E.Ü. Su Ürünleri Dergisi 2006
E.U. Journal of Fisheries & Aquatic Sciences 2006
Cilt/Volume 23, Sayı/Issue (3-4): 263–267

ISSN 1300 - 1590
http://jfas.ege.edu.tr/

Apoptosis in Postovulatory Follicles of *Chalcalburnus tarichi* Pallas, 1811*

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Özet: ***İnci kefali*** Chalcalburnus tarichi***, Pallas, 1811 postovulasyon foliküllerinde apoptoz.*** Bu çalışmada, inci kefali (*Chalcalburnus tarichi*) postovulasyon foliküllerinde apoptoz, TUNEL boyama ve agaroz jel elektroforez metodları ile araştırıldı ve plazma 17β-estradiol (E_2) seviyesi ovulasyondan sonra 1., 3. ve 5. günlerde belirlendi. Postovulasyon foliküllerinde apoptotik hücreler, histolojik kesitlerde, ovulasyondan sonra 1. günde işaretlendi ve apoptozun çoğunlukla granuloza nadiren de teka hücrelerinde meydana geldiği belirlendi. Apoptozun işareti olan oligonükleozomal büyüklükteki DNA parçalanması ovulasyondan sonra 1., 3. ve 5. günlerde gözlendi. Ovulasyon anında oligonükleozomal DNA parçalanması gözlenmezken, 3. ve 5. günlerde bu parçalanmanın belirgin olduğu görüldü. Plazma E_2 seviyesi ovulasyondan sonra dereceli olarak düştü. Bu düşüş 3. ve 5. günlerde önemliydi ($P<0.05$). Bu sonuçlar, inci kefalinde apoptozun postovulasyon foliküllerinin ortadan kadırılmasından sorumlu olduğunu gösterir bununla birlikte ovulasyondan sonra düşük E_2 seviyesinin postovulasyon folikül apoptozunu hızlandırdığı düşünülmektedir.

Anahtar Kelimeler: Apoptoz, *Chalcalburnus tarichi*, Postovulasyon Folikülü, Van Gölü, 17β-östradiol.

Abstract: In the present study, apoptosis in postovulatory follicle was investigated with TUNEL staining and agarose gel electrophoresis methods, and plasma 17β-estradiol (E_2) level was determined at the 1st, 3rd and 5th day after ovulation in *Chalcalburnus tarichi*. Apoptotic cells were labelled in postovulatory follicles in histological sections on the 1st day after ovulation and it is indicated that apoptosis was observed mainly in granulosa cells and scarcely in theca cells. Oligonucleosomal length DNA fragmentation which is hallmark of apoptosis was observed on the days 1, 3 and 5 after ovulation. While no oligonucleosomal DNA fragmentation was observed on the ovulation time, it was observed that this fragmentation was remarkable on the days 3 and 5. Plasma E_2 level decreased gradually after ovulastion. This decrease was significant on the days 3 and 5 ($P<0.05$). These results demonstrate that apoptosis is responsible for elimination of postovulatory follicles however it was considered that low level of E_2 after ovulation, accelerates the postovulatory follicle apoptosis in *Chalcalburnus tarichi*.

Key Words: Apoptosis, *Chalcalburnus tarichi,* Postovulatory Follicle, Lake Van, 17β-estradiol.

** This study is a MSc thesis and was supported by a Grant from the Scientific Research Projects Presidency of Yüzüncü Yıl University (2003-FED-044).*

Introduction

C. tarichi is an endemic cyprinid species of the lake Van basin in Turkey. Having an anadromous character, this carp migrates to the streams pouring into the lake in order to spawn.

Apoptosis is a physiological type of cell death which plays role in the renewing and remodelling of the tissues (Kerr *et al*. 1972; Schwartzman and Cidlowski 1993). The internucleosomal DNA fragmentation which occurs as a result of Ca^{++}/Mg^{++}-dependent endonuclease activity is the most important biochemical hallmark of apoptosis (Wyllie 1980).

