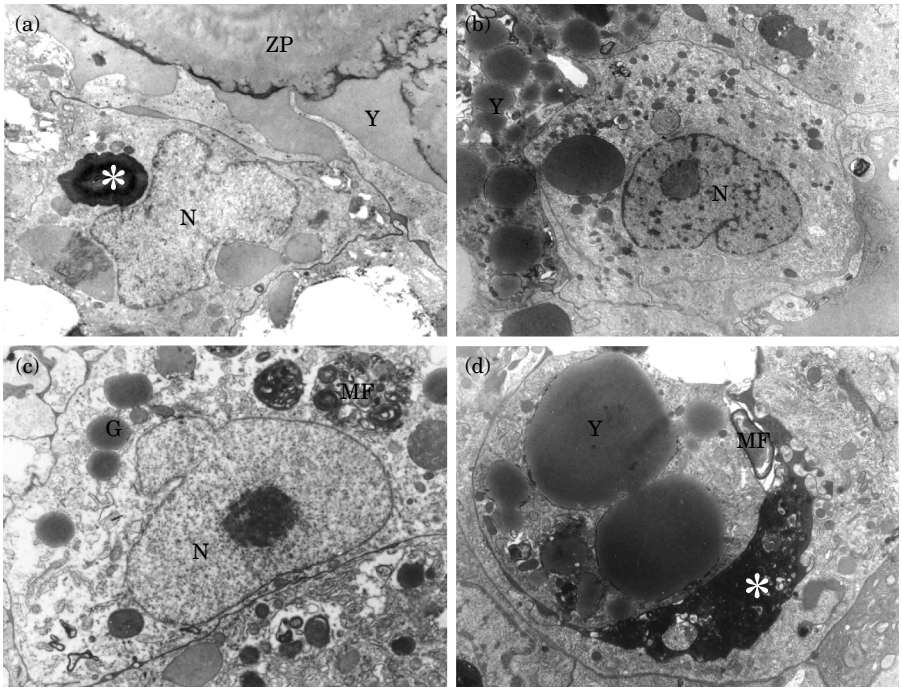

FIG. 4. Ultrastructure of atretic follicles in *Leporinus taeniatus*. (a) Early atresia with fractured zona pellucida, released yolk and granulosa cell in initial resorption of yolk, ×3796. (b) Granulosa cell showing endocytic activity in advanced atresia, ×2770. (c) Granulosa with characteristic cell secretion and with myelin figures in the cytoplasm during advanced atresia, ×4070. (d) Cellular degeneration such as an apoptotic body in the granulosa, ×3440. ZP, fractured zona pellucida; Y, yolk; N, nucleus; ✻, electron-dense structure; G, electron-dense granule; MF, myelin figure.

TUNEL

In the postovulatory follicles, TUNEL-positive cells were mainly observed in the granulosa [Fig. 5(a)]. Cell fragments such as apoptotic bodies in the follicular lumen and theca cells were also labelled by TUNEL technique at 48 and 72 h postspawning. During follicular atresia, TUNEL-positive granulosa cells were most frequently observed in the late stages of regression [Fig. 5 (b)].

FOLLICULAR AREA AND APOPTOTIC INDEX

In the postovulatory follicles, both area and I_A were time-dependent. The mean area of these follicles decreased *c.* 16% at 6 h and *c.* 60% at 72 h after spawning, and there was no significant difference from 12 to 48 h postspawning [Fig. 6(a)]. The I_A of the granulosa increased significantly at 12 h, with *c.* 60% enhancement at 72 h after spawning as compared to recently ovulated follicles, and no significant difference was detected from recently spawned follicles to 6 h after spawning. A negative correlation was obtained between area and time ($r = -0{\cdot}41$) and positive between I_A and time ($r = 0{\cdot}54$) in these follicles.

