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RESEARCH PAPER

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Immune Effects of Alarelin on Ovarian Development and GnRH Receptor mRNA Expression Levels of Pituitary in Tilapia (*Oreochromis niloticus*)

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Abstract

The effects of a gonadotropin releasing hormone analogue (GnRH-a, alarelin) on ovarian development and the associated gene expression in the tilapia (*Oreochromis niloticus*) pituitary gland were investigated for 60 days. Eighty sexually mature female tilapia were randomly divided into group I (GI, 10 μg alarelin), group II (GII, 15 μg alarelin), group III (GIII, 20 μg alarelin), and control group (GC). Anti-GnRH antibody levels peaked on day 30 in GI, days 40–50 in GII, and day 40 in GIII, and were highest in GII ($P<0.05$). The highest follicle stimulating hormone β (FSH-β) and luteinizing hormone β (LH-β) concentrations occurred in GII and GIII on day 40, and were higher in GII than in the other groups ($P<0.05$). No significant differences in FSH-β or LH-β concentrations were observed between GI and CG. FSH-β and GnRH receptor mRNA levels declined significantly in the pituitary after alarelin treatment, whereas LH-β mRNA increased by day 60. Alarelin treatment promoted ovarian and follicular development, which has implications for novel GnRH-based techniques established to regulate sexual maturation in tilapia.

Introduction

Gonadotropin releasing hormone analogues (GnRH-a) induce the secretion of luteinizing hormone β (LH-β) and follicle stimulating hormone β (FSH-β), and these hormones function directly on the ovary to stimulate steroidogenesis and gametogenesis (Crawford & McNeilly, 2002; Schneider et al., 2006). Recent studies have shown that there are eight types of GnRH in teleosts (Somoza et al., 2002), indicating that teleosts contain the most diverse group of GnRHs in extant vertebrates (Nelson, 1994). As well as different forms of GnRH, multiple forms of the GnRH receptor (GnRHR) were found. GnRHRs bind to GnRH and initiate intracellular signaling (Jodo et al., 2003). Because the limited amount of natural GnRH present in individual organisms led to it is difficult to extract. So GnRH analogues (GnRH-a) are often used experimentally in its stead. Several studies have reported that the binding affinity of GnRH for GnRHR is 100–200 times lower than the affinity between its analogue and the receptor (Cheng & Leung, 2005). Many studies have indicated that GnRH-a increase the releases of FSH-β and LH-β by the animal pituitary gland, which is relative to the effect of native GnRH through its stronger affinity for the receptor (Wang, 1995; Tarlatzis, & Kolibianakis, 2007). Injection of GnRH-a is associated with increased blood concentrations of LH-β and FSH-β (Wang, 1995; Tarlatzis, & Kolibianakis, 2007; Birhanu, Berihu, Solmon, Alemselam, & Gebrhiwot, 2015). The level of LH-β expression was significantly increased in the

plasma of striped bass (*Morone saxatilis*) treated with GnRH-a, which indicates that LH can be induced by exogenous GnRH (Mylonas et a1., 1997). An analysis of the genes that express LH-β and FSH-β during the development of the gonad in the male striped bass showed the LH-β expression was lower in the testes of immature males than those of mature males, and that the mRNA levels of FSH--β were elevated during early spermatogenesis in maturing males, whereas LH--β mRNA levels peaked during spermiation (Hassin et al., 2000). An mRNA analysis of ovarian fragments of the red seabream (*Pagrus major*) indicated that LH, but not FSH, stimulates estradiol-17β production and CYP19a1 gene expression, and that insulin-like growth factor (IGF-I) enhances LH-stimulated CYP19a1 gene expression. This suggests that LH-β is a major factor promoting ovarian maturation and synthesis of steroid hormones (Kagawa et al., 2003).