Follicle apoptosis occured in the ovary is under the control of hormones (Hsueh *et al*. 1994; Tilly 1996; Chun and Hsueh 1998). Estrogens play a role in the inhibition of apoptosis in ovary (Billig *et al*. 1993). It has been reported that luteal cell apoptosis increased depending on time as a result of depletion of exogenous estradiol and the supression of endogenous estradiol in the rabbit ovary (Goodman *et al*. 1998). Endonuclease activity increasing with luteinization of granulosa cells differentiated after ovulation in the rat ovary was been determined (Zeleznik *et al*. 1989). Tilly *et al*. (1991), identified an internuclesomal DNA fragmentation during the postovulation regression in the chicken ovary. In the subsequent studies, researchers have shown that apoptosis plays a role in the regression of postovulation in numerous mammalian species (Jennifer *et al*. 1993; Matsuyama *et al*. 1996; McCromark *et al*. 1998).

Apoptosis was investigated in the previtellogenic and preovulatory follicles of rainbow trout. Based on these investigations, it was reported that apoptosis was associated with different stages of follicle development and that the spontaneous apoptosis occured in preovulatory follicles incubated in serum-free medium was supressed by gonadotropins, epidermal growth factor (EGF) and 17β-estradiol (E_2) (Janz and Van Der Kraak 1997). Apoptosis was found to be a basic mechanism responsible for the elimination of postovulatory follicles *Astyanax bimaculatus lacustris* (Drummond *et al*. 2000). In the studies conducted of forwards apoptosis was related with normal ovary development and it was played a role in postovulatory follicle regression in teleosts; however as it is the case with mammals, it does not occur as an earlier event in teleost follicle atresia (Wood and Van Der Kraak 2001). The effects of salmon gonadotropin (SG-100), EGF, insuline like growth factor and the differrent concentrations of E_2 on spontaneous apoptosis occuring in

vitellogenic follicles cultured under serum-free medium in rainbow trout were investigated, and it was reported that rainbow trout varied from mammals in terms of the cell types susceptible to apoptosis and its unresponsiveness to specific growth factors in the inhibition of apoptosis (Wood and Van Der Kraak 2002).

This study was conducted in order to demonstrate postovulatory follicle apoptosis and measure the plasma E_2 level during five day period after ovulation in *C. tarichi*.

Materials and Methods

Adult fishes used in this study were caught from Karasu river which pours into lake Van. The live fish brought to the laboratoary were put to 90x90x250 cm fiberglass tanks where there was a water flow under natural photoperiod with a water temperature 16-18 ^oC. Fish which were ready for spawning were stripped and then put into seperate tanks and they were fed with live zooplankton. The stripping or ovulation day was defined as 0 day. The fish were anesthesied with methanesulphonate (MS-222) and then the tissue and blood samples were taken. The blood samples from the fish were taken with heparanized enjectors by cutting their tail on the post-stripping days of 1st, 3rd and 5th. Ovaries were removed from the fish for histological examination and the remaining parts were stored in -76 ^oC deep-freeze for DNA extraction. Three fish were used for each analysis. The plasms obtained from the blood samples were kept in -76 ^oC deep-freeze untill analysis.

The ovaries were removed from fish which were kept in fiberglass tanks for one day and ovaries fixed in 4% paraformaldehyde were dehydrated and then embedded in parafin. The sections taken from the tissues (5 μm) were mounted on polylysine coated slides (Menzel Gläser, Germany) and then dried in 37 ^oC. Some section were stained with Haematoxylin and eosin and the others were used for histochemical detection of fragmented DNA.

TUNEL method was preferred in order to determine the apoptotic cells in postovulatory follicles. For this purpose, two different commercial kits, TdT-FragEL and Fleurescein-FragEL DNA fragentation kit (Oncogene Research Products, USA), were used. Sections taken from ovaries were stained with TUNEL Kits according to manufacture's protocols. Two slides for two different TUNEL kits were used as positive and negative controls. In the sections of negative controls, bidistilled water was used in place of TdT enzyme. As for the positive control sections, they were incubated with DNAase-I (1 μg/ml, Applichem) for 20 minutes and then exposed to TUNEL reagents. The sections applied to TdT-FragEL kit were stained with 3% Methyl Green for counterstaining. The preparations were examined on the Nicon Eclipse E600 microsope and then photos were taken. Some other sections stained with Fleurescein-FragEL kit were mounted with the mounting media mixed with propodium iodid (Oncogene Researc Pro., USA). The preparations were scanned with a Confocal Laser Scanning Microscope (Zeiss LSM-510 Meta, Germany) at multitract mode with Ar (488 nm) and with He-Ne (543 nm) lasers on, using a plan-neofluar X40 (N.A.1.3) oil immersion objective and then photos were taken.

