

INSULIN ASPART CAN LEAD MEIOTIC RESUMPTION IN CATFISH, *Clarias Grapienus* OOCYTES

Insulin Aspart Memengaruhi Resumsi Meiosis pada Oosit Ikan Lele (Clarias grapienus)

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ABSTRACT

This study directly tested the hypothesis that the induction of oocyte maturation on catfish *Clarias grapienus* by insulin aspart resulted in a transient decrease of oocyte cyclic AMP (cAMP) level due to the increase of phosphodiesterase (PDE) activity. Under the influence of insulin the germinal vesicle (GV) of the oocyte migrated towards the animal pole (GVM), reached the micropyle, and then dissolved (GVBD). By using different concentrations of insulin aspart i.e., 0 U/ml, 0.01 U/ml, 0.1 U/ml, 1 U/ml and 10 U/ml, optimum amount required was found to be 1 U/ml. The number of oocytes incubated for 24 hours with insulin aspart treatment that undergo meiotic re-initiation can reach the percentage of meiotic resumption oocytes was 82%. The results suggest that catfish oocytes have responsive to sensitive adenylate cyclase and also an active phosphodiesterase system. Addition of the maturation-inducing steroid, a progesterone, stimulated ($P < 0.01$) GVBD of catfish oocytes and its combination with insulin aspart showed an additive effect. This information will be important in hormonal manipulation during induced breeding of Indonesian commercial catfish.

Key words: insulin, aspart, cAMP-level, resumption, GVBD, catfish

ABSTRAK

Penelitian ini menguji secara langsung hipotesis bahwa induksi pematangan oosit pada ikan lele *Clarias grapienus* oleh insulin aspart akan diikuti dengan penurunan kadar cAMP yang disebabkan oleh peningkatan aktivitas phosphodiesterase (PDE). Insulin pada akhirnya memengaruhi timbulnya pergerakan inti atau germinal vesicle migration (GVM) dalam oosit sampai inti berpindah ke arah kutub anima mencapai lubang mikropil dan akhirnya kemudian terjadi germinal vesicle breakdown (GVBD). Dengan perlakuan konsentrasi insulin aspart yang meningkat yaitu, 0 U/ml; 0,01 U/ml; 0,1 U/ml; 1 U/ml; dan 10 U/ml, ternyata dapat memengaruhi timbulnya proses resumsi dalam oosit ikan lele secara sangat signifikan ($P > 0,01$). Hasil penelitian menunjukkan bahwa jumlah oosit yang mengalami resumsi paling tinggi sebesar 82% pada perlakuan 1 U/ml. Hasil elektroforesis menunjukkan bahwa insulin aspart yang dipaparkan kepada oosit ikan lele bersifat responsif terhadap adenilatsiklase dan juga sistem fosfodiesterase aktif. Penambahan konsentrasi insulin menjadi 10 U/ml tidak memberikan pengaruh yang berbeda dari perlakuan dengan konsentrasi 1 U/ml sehingga disimpulkan bahwa konsentrasi optimum insulin aspart terhadap respon maturasi oosit ikan lele adalah 1 U/ml.

Kata kunci: insulin aspart, level cAMP, resumsi, GVBD

INTRODUCTION

Insulin is a hormone central to regulate carbohydrate and fat metabolism in the body. In target cells, insulin initiates a signal transduction which has the effect of increasing glucose uptake and storage, and finally when insulin is degraded the response is terminated. Previous studies have been report that insulin or insulin growth factor-1 (IGF-1) also can induce the maturation of fish oocytes (Weber and Sullivan, 2000; Weber and Sullivan, 2005; Mukherjee et al., 2006) but direct evidence for the role of insulin activity is still lacking. Involvement of insulin in withdrawn of the meiosis resumption stimulates the phosphodiesterase activity (Sadler and Maller, 1987) and mediated by actions of *ras* oncogene products (Sadler et al., 1990). Insulin initiates its biological effects by activation of receptors containing protein-tyrosine kinase activity (Chuang et al., 1993). The binding of insulin to its receptor will generates the signal forwarded into oocytes cytoplasm. To elicit the final cellular response is need a docking protein termed insulin receptor substrate 1 (IRS-1) to associates with insulin signal. IRS-1 serves a critical role in linking

IGF-I and insulin to their final cellular responses (Chuang et al., 1993). Further signal of IRS-1 would enable subsequent cascade particularly phosphatidylinositol 3-kinase (PI3 kinase) and mitogen activated protein kinase (MAPK) (Mukherjee et al., 2006; Paul et al., 2009), p21 ras (Sadler et al., 1990), Phosphodiesterase (PDE) (Sadler and Maller, 1987) which in turn will activate the maturation-promoting factor (MPF) to achieve germinal vesicle breakdown (GVBD) (Nagahama et al., 1994; Masui, 2001), a marker for resumption of meiosis, in oocytes of fish.