Previous studies have shown that GnRH increases the number of oocytes recovered during follicular development in heifers and ewes, and promotes oocyte maturation in rats (Wei et al., 2012). Active immunization with a GnRH agonist downregulated GnRH responsiveness to anestrus in cows and rabbits (Leung, Cheng, & Zhu, 2003; Wei et al., 2012; Wei, Gong, & Wei, 2011). The changes in the expression of GnRH during early development and the periods of gonadal sex differentiation were analyzed in the chub mackerel (*Scomber japonicus*) and suggested the possible involvement of GnRH during the early development and gonadal sex differentiation of this fish (Selvaraj et al., 2015). An analysis of the presence of GnRH2 and GnRH3 in the ovary during ovarian maturation in the zebrafish (*Danio rerio*) suggested that GnRH plays a paracrine role in the ovary (Corchuelo et al., 2017). A period of downregulation in the endocrine and ovarian follicular systems of domestic animals was detected after the administration of GnRH-a, but much less information has been reported regarding their restoration to normal levels (Docchio et al., 2000). Large doses of GnRH-a produce a contraceptive effect by inhibiting gonadal development (Leung, Cheng, & Zhu, 2003). Therefore, the exact effects and mechanisms of GnRH-a on reproductive performance in animals remain unclear (Bertschinger, Jago, Nothling, & Human, 2006). Treatment with GnRH-a can induce a failure of steroidogenesis in follicular cells and prevent the development of preovulatory follicles and ovulation (Gong et al., 1996; Rajamahendran et al. 1998; Garverick et al. 2002; Hampton et al. 2004).

Alarelin is a GnRH-a that consists of nine amino acids. It is usually used as an agonist to regulate reproduction in humans and animals because it is more readily synthesized and cheaper than other agonists (Wei, Gong, & Wei, 2011). The efficacy of alarelin is also 15–20 times higher than that of native GnRH (Wei et al., 2012). However, it is unclear whether alarelin influences gonadotropin secretion and the reproductive functions in tilapia, *Oreochromis niloticus.* Its effects on the number and quality of oocytes are particularly important if it is to be used as a novel profertility drug to enhance production (Docchio et al., 2000; Schneider et al., 2006). In this study, the effects of alarelin on ovarian development and the expression of the associated genes in female tilapia were investigated to allow the establishment of a new technique to control the reproductive functions of this species.

Materials and Methods

Experimental Animals

Eighty sexually mature female tilapia (mean body weight, 614±62.89 g; mean body length, 21±0.86 cm) were collected from the National Tilapia Breeding Ground, Nanning, Guangxi Province, and rapidly transported to our laboratory. The fish were randomly divided into four groups (n=20) and kept in four well-aerated freshwater aquaria containing 800 L water in a naturally ventilated room: group I (GI), group II (GII), group III (GIII), and the control group (CG). In this experiment, all the fish were raised with special feed (Guangdong Taihua Feed Co. Ltd, Guangdong, China) containing 125 g of crude fiber and 185 g of crude protein per kg.

Preparation of the Alarelin Antigen

The alarelin antigen was prepared according to the method of Wei, Gong, and Wei (2011). A 10 mg portion of bovine serum albumin (BSA; Sigma, St. Louis, MO, USA) and 10 mg of alarelin acetate (Zhejiang Biochemistry Company, Zhejiang Sheng, China) were thoroughly mixed and dissolved in double-distilled H_2O (ddH_2O) to prepare an alarelin–BSA solution. Then 250 mg of dimethylaminopropyl-3-ethylcarbodiimide hydrochloride (EDC-HCL, coupling reagent; Zhejiang Biochemical Reagent Co., Zhejiang Sheng, China) was added to the mixture, which was then dialyzed against ddH_2O for 50 h in a dialysis bag (Sigma). Freund's incomplete adjuvant (Shanghai Biochemical Sci-Tech Co. Ltd, Shanghai, China) was then added. The physical properties of the antigen were determined according to *Veterinary Biological Product Quality Inspection* (Wang, 2002).

Injection of the Alarelin Antigen and Collection of Samples

The alarelin antigen (10, 15, and 20 μg) was subcutaneously injected into the fish in the GI, GII, and GIII groups, respectively. The fish in the control group (CG) were injected with vehicle as the blank control. Samples of blood were removed from the tail veins at 10 day intervals from day 0 to day 60. The sera were

separated by centrifugation and stored at −20°C. The pituitary glands and ovaries were removed and fixed in 3% glutaraldehyde and 10% formaldehyde, respectively. These steps were performed under aseptic conditions.

Measurement of Gene Expression with Reverse Transcription–Real-Time Quantitative PCR (RT–Qpcr)

Primer Design

TaqMan probes and primers were designed for GnRHR, glyceraldehyde 3-phosphate dehydrogenase (GAPDH), FSH-β, and LH-β using the Primer Premier 5.0 software (Rozen & Skaletsky, 2000), and the sequences were Gn-RHR (GenBank accession number NM-001082738), *gapdh* (GenBank accession number NC-013676.1), FSH-β (GenBank accession number FJ-887900), and LH-β (GenBank accession number NM-001082695). The TaqMan probes were labeled at the 5′ and 3′ ends with the fluorescent reporter FAM and the fluorescent quencher BHQ1, respectively. Four concentrations of the primers (100, 200, 300, and 400 nM) and the production of primer dimers (Saibo Bio, Guangzhou, China) were evaluated with a melting curve analysis. The primers that did not generate dimer reactions were selected for the subsequent analysis. The primers and probes are shown in Table 1.