DNA extraction and agarose gel electrophoresis

25 mg postovulatory follicle excised under stereo microscope from three fish pertained to each day (0, 1, 3 and 5 day after ovulation) were used for DNA extraction. Total genomic DNA was extracted with DNA Exraction Kit (Qiagen, Germany) according to kit protocols. After this procedure, purity of samples were quantified photometrically at A_{260}. The total yield from each extract was about 60-80 μg/ml then samples were stored at -20 ^oC until agarose gel electrophoresis was carried out.

The DNA extract was separated in 1% agarose gel prepared with Tris-Acetate EDTA (TAE: 0.04 M Tris Acetate, 1 mM EDTA) buffer. TAE buffer was also used as a running buffer. Each 15 μl DNA extract mixed with 5 μl 6X Loading Buffer (30% glycerol, 0.25% bromophenol blue, 0.25% xylene cyanol) was loaded onto agarose gel and run for 50 min at 100 V. "100 bp. Ladder" was used as a standart (100-1500 bp, Fermantas). The gel was stained, approximately for 20 min with ethidium bromide solution (1 μg/ml). The stained gels were examined with Syngene (Synoptics Ltd., Cambridge, GB) gel imaging and analysis system.

Three plasma samples pertained to each day (1st, 3rd and 5th) after ovulation were passed through Sep-Pak C-18 cartridges (Waters) preconditioned with methanol-bidistilled water mixture prepared in a proportion of 8:5. Cartridge adsorbing hormones were eluated with 2.5 ml methanol and eluation was injected into HPLC (Shimadzu, LC-10 AD). Stereoids were seperated by chromatographic system under isocratic conditions at a flow rate of 1.4 ml/min: μBondopak column with asetonitrile (35.5 %) as a mobile phase. The pressure and wavelength were 2800 psi and 254 nm, respectively. The retention time for E_2 was determined to be 14.4 min. The data were analyzed by analysis of variance (ANOVA). The significance level for differences was set at 0.05.

Results

In *Chalcalburnus tarichi,* a multidute of shrunk empty follicles (postovulatory follicle) and a small number previtellogenic oocytes were seen in ovarian sections (Figure 1).

Whereas the nuclei of non-apoptotic cells in the sections applied with TdT FragEL DNA fragmentation detection kit were stained in green colour, all apoptotic nuclei were stained in brown (Figure 2a,b). The nuclei of propodium iodid stained cells in the sections, applied with Florescein FragEL DNA fragmentation detection kit, were stained in red, whereas apoptotic nuclei were stained in bright green (Figure 3).

The apoptotic cells were observed to be scattered in postovulatory follicles. Apoptosis in postovulatory follicles were observed both in granulosa and theca cells one day after ovulation. Apoptosis was observed on a large scale in granulosa cells (Figure 2a,b and 3), but it was observed on a

small scale only in theca cell layer of some follicles (Figure 4).

In apoptotic cells of postovulatory follicles stained with TdT FragEL kit, the condensation of chromatin to perinuclear area as a crescent shaped and entity of apoptotic bodies were observed (Figure 5 a, b).

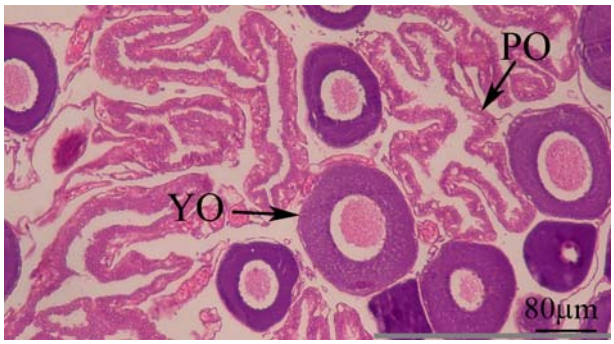
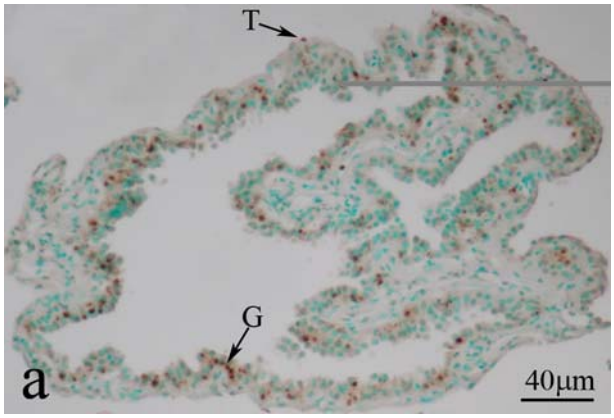


Figure 1. The ovary section after one day ovulation in *C. tarichi*. (PO), postovulatory follicle; (YO), previtellogenic oocyte (Haematoxylin and eosin).