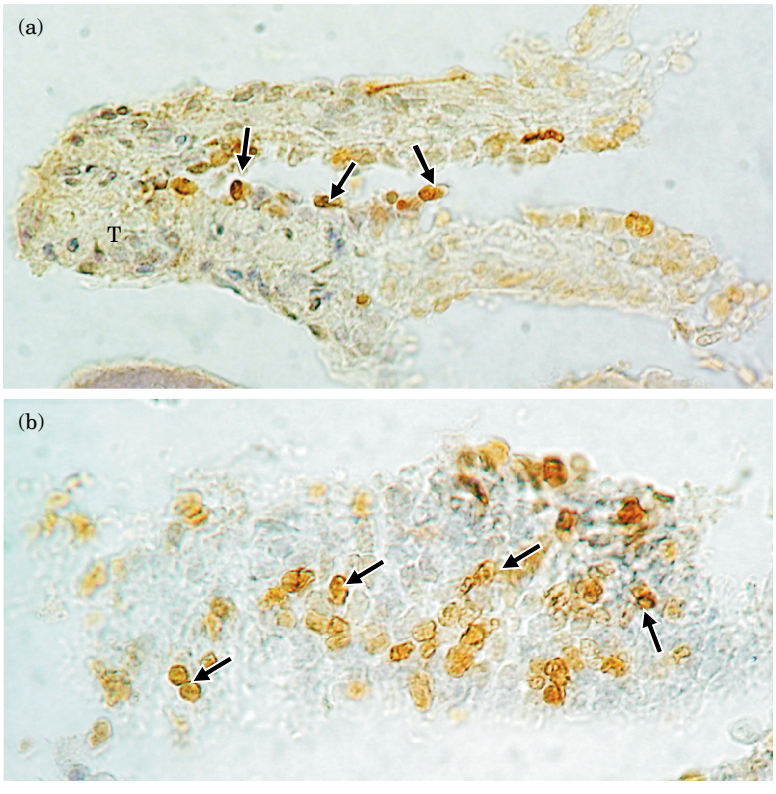

FIG. 5. TUNEL-positive reactions (➡) in apoptotic granulosa cells of (a) a postovulatory (×759) and (b) an atretic follicle (×1327) in *Leporinus taeniatus*. Counter-staining with haematoxylin.

In the atretic follicles, the areas diminished significantly during the regression stages as compared to the non-atretic follicles, reaching up to 50% reduction during the early stage, 80% in the advanced and >90% in the late stage. The I_A increased significantly during the regression, however, the rates were inferior to those calculated for postovulatory follicles [Fig. 6(b)]. A highly negative correlation ($r = -0{\cdot}70$) was detected between I_A and area during follicular atresia and a low correlation ($r = -0{\cdot}21$) was obtained during regression of the postovulatory follicles.

DISCUSSION

Ovulation was attained in most females, since their oocytes showed germinal vesicle migration towards the micropyle, an important event of the final oocyte maturation (Selman & Wallace, 1989).

During postspawning ovarian regression, shrinkage and resorption of both postovulatory and atretic follicles occurs and the ovaries return to the resting period to initiate another reproductive cycle. In general, postovulatory follicles are rapidly reabsorbed without leaving signs in the connective stroma (Isaac-Nahum *et al.*, 1988). In the present study, postovulatory follicles in late

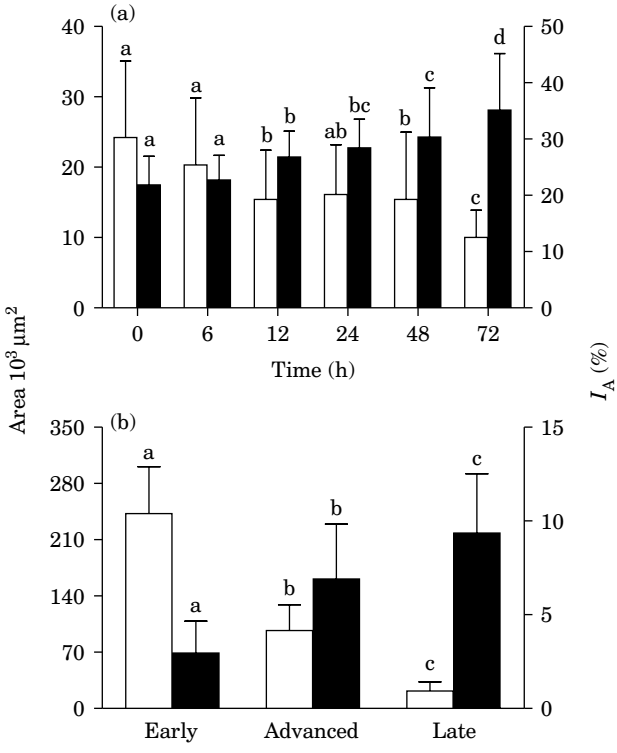

FIG. 6. Areas (□) and apoptotic indicies (■) of granulosa in (a) postovulatory follicles (POF) at different times postspawning and (b) during early, advanced and late stages of the follicular atresia (AF). Values represent means ± S.D. ($n = 60$ POFs at each time and $n = 60$ AF at each phase). Common lower case letters indicate that means do not differ significantly ($P > 0{\cdot}05$).