RNA Extraction and cDNA Synthesis

Total cellular RNA was extracted from 100 mg of each pituitary gland with TRIzol Reagent (Life Technologies, Carlsbad, CA, USA), according to the manufacturer's instructions. Genomic DNA (gDNA) was removed with the gDNA Wipeout Buffer (Qiagen, Hilden, Germany). After electrophoresis was performed on a 1.2% denaturing formaldehyde (agarose) gel containing ethidium bromide, the RNA was analyzed with the Bio-BEST 140E (SIM) imaging system (Shanghai Biochemical Sci-Tech Co. Ltd). The RNAs were quantified spectrophotometrically with a NanoDrop spectrophotometer (Shanghai Biochemical Sci-Tech Co. Ltd). The 260/280 nm absorbance ratios of the samples were calculated and those > 1.9 indicated high-purity RNA. cDNA was synthesized with the Superscript™ III First Strand Synthesis System for RT–qPCR (Shanghai Biochemical Sci-Tech Co. Ltd), according to the manufacturer's instructions. A NanoDrop spectrophotometer was used to quantify the resulting single-stranded cDNAs, and the products were diluted 50-fold with ultrapure water.

TaqMan qPCR

Gene amplification was performed with qPCR in 96-well plates using a SLAN® thermocycler (Shanghai Biochemical Sci-Tech Co. Ltd). Each reaction (25 µL) consisted of the diluted cDNA (4 µl) as the template, a primer pair (10 µM, 1 µl), probe (10 µM, 0.25 µl), and TaqMan Universal PCR Master Mix (10-fold dilution, 12.5 µl) containing buffer, dNTP, DNA polymerase, and SYBR® Green II (Promega, Beijing, China). Adhesive optical film (Promega) was used to seal the 96-well plates before the initial denaturation step at 95°C for 15 min, which was followed by 45 cycles of amplification in accordance with the following thermocycling profile: denaturation at 95°C for 30 s, annealing at 60°C for 20 s, and extension at 72°C for 20 s. The final fluorescence data were obtained, and the specificity of the qPCR system and the presence of primer dimers were investigated with a dissociation protocol using a gradient from 65 °C to 97 °C. The cycle threshold (C_T) values correspond to the number of cycles at the fluorescent signal that can be detected above a threshold value, which was arbitrarily set to 0.3. GAPDH was used as the endogenous control. The relative changes in mRNA abundance were determined with the $2^{-\Delta\Delta Ct}$ method and compared with the control.

Detection of FSH, LH and Anti-Gnrh Antibodies in Sera

The serum FSH and LH concentrations were quantified with FSH and LH detection kits (for fish), according to the manufacturer's instructions (Shanghai Biochemical Sci-Tech Co. Ltd). Anti-GnRH antibodies were detected with the anti-GNRH Kit (for fish; Sigma), according to the manufacturer's instructions (Ohlsson

Table 1. Nucleotide sequences of primers and TaqMan probes used for real-time TaqMan PCR and product sizes (forward primers are listed first)

Gene	TaqMan Probe(5′-3′)	Primer(5′-3′)	Prodside(bp)
GnRHR	TGGCTTGCCGGAGACTTTGCCTGCA	F-TTGTCAACCTGACGATGGCG R-GGTTGAGGATGGCTGACTGC	198
GAPDH	CGCCTGGTCCTGAGGGCTTGCCTTC	F-GAGGTTACTCCGCACTCCCT R-GCCACAACCTTGATGCCCTT	126
FSH-β	AGCAAGACTGCAGCTCCGGCTGTCG	F-TGGTTGTCATGGCAGCAGTG R-AATGGTGGTGTCGACGAAGC	119
LH-β	AGCTTCCCGATTGCCCACCTGGTGT	F-CCCAGCTGTCACCCAGTAGA R-GGTAGGTGACGATCGGGTCT	178

Note: TaqMan probes and primers were designed based on known sequences of *gnrhr* (GenBank accession no. AB111356.2), *gapdh* (JN381952.1), *fshb* (AY294015.1), and *lhb* (AY294016.1) of tilapia (*O. niloticus*)

et al., 2007), and the optical density of each sample at 450 nm (OD_{450}) was recorded with an MK-3 type enzyme-label instrument (Rayto, Shanghai, China).