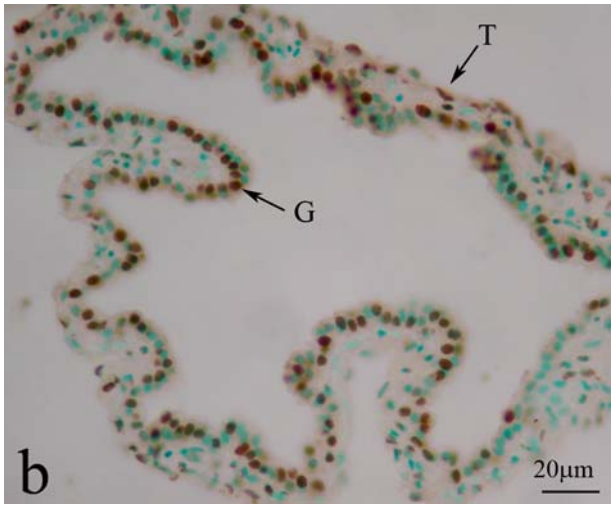


Figure 2. The apoptotic cells stained with TUNEL in the postovulatory follicles after one day ovulation. (a) Low magnification of the post ovulatory follicle. (b) Higher magnification of postovulatory follicle. G, granulosa cell; T, theca cell.

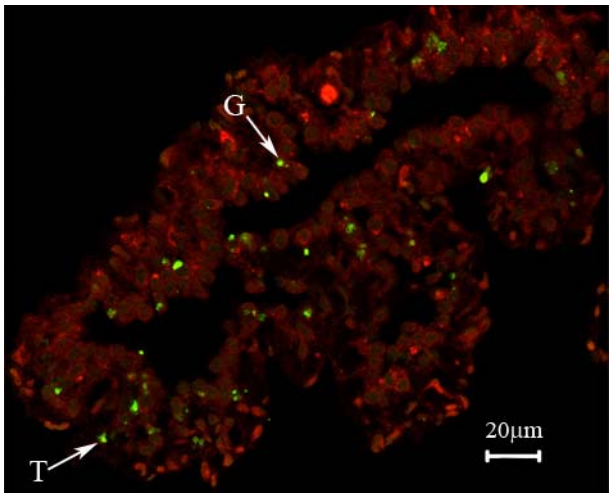


Figure 3. The apoptotic cells with green nuclei in laser scanning confocal micrograph stained with TUNEL of the postovulatory follicles after one day ovulation. (G), granulosa cell; (T) theca cell.

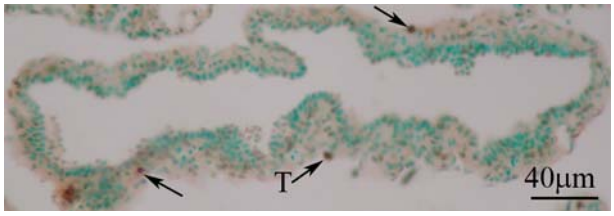
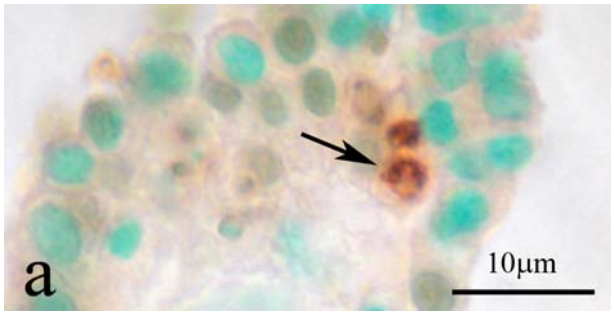


Figure 4. Apoptosis in postovulatory follicles stained with TUNEL after one day ovulation. Apoptosis was observed only in theca cells (T and arrows).