Fish oocyte maturation, a final oocyte maturation, starts when withdrawn arrested prophase-I to reinitiation and completion of the first meiotic division until they stop again in metaphase II (Yamashita, 2000; Masui, 2001; Suwa and Yamashita, 2007; Nagahama and Yamashita, 2008). The peak of achievement of sequential action of hormones to induce the maturation is activation of MPF (Nagahama et al., 1995). The ripening of teleost oocytes are controlled by the combination of exogenous and endogenous factors. Environmental stimuli translated by the brain into neural signals which result in release of gonadotropin releasing

hormone (GnRH) and/or inhibition of release of gonadotropin release inhibiting factor (GnRIF) causing the pituitary to secrete gonadotropins (GTHs) (Peter et al., 1988). When amount of GTH is enough, vitellogenic oocytes will undergo final oocyte maturation process, germinal vesicle migrates to periphery region, theca and granulosa cells of the follicle are stimulated to secrete a maturation-inducing steroid (MIS), and the MIS induces germinal vesicle breakdown (GVBD) (Fostier and Jaubert, 1982; Goetz, 1983; Nagahama et al., 1995). A period of oocyte growth is followed by a process called oocyte maturation which occurs prior to ovulation and is a prerequisite for successful fertilization. Oocyte maturation is defined as the reinitiation and completion of the first meiotic division, subsequent progression to metaphase II (Masui, 2001). In some species such as goldfish, the first visible event of oocyte maturation is the migration of the germinal vesicle (GV) toward the animal pole and is often used as an indicator of the onset of oocyte maturation (Yamashita, 2000). Following the migration of the GV, several processes occur in the nucleus and cytoplasm of the oocyte, including the breakdown of the germinal vesicle (GVBD) (Suwa and Yamashita, 2007) (which indicates the end of prophase I), condensation of the chromosomes, formation of a spindle, and extrusion of the first polar body (which marks the completion of meiosis I) (Goetz, 1983; Masui, 2001; Nagahama and Yamashita, 2008).

Depressing the production of cAMP by cyclic adenylate cyclase (AC), providing a possible mechanism for the process of oocytes maturation (Scordert-Slatkine et al., 1982; Vivarelli et al., 1983; Haider and Baqri, 2002). Thus, the exposure to the other substances i.e. cyanoketone and forskolin, an activator of adenylate cyclase (LaRosa and Downs, 2005), sodium arsenite (Corton et al., 1994), sodium azide (Barnes et al., 2002), menadione (Jin et al., 2004), sorbitol (Hardie and Pan, 2002), dinitrophenol (Pelleiter et al., 2005), hydrogen peroxide (Toyada et al., 2004), 8-bromo-adenosine and 5-aminoimidazole-4-carboxamide 1- β -D-ribofuranoside (AICAR) (Downs and Chen, 2006) can decrease the level of cAMP and those substances can be considered to be inducer for initiate the invitro oocytes maturation.

The hypothesized that insulin can decrease the cAMP level most prominent among transducer are inhibition of adenylate cyclase by activated the PDE in membrane peripheral around. Therefore in this recent study we exposed the catfish oocyte by insulin aspart, a commercial drug which more cheap and easy to get.

MATERIALS AND METHODS

Animals

Clarias grapienus shows a seasonal gonadal maturation which is usually associated with the rainy season. The maturation processes of *C. grapienus* are influenced by annual changes in water temperature and photoperiodicity and the final triggering of spawning is caused by a rise in water level due to rainfall (de Graaf et al., 1995). Naturally they spawn in July until

November, thereafter the oocytes gradually maturing and becoming ripe again in March. Catfish were used for all the experiments are 700-1200 g body wt.; 400-600 mm in body length were acclimatized in large laboratory cement tank aeration at $29 \pm 2^\circ \text{C}$ at least for one week prior to the experiment. They were fed *ad libitum* with commercial fish food (Hi-ProVit: 786 produced by: PT. Central Pangan Pertiwi, Indonesia).