Measurement of Ovarian Parameters

The 10% formaldehyde-treated ovaries were embedded in paraffin wax and sliced into five 6 µm sections in each group. The sections were stained with hematoxylin and eosin and observed under a light microscope (Leica, Tokyo, Japan). The data for ovarian cortex thickness (OCT), primary follicle vertical diameter (PFV), primary follicle transverse diameter (PFTD), primary follicle transverse diameter (PFTD), primary follicle wall thickness (PFWT), primary follicle external thecal thickness (PFET), and primary follicle internal thecal thickness were (PFIT) were determined with the Image-analyst 1.0 software.

Statistical Analysis

The data are presented as means ± standard errors and were analyzed statistically with the Origin 7.0 software (OriginLab, Northampton, MA, USA). Square root transformation was performed before one-way analysis of variance. Significant differences were detected with Tukey's *post hoc* test. P values < 0.05 were considered significant.

Results

Effects of Anti-Gnrh Antibodies on Female Tilapia Treated with Alarelin for 60 Days

The concentrations of anti-GnRH antibodies in alarelin-treated GI and GII were the same after 10, 20, and 30 days, and were significantly higher than those in GIII at the same sampling times (P<0.05; Table 2). No anti-GnRH antibodies were detected in CG on days 0–60. The peak concentrations of anti-GnRH antibodies in GI, GII, and GIII occurred on days 30, 40–50, and 40, respectively. Although the anti-GnRH antibody concentrations decreased after 40 days in GI, 60 days in GII, and 50 days GIII, the values in these three groups were still significantly higher at those sampling times than in CG (P<0.05). The concentrations were significantly higher in GII than in GI or GIII from day 40 to day 60 (P<0.05).

Effects of Treatment with Alarelin for 60 Days on Serum FSH-B and LH-B in Female Tilapia

The serum concentrations of FSH-β in alarelin-treated GII and GIII began to increase after the initial injection; equal peak levels were detected on day 40 (P<0.05), and then the levels returned to normal (Table 3). The values in GII and GIII increased significantly from day 30 to day 40 compared with the FSH concentrations in GI and CG (P<0.05). No significant differences were detected between GI and CG or between GII and GIII (P<0.05). These results suggest that the synthesis and secretion of FSH induced by the injection of alarelin was dose-dependent from day 30 to day 40.

The serum concentrations of LH in alarelin-treated GI and CG remained stable from day 0 to day 20 (Table 4). The LH concentration in GII was significantly higher than those in GI, GIII, and CG on days 30–40 (P<0.05), and the peak level occurred on day 40 in GII. These results indicate that the synthesis and secretion of serum LH increased from day 30 to day 40 after the injection of a further 15 µg of alarelin.

Table 2. Detection of anti-GnRH antibodies in female tilapia (*O. niloticus*) treated with alarelin

Ggroup (n = 6)	Time (days)						
	0	10	20	30	40	50	60
CG	0	0	0	0	0	0	0
GI	0	1:150*	1:300*	1:600*	1:300*	1:150*	1:100*
GII	0	1:150*	1:300*	1:600*	1:1200**	1:1200**	1:600**
GIII	0	1:100**	1:200**	1:400**	1:800***	1:600***	1:400***

Note: Different asterisks in the same column express significant differences between groups (P<0.05)

Table 3. Concentrations of serum FSH in female tilapia (*O. niloticus*) treated with alarelin (mIU/ml)

Ggroup	Time (days)						
(n = 6)	0	10	20	30	40	50	60
CG	1.36±0.22	1.38±0.08	1.38±0.21	1.34±0.25	1.35±0.21	1.34±0.25	1.40±0.23
GI	1.35±0.07	1.42±0.11	1.41±0.21	1.36±0.21	1.33±0.11	1.35±0.17	1.36±0.18
GII	1.32±0.08	1.41±0.21	1.43±0.28	1.50±0.38*	1.70±0.31*	1.47±0.21	1.46±0.17
GIII	1.41±0.12	1.42±0.19	1.40±0.29	1.52±0.38*	1.62±0.34*	1.44±0.22	1.34±0.21

Note: Single asterisks in the same column indicate significant differences between groups (P<0.05)

Effects Alarelin Treatment for 60 Days on GnRHR, FSH-β, and LH-β mRNA Expression in the Pituitary Glands of Female Tilapia

The expression of the FSH-β, LH-β and GnRHR genes was examined in the tilapia pituitary glands of the GI, GII, and GIII groups, and was normalized to that in the CG group. The expression of FSH-β decreased significantly compared with the CG group (by 81% in GI, 97% in GII, and 72% in GIII), whereas LH-β mRNA levels increased significantly (2.18 times in GI, 1.36 times in GII, and 2.71 times in GIII) (P<0.05) (Figure 1). GnRHR expression increased significantly in GI (2.16 times) (P<0.05), whereas it decreased significantly in GII (to 39%) and GIII (to 76%) (P<0.05).