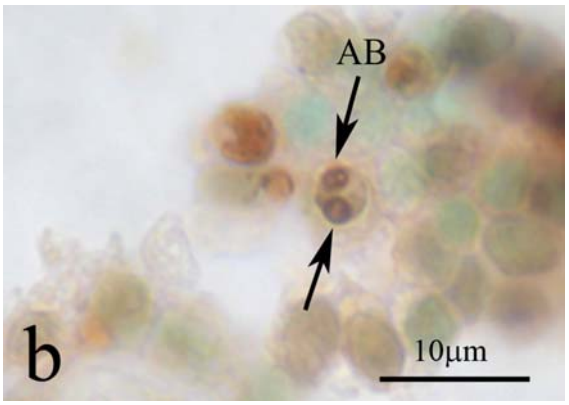


Figure 5. Apoptotic cells in the postovulatory follicle. (a) Condensation of chromatin in perinuclear area in a crescent shape (arrow). (b) Apoptotic bodies (AB and arrow).

Internucleosomal DNA fragmentation, in total DNA extracted from the postovulatory follicle, was not observed on the stripping day whereas a slight staining was observed on the first day and a remarkable staining on the 3rd and 5th days with ethidium bromide (Figure 6).

Plasma E_2 level on 1, 3 and 5 days after ovulation was measured of 4,83 ng/ml, 1,01 ng/ml, 0,64 ng/ml respectively. Plasma E_2 level on the 3rd day showed a significant decrease compared to the first day (P<0.05). As for the 5th day, such a decrease was insignificant compared to the third day but this decrease was not statically significant (P>0.05). The plasma E_2 levels in the postovulatory days of 1st, 3rd and 5th are shown in Figure 7.

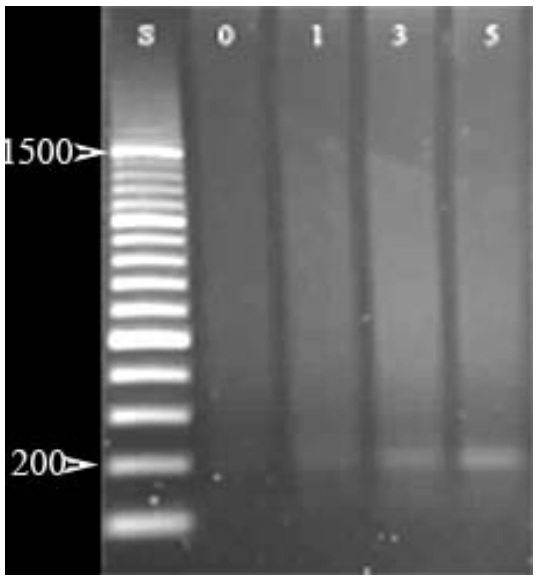


Figure 6. Ethidium bromide stained agarose gel illustration of DNA extracted from postovulatory follicles. No oligonucleosomal DNA fragmentation was observed on day 0 (ovulation day), a slight fragmentation on day 1 and remarkable fragmentations were observed on days 3 and 5. 100 bp ladder (S) were used as a standart. Days were indicated on the top of each lane and size of fragments were indicated on the left panel.

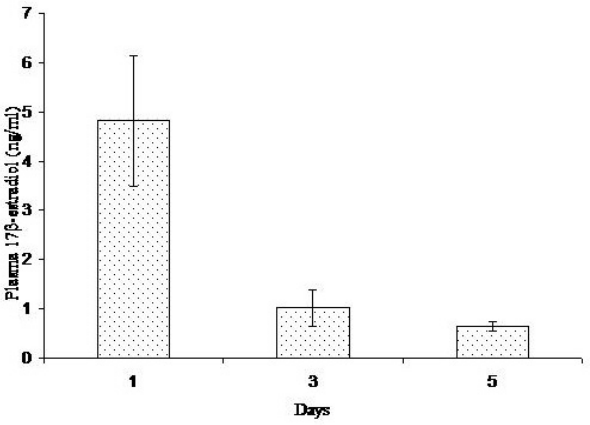


Figure 7. Plasma 17β-estradiol levels between 1st and 5th days after ovulation.

Discussion

Through this investigation it is shown that postovulatory follicles were eliminated with apoptosis by TUNEL methods and agarose gel electrophoresis, and plasma E_2 on the postovulatory 1st, 3rd and 5th days were measured.