Chemicals

Insulin aspart (Plexpen 70-250 Unit/tube) were purchased from public hospital pharmacies in Malang, Indonesia. $17\alpha, 20\beta$ -dihydroxy-4-pregnen-3-one (DHP), ELISA-kit for cAMP and Okadaic Acid were purchased from Sigma Aldrich via CV. Gamma Biolab, an agency of chemical supplier in Malang-Indonesia, Medium Hanks-Basic Solution from CV. Sari Kimia Raya, a local chemical store in Malang-Indonesia. Clearing solution was made by mixing the ethanol, formalin, and acetic acid (6:3:1 v/v) following the method of Lessman (1984). Then DHP was dissolved in ethanol and transferred to medium until the desired concentration each treatment.

Incubation for *In vitro* Maturation

Ovaries were dissected from the carp killed by decapitation and immediately placed in ice-cold oxygenated Hank's Medium obtained from CV Gamma Bio lab, Malang, Indonesia. The mes ovarian covering of the ovary was cut from the posterior to the anterior region with the help of fine scissors and forceps and peeled off to collect the follicles, which were then immediately immersed in the medium. Isolated follicles were pooled and screened to remove damaged follicles. Fully-grown follicles were then washed five times with medium and about 2,400 of them were placed in each $\varnothing 7$ cm petridish containing 10 ml of medium. The medium was supplemented with penicillin (100 U/ml) and streptomycin (100 ng/ml) and was gassed with 95% O_2 /5% CO_2 . Viability of ovarian follicles was detected using 0.1% trypan blue dye exclusion. Each experiment with either the same or different chemical treatment was carried out with the follicles from one fish. Incubation of ovarian follicles was performed at $30 \pm 1^\circ \text{C}$ with gentle shaking under an atmosphere of air. Follicles were preincubated for 2 h before insulin and other chemicals were added. Two hours pre incubation time appears to be all right to waive the surgical shock as determined earlier. Follicles were incubated at different time period or till 24 h at $30 \pm 1^\circ \text{C}$ in the absence (control) or presence of hormones. GVM and GVBD were monitored every hour and expressed as a percentage of total number. Maturation processes were assessed by immersing the oosit in a clearing solution containing ethanol/formalin/acetic acid; 6:3:1 (Levavi and Yaron, 1986). This clearing solution was applied each taking sample to observe which gave a transparency through follicles, thus enabling an easy microscopic examination of the following maturational stages of

oocytes such as central germinal vesicle (GV), initiation of GV migration, peripheral migration of GV, and GVBD.

cAMP Assay

Cyclic AMP concentration was measured using an ELISA-kit, following the method of Pradelles et al. (Pradelles et al., 1989). On the day of the analysis, the samples were diluted with assay buffer (provided with the kit). Samples (50 µl) were reacted with 50 µl each of cAMP-acetylcholine esterase (AChE) tracer and cAMP antibody in mouse monoclonal anti-rabbit IgG precoated wells, followed by 18 h incubation at 4°C. After the incubations, the solutions were decanted and the wells were rinsed five times with the wash buffer. 200 µl Ellman's Reagent (provided with the kit) was added to the wells and incubated in dark for 90–120 min for optimum color development (0.3–0.8 a.u., after blank subtraction). The developed plate was read at 405 nm in an ELISA reader. The concentration of total cAMP was calculated as pmol/mg oocyte.

Statistical Analysis

Data were analysed by one-way analysis of variance (ANOVA). Where *F* values indicated significance, means were compared by multiple range test. All values are expressed as means ± SEM.

RESULTS AND DISCUSSION

Figure 1 shows the migration of GV in one oocyte in response to insulin. It could be seen from figure 1a and 1d that GV was located at the centre of oocyte and on addition of insulin it started to migrate towards micropyle at peripheral site at 6 h (Figure 1b-c). After 6 h incubation few oocytes had begun change of lies of dark blot which is considered as a germinal vesicle

(GV). GV reached the micropyle region of the animal pole at 16 to 20 hr (Figure 1d) and initiated to dissolve (Figure 1e) than completely dissolved i.e. GVBD occurred at 20-24 hr (Figure 1f). Visualizing of germinal movement and germinal vesicle breakdown as confirmed by immersed of oocytes for 10 to 15 minutes in clearing solution. In control oocytes which were incubated *in vitro* without insulin GV remained at the centre at the end of 24 hr without any sign of migration.