regression were recorded within 7 days after spawning. In the lambari *Astyanax bimaculatus* (L.) submitted to the same conditions as the present study, resorption of the postovulatory follicles was completed at 11 days after spawning (Drummond *et al.*, 2000). Results varying from 2 days in *Tilapia zilli* (Gervais) (Coward & Bromage, 1998) and *Vinciguerria nimbaria* (Jordan & Williams) (Stequert *et al.*, 2003) to 28 days in *Perca fluviatilis* L. (Lang, 1981*a*) could be due to the reproductive strategies of the different species and temperature during ovarian regression. In contrast, the degeneration and resorption of the atretic follicles is a prolonged process, varying from 4 to 7 months and, frequently, leaving signs in the ovary that can remain along of the next reproductive cycle (Rizzo & Bazzoli, 1995; Miranda *et al.*, 1999). In the present study, the ovarian regression was monitored during 7 days only and the atretic follicles were very active at this time.

Granulosa cells became highly columnar during the onset of postovulatory regression, as also recorded in other species (Rizzo & Bazzoli, 1993; Drummond *et al.*, 2000). As apoptosis is an energy-dependent process that needs specific enzymes (Thompson, 1995; Robertson & Orrenius, 2000), the cells acquire characteristics of protein synthesis before the beginning of the apoptosis as observed in *L. taeniatus*. In contrast, during follicular atresia, the hypertrophied

granulosa develop intense endocytic activity to ingest and degrade the yolk and the zona pellucida remains. Cells of the immune system, granulocytes and macrophages act synergistically with the granulosa in yolk resorption (Besseau & Faliex, 1994). According to Wood & Van Der Kraak (2003) a novel biochemical mechanism involving L-cathepsin mediating yolk proteolysis promotes the follicular atresia in oviparous vertebrates, which do not induce widespread follicular apoptosis as occurs in mammals.

In the present study, the increase in the granulosa I_A was coupled with an area reduction of both postovulatory and atretic follicles. In addition, the reduction of the I_G after spawning was associated with the increase of the apoptosis rate during follicular atresia in the white sucker *Catostomus commersoni* (Lacépède) (Janz *et al.*, 2001). Together these results indicate the apoptosis as an important mechanism for ovarian regression in teleosts. The increase of the I_A after spawning could be attributed to the decline in the hormonal levels, since oestradiol seems to act as anti-apoptotic agent, being responsible for the survival of ovarian follicles during gonadal maturation (Janz & Van Der Kraak, 1997). Moreover, ß1-integrin protects epithelial cells from apoptosis and when the basement membrane is altered, it suppresses the anti-apoptotic pathway, constituted by members of Bcl-2 family (Tibério *et al.*, 2002). Since the basement membrane was altered preceding the increase of the apoptosis rate in the present study, then the Bcl-2 pathway may be involved in the apoptosis of the granulosa in *L. taeniatus*.

The presence of the micropylar cell in the lumen of the postovulatory follicles of *L. taeniatus* was also observed in *A. bimaculatus* (Drummond *et al.*, 2000). The presence of this cell during follicular atresia has not been found in other species. Desmosomes and probably nexus join the micropylar cell to the neighbouring cells during oocyte maturation (Nakashima & Iwamatsu, 1989) and this coupling could maintain the integrity of the micropylar cell until the late stage of the follicular atresia. Ultrastructural studies showed that the micropylar cell acts not only as plug of the micropyle but also as a secretory cell (Nakashima & Iwamatsu, 1989), however, the function of this cell is unknown after spwaning.

The major morphological events of follicular atresia are similar in oviparous teleosts (Saidapur, 1978; Lang, 1981*b*; Miranda *et al.*, 1999; Bromley *et al.*, 2000). In mammals, the apoptotic pathway is activated soon after the suppression of cell survival factors, constituting a primary event of the follicular atresia (Hsueh *et al.*, 1994; Kaipia & Hsueh, 1997; Chun & Hsueh, 1998). Since the granulosa cells are important in proteolysis and resorption of the yolk in teleosts, apoptosis is increased only in late stages of follicular atresia, as observed in the present study and in *O. mykiss* (Wood & Van Der Kraak, 2003). Thus, autocrine and paracrine mechanisms must be acting in the teleost ovary after spawning contributing to the survival of granulosa cells during yolk resorption in atretic follicles.

The present findings support the conclusion that apoptosis accounts for deletion of the granulosa during resorption of both postovulatory and atretic follicles of *L. taeniatus*, thus performing an important role on postspawning ovarian recovery in teleosts. In addition, the results suggest that the granulosa cell death may be due to the inhibition of the anti-apoptotic Bcl-2 pathway as occurs in the ovarian follicles of mammals and birds.

We thank the Hydrobiology and Hatchery Station of Três Marias, CODEVASF for assistance during this investigation. The study was supported by grants from FAPEMIG, CNPq and CAPES.

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