Drummond *et al*. (2000), reported that apoptosis was a preliminary mechanism responsible for the elimination of postovulatory follicles in *Astyanax bimaculatus lacustris*. Wood and Van Der Kraak (2001) demonstrated through TUNEL method that apoptosis was associated with postovulatory regression in the rainbow trout *Onchorhyncus mykiss*. In applying TUNEL method in *C. tarichi*, we also found that postovulatory follicles are eliminated with apoptosis.

Apoptosis in the postovulatory follicles, first day after the ovulation, was observed to have commonly occured in granulosa cells and rarely in theca cells in *C. tarichi*. Similar results were reported from rainbow trout (Wood and Van Der Kraak 2001). Wood and Van Der Kraak (2001), reported that programmed cell death in vitellogenic and postovulatory follicles in rainbow trout, which oocytectomized and incubated under serum-free medium, was more intense than the follicles which did not undergo any such procedure of oocytectomy. However it was further pointed out that apoptosis in vitellogenic follicles could be supressed by oocyte entity and that follicle-oocyte communication could affect the suscebtibility to apoptosis. This may imply that little or no apoptosis in the granulosa cells of some follicles in *C. tarichi* can be explained in that the oocytes in these follicles are split from the follicles a bit earlier. The apparence of apoptosis especially in the granulosa cell layer may be due to the disconnection between the cells and the oocyte.

Internucleosomal DNA fragmentation was observed on the 1st, 3rd and 5th day after ovulation in *C. tarichi*. It has also been noted that the internucleosomal DNA fragmentation existed during luteal regression in chicken (Tilly *et al*. 1991), cattle (Jennifer *et al*. 1993), rat (Nitsuyama *et al*.1996) and hamster (McCormarck *et al*. 1998). It was also reported that endonuclease activity increased in granulosa cells after ovulation in rat ovary and that such increase continued together with luteinization in granulosa cells (Zeleznik *et al*. 1989). In our study, internucleosomal DNA fragmentation was not observed in postovulatory follicles on stripping day but a slight fragmentation was observed on the first day. This fragmentation gradually became more evident on the 3rd and 5th days. Although our findings, with regard to agarose gel electrophoresis, were not quantitative, it could be interpreted that the apoptotic cell death started on the first day after ovulation and increased on the 3rd and 5th days.

In our previous study (Ünal *et al*., 2005), ovary E_2 level was reported to have decreased before ovulation and such decrease continued after ovulation in *C. tarichi*. Similarly in this study, it was observed that the plasma E_2 level gradually decreased after ovulation. These results are in aggreement with results of other studies on *Salvenius leucomanis* (Kagawa *et al*. 1981), *Oncorhyncus rhodurus* (Kagawa *et al*.

1983), *Catosmus commersani* (Scott *et al*. 1984) and *Anchellgnathus rhombdea* (Shimizu *et al*. 1985).

Apoptosis was observed to have increased in a time-dependent manner as estradiol decrease in rabbit ovary, *in vivo* (Goodman *et al*. 1998). It was reported that estrogens inhibit internucleosomal DNA fragmentation in rat ovary (Billig *et al*. 1993) and that E_2 supplemented medium in rainbow trout (*Oncorhyncus mykiss*) inhibitted apoptosis in follicles in vitellogenic and preovulatory period (Janz and Van Der Kraak 1997; Wood and Van Der Kraak 2002). Based on these findings, E_2 is a crucially important factor for follicle cells in postovulation. As plasma E_2 level decreased after ovulation in *C. tarichi*, an oligonucleosomal DNA fragmentation was remarkable in 200 bp. region through electrophoresis. In accordance with the findings obtained by Goodman *et al*. (1998), plasma E_2 level in postovulatory period in *C. tarichi* decreased and internucleosomal DNA fragmentation becomed remarkable on the 5th day in our study. These results indicate that apoptosis which occurs in postovulatory follicles in *C. tarichi* may be accelerated depending on decrease in plasma E_2 level but we have to state that more detailed experimental study must be carried out for exact expression.

In conclusion, in *C. tarichi,* apoptosis is a basic mechanism responsible in elimination of postovulatory follicles and it was considered that low levels of E_2 accelerates the postovulatory follicle apoptosis.

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