The effect of insulin aspart on meiotic resumption, with and without concurrent treatment ranging from 0.01 to 10 U/ml medium demonstrates the effect of varies different concentrations of insulin aspart on oocyte GVBD at different time levels (Fig. 2). The data shown are for oocytes from a local reared fish from Malang-Indonesia following 24 hours incubation after addition of hormones. Best results were obtained with 1.0 U/ml which also responded well to progesterone so we combined with 100M progesterone. The combine treatment reached the highest percentages GVBD (74.66%, Fig 3) of oocytes stimulation. Treatment of the oocytes with the insulin aspart, was augmented to progesterone and DHP at least 16-20 hr. Lowest concentration at which insulin was 0.01U/ml did not potentiate stimulation maturation, suggesting that they were not sufficient to induce meiotic resumption. Highest effect of insulin alone induced meiotic resumption observed with 0.01 U/ml was the significant increase in meiotic resumption as compared with control ($P < 0.01$) could be detected at 10 hr which linearly increased till 16 hr. Insulin treatment (1 U/ml) appears to induce faster than the others but up to 20 hr were no addition effects on meiotic resumption occur any longer. In all the cases did not produce significant additional effect on meiotic resumption over 20 hr. Figure 3 shows the comparison between insulin alone

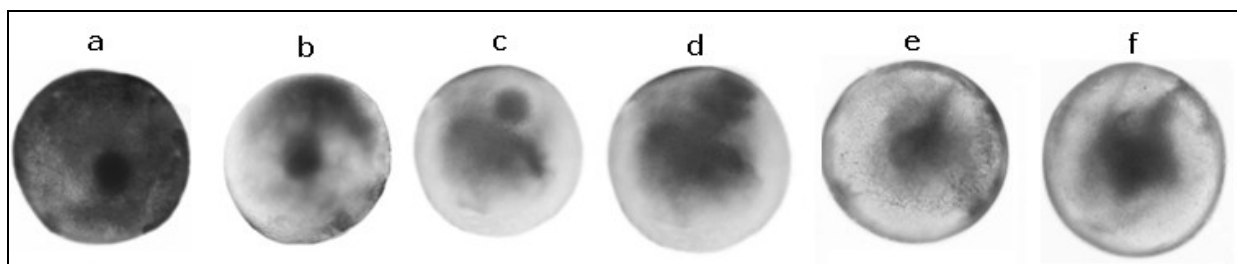


Figure 1. Photograph of catfish oocytes displaying the changes of germinal vesicle (GV), a dark blot as a visual sign of meiotic resumption in oocyte. *a*: immature oocyte with centrally GV, *b*: starting GV movement indicates by increase of GV/oocyte ratio in diameter, *c*: germinal migration stage which GV move toward peripheral, *d*: GV reach the peripheral site, *e*: GV starting to dissolve initiate germinal vesicle breakdown (GVBD), *f*: full dissolved of GV as sign of GVBD complete.

Table 1. Percentage (%) of total number catfish oocytes (n=20) which undergo meiotic resumption after incubated for 24 hours

Treatment	Time incubation (hours)							
	6	8	10	12	14	16	20	24
0.0 U/ml	0	0	0.33	0.33	0	1.67	1.67	0
0.01 U/ml	0	5.00	5.00	5.00	5.00	11.7	18.33	14.00
0.1 U/ml	0.00	5.00	8.33	16.67	36.67	38.30	45.00	46.00
1.0 U/ml	0.00	10.00	20.00	31.67	36.67	75.00	78.33	81.00
10 U/ml	1.67	10.00	20.00	30.00	38.33	73.3	80.00	82.00