Effects of treatment with alarelin for 60 days on the ovaries and follicles of female tilapia

OCT, PFVD, PFTD, and PFWT in the alarelin-treated GI, GII, and GIII groups increased significantly compared with those in GC (P<0.05), and the highest values for OCT, PFWT, PFVD, and PFTD occurred in GII (Table 5). The lowest values for PFWT and OCT occurred in GI, and the lowest values for PFVD and PFTD occurred in GIII. However, no significant

Table 4. Serum LH concentrations in female tilapia (*O. niloticus*) treated with alarelin (mIU/ml)

Ggroup (n = 6)	Time (days)						
	0	10	20	30	40	50	60
CG	1.47±0.13	1.47±0.26	1.48±0.27	1.46±0.22	1.46±0.21	1.45±0.31	1.47±0.29
GI	1.47±0.15	1.49±0.20	1.47±0.23	1.46±0.31	1.47±0.30	1.47±0.30	1.47±0.27
GII	1.46±0.11	1.47±0.21	1.47±0.26	1.59±0.31*	1.60±0.27*	1.46±0.27	1.47±0.26
GIII	1.47±0.13	1.48±0.21	1.47±0.27	1.46±0.30	1.55±0.30*	1.48±0.31	1.46±0.28

Note: Single asterisks in the same column indicate significant differences between groups (P<0.05)

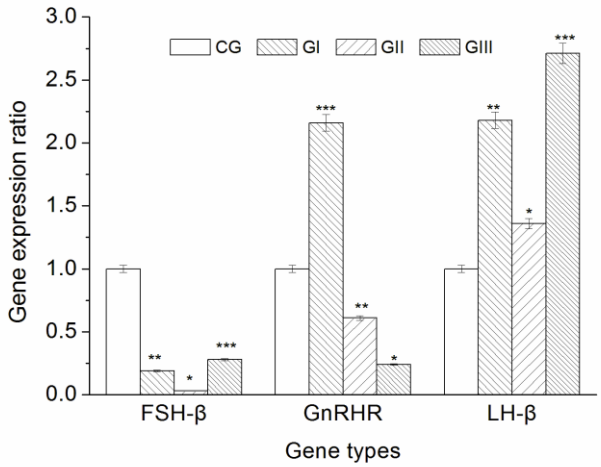

Figure 1. Expression of genes GnRHR, FSH-β, and LH- β in the pituitary gland. Different asterisks indicate significant differences between groups (P < 0.05).

Table 5. Measurements (µm) of OCT, PFV, and PET in female tilapia (*O. niloticus*) treated with alarelin

Ggroup (n = 6)	OCT	PFVD	PFTD	PFWT	PFET	PFIT
CG	28.12±5.13	244.12±89.13	173.48 ±62.27	81.86±14.22	40.46±9.21	40.45±9.31
GI	31.37±4.15	371.37±106.15**	271.97±122.33**	84.46±11.31*	40.67±10.03	40.97±6.13
GII	35.42±5.11*	389.52±97.11**	288.77±68.56**	89.59±12.31**	41.56±11.57	41.60±5.44
GIII	33.11±7.13*	264.11±87.13*	191.67±30.47*	86.46±16.30*	43.75±9.80	44.28±7.31

Note 1: OCT, ovarian cortex thickness; PFVD, primary follicle vertical diameter; PFTD, primary follicle transverse diameter; PFWT, primary follicle wall thickness; PFET, primary follicle external theca thickness; PFIT, primary follicle internal theca thickness.

Note 2: Different asterisks in the same column indicate significant differences between groups (P<0.05).

differences were detected in FETD or FITD in any of the groups. These results suggest that alarelin enhances the development of the ovaries and follicles in the female tilapia, and that an injected dose of 15 μg was significantly more effective than the 10 μg or 20 μg treatment.