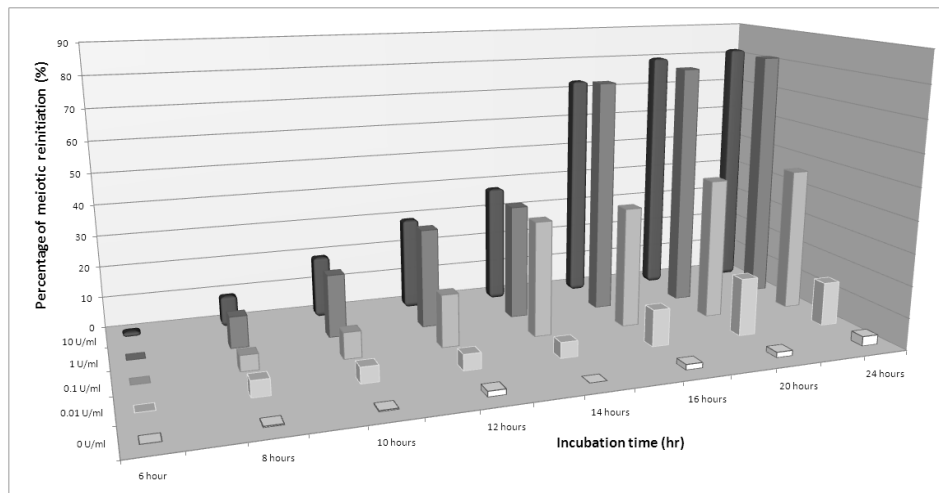


Figure 2. Time course responses histogram of insulin aspart effect for catfish meiotic resumption

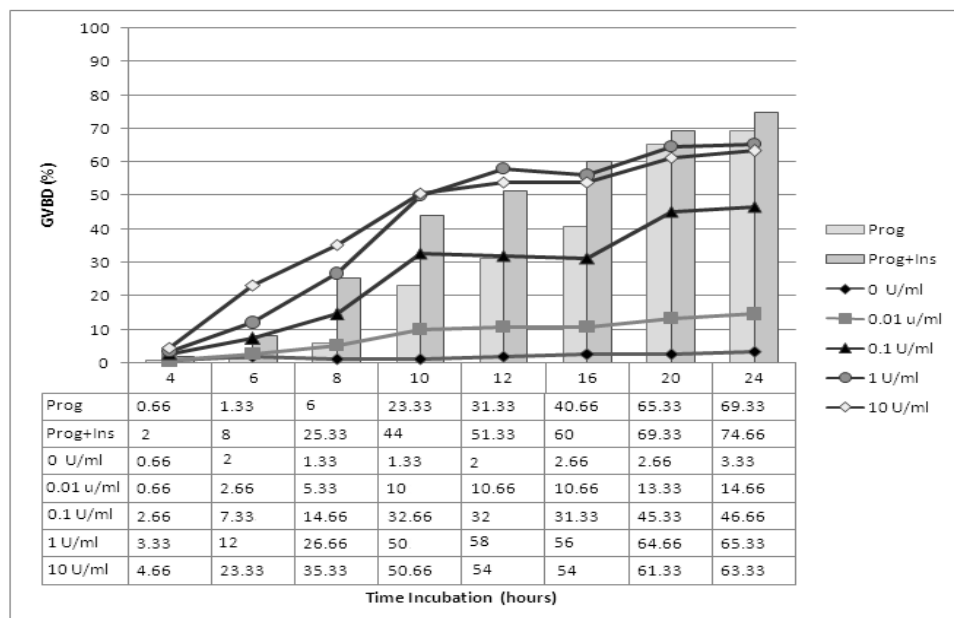


Figure 3. Time course responses curve of insulin aspart effect for *germinal vesicle breakdown* (GVBD). Three of four doses could induce maturation and the most effective dose dependent to induce in vitro maturation of catfish oocytes at 1 U/ml

treatment and in combination with progesterone effective to stimulate the GVBD of catfish oocytes.

The incubation of the follicles with insulin added by progesterone decreased cAMP significantly in a duration-dependent manner. The decrease was significant at all intervals compared with the control (Data not shown).

It has been long time established that the decrease in cAMP levels is associated with reinitiation of oocyte maturation. It has also been shown in fish and amphibian that following hormonal stimulation, the level of intraoocyte cAMP decreased via the inhibition of adenylate cyclase activity (Sadler and Mailer 1983; Jordana et al. 1984; Sadler and Maller, 1987; Mishra and Joy, 2006). The data of the present study demonstrate the involvement insulin alone can induced oocyte maturation in the catfish and that indicate causing the withdrawal of meiotic arrest in catfish

oocyte in a dose dependent manner. The time course study showed that the effect on GVM and the GVBD was evident begin time interval of 4 to 6 h incubation (the first sampling interval) and was maximal at 30 h (the end sampling time). Figure 3 shows that 65% GVBD is induced by 1 U/ml dose, but 0.01 U/ml is incapable of producing any effect. When we was increasing the dose to 10 U/ml, we found catfish oocytes undergo GVBD is similar to 1 U/ml This indicates selection of dose has no additional effect over 1 U/ml and may be that dose is crucial in getting maturational effect of insulin. Some research has been report that insulin can enhance maturational in red seabream (Kagawa et al., 1994), a daily spawning teleost, little or no activity was reported in goldfish (Lessman and Kayumpurath, 1984), striped bass (Weber and Sullivan, 2000) and in carp (Dasgupta et al., 2001) respectively. Our report shows insulin aspart,

a commercial human analog can cause withdrawal of meiotic arrest in catfish oocyte. Insulin aspart is homologous with regular human insulin with the exception of a single substitution of the amino acid proline by aspartic acid in position B28, and is produced by recombinant DNA technology utilizing *Saccharomyces cerevisiae* as the production organism. We have incubated catfish ovarian follicles with insulin aspart *in vitro* where no interference by other factors is expected. To monitor the maturational event occurring in the oocyte, clearing solution was used which enabled the visibility of the germinal vesicle. Insulin clearly effected meiotic resumption and since no other hormones or factors were present, induction of meiotic maturation by this type of insulin appears to be specific. We have shown that insulin causes migration of carp oocyte GV and then dissolution of vesicle on reaching the micropyle region (GVBD). Insulin aspart has the same clear effect of insulin bovine and porcine on the withdrawal of meiotic arrest on carp (Dasgupta et al., 2001).

Our experiment with catfish oocyte with various doses of insulin aspartat different time levels provides coleration to cAMP level in oocytes. The data of the present study demonstrate the involvement of cAMP in insulin and progesteron-induced oocyte maturation in the catfish. Total oocyte cAMP levels decreased significantly and persistently concomitant with the increase in GVBD. The changes were similar to those reported in catfish (*Clarias batrachus*) oocytes after 17.20-DP treatment (Haider and Chaube, 1995; Haider and Baqri, 2000). These workers correlated the changes in cAMP level with the morphological stages of the oocytes, it was high in centrally located GV oocytes, decreased in relation to GV migration and lower still in oocytes that had undergone GVBD. A 10-21% decrease in cAMP level was sufficient to induce GVBD in *Xenopus* (Huchon et al., 1981; Cicirelli and Smith, 1985).

Many studies have reported a 10-50 percent drop in cAMP levels during maturation. Cicirelli and Smith (1983) found a 20% decrease in the cAMP content of *Xenopus* during the first 2-50 min following progesterone addition. Mailer (1985) reviewed that within 0.1 GVBD, a rapid drop occurs in the level of cAMP to about 40-60% of basal after progesterone addition (Jalabert and Finet, 1986) has also observed a significant decrease in cAMP levels in rainbow trout oocytes incubated with 3 μ M 17,20 P. The magnitude of cAMP decrease is still being disputed currently. Some authors contend that the reported drop in cAMP levels is small compared to those associated with the regulation of cAMP by hormones in somatic cells. But, Thibier et al. (1982) have calculated that 10-20% decrease in cAMP levels would be sufficient to trigger oocyte maturation.

Progesteron and DHP are also a highly potent stimulator of meiotic reinitiation in catfish oocytes, they acts in much lower doses and produces far greater effect than insulin. This is expected as progesterone

and insulin have two different classes of receptors in fish ovary. But our objective is different; we intend to observe the lone effect of insulin aspart for its use in aquaculture practice. In the field, induced breeding of economically important fish would be practically impossible by combining synthetic gonadotropin. Insulin aspart on the other hand, is not that expensive and easily available. Gonadotrophin releasing hormone binding to pituitary gonadotropin cell stimulates GtH release and GtH in turn induces the production of progestins from ovarian follicular cells, a highly potent agent for meiotic arrest withdrawal. We have found progestine maturation activity in catfish oocyte is significantly augmented by insulin aspart. This has an impact on aquaculture practice where hormonal induction of ovulation depends on meiotic arrest withdrawal.

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