Discussion

The GnRH-a antigen showed good antigenicity and immunogenicity in female tilapia in that it induced anti-GnRH antibodies when combined with an intrinsic macromolecular immunogenic protein (Hosseini, Aleyasin, Saeedi, & Mahdavi, 2010). Induced serum anti-GnRH antibodies were detected on day 10 in GI, GII, and GIII after the alarelin treatment, and the peak GnRH antibody concentrations were detected on days 30, 40–50, and 40, respectively. This phenomenon has also been reported in in other fish species, domestic animals, and humans (Wei et al., 2012; Carvalho, Wiltbank, & Fricke, 2015; John, Chang, & Joshua, 2017; Barlev, Harris, Tomi, Stojilkovic, & Blumenfeld, 2015). Our results indicated that the injection of alarelin strengthened immunity and caused high antibody levels to be maintained in the serum. Antibodies specifically directed against endogenous active substances, such as GnRH, obstructed the activities of those substances, subsequently caused considerable metabolic disturbance (Clarke & Brown, 1998). Reproductive performance was suppressed by an anti-GnRH antibody that specifically neutralized GnRH in animals (Busby, Soeta, Sherwood, & Johnston, 2014). A reduction in the amount of the gonadal LH-β receptor was detected, but no change in receptor affinity (Peter & Yu, 1997). Gonadotropin production ceases in young and adult male and female animals immunized against GnRH (Brown & Mattner, 1995). Fish treated with an anti-GnRH antibody early in life suffer from the long-term suppression of ovarian development, indicating that anti-GnRH immunization impairs the hypothalamic–pituitary function in fish (Elham & Amani, 2017). In the present study, the FSH-β and LH-β concentrations remained unchanged in the control fish, whereas the injection of alarelin significantly increased the FSH-β and LH-β concentrations from day 30 to day 40, although they were restored to normal levels 50 days after GnRH immunization, indicating that the synthesis of FSH-β and LH-β is suppressed by anti-GnRH antibodies.

After the GnRH-a treatment, the expression of LH-β mRNA and GnRH mRNA by the pituitary gland decreased significantly in pigs and cows (Avtanski et al., 2014), wild animals, such as koalas (Busby, Soeta, Sherwood, & Johnston, 2014), and teleosts, such as goldfish and zebrafish (Golan, Hollander, & Levavi, 2016; Karigo & Oka, 2013; Golan, Zelinger, Zohar, & Levavi, 2015). The expression of FSH-β mRNA and LH-β mRNA also decreased in GnRH-a-treated cultured adenohypophysis cells from preovulated pigs (Avtanski et al., 2014). The amount of GnRHR was markedly reduced in goldfish immunized against GnRH and its analogues (Karigo & Oka, 2013). GnRH maintains GnRHR mRNA synthesis in the pituitary gland (Hapgood et al., 2005). In the present study, the expression levels of FSH-β mRNA (GI, GII, GIII) and GnRHR mRNA (in GII, GIII) were significantly lower than those in GC, but the expression level of GnRHR mRNA in GI significantly higher than that of GC, which is consistent with the results of Schirman et al. (2005) and Jan et al. (1979). LH-β mRNA increased significantly after the alarelin treatment in this study.

GnRH-a is commonly used to stimulate fish ovarian development (Zanagnolo, Dharmarajan, Hesla, & Wallach, 1996; Tarlatzis & Kolibianakis, 2007). Follicular development, oocyte maturation, and the establishment of the corpus luteum are stimulated by exogenous gonadotropins, and this process is directly induced by GnRH-a (Zanagnolo, Dharmarajan, Hesla, & Wallach, 1996). GnRH-a treatment induces the resumption of meiosis by mimicking LH action, and isolated ovarian follicles exposed to GnRH-a *in vitro* undergo oocyte maturation (Tarlatzis & Kolibianakis, 2007). Once the preovulatory-follicle-enclosed oocytes are stimulated with GnRH-a, progesterone synthesis begins in the granulosa cells (Srivastava, Krishna, & Sridaran, 1995). The development and maturation of the follicles depend on the successive actions of gonadotropins (Leung, Cheng, & Zhu, 2003; Wei, Gong, & Wei, 2011). Follicle growth of goldfish was affected dose-dependently by GnRH-a (Peter & Yu, 1997). The present study demonstrates that a 15 μg dose of alarelin strongly induced follicular development and growth in female tilapia, and the increases in ovarian weight and morphometric parameters correlated with the increase in serum FSH-β concentrations.

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