Revised

Production of live larvae following *in vitro* maturation of zebrafish oocytes

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Abstract

This study aimed to assess the effects of carp pituitary extract (CPE), follicle stimulating hormone (FSH) and luteinizing hormone (LH) on zebrafish oocyte maturation and the ability of these mature oocytes to be fertilized and developed until hatching. Stage III follicles were matured in eight treatments: five concentrations of CPE (16, 32, 48, 64 and 80 µg/mL), one of FSH (0.5 µg/mL), one of LH (0.5 µg/mL), or one combination of FSH (0.5 µg/mL) and LH (0.5 µg/mL). Maturation rates in CPE treatments were 12.8% (16 µg/mL), 24.8% (32 µg/mL), 27.0% (48 µg/mL), 22.7% (64 µg/mL) and 9.6% (80 µg/mL); in FSH was 15.7% (0.5 µg/mL), in LH was 31.8% (0.5 µg/mL) and in FSH (0.5 µg/mL) combined with LH (0.5 µg/mL) it was 50.4%. *In vitro* fertilization was performed in all treatments; however, only the treatment combining FSH and LH resulted in fertilized oocytes. After maturation using FSH combined with LH, the cleavage rate was 33.3% and hatching rate of live larvae was 20.0%. These results showed that FSH combined with LH was effective in IVM of zebrafish oocyte.

Keywords: *in vitro* maturation; *Danio rerio*; ovarian follicles; carp pituitary; pituitary gonadotropins.

1. Introduction

Maturation process, in teleost fish, is characterized by endocytosis reduction or interruption, meiosis resumption, germinal vesicle breakdown (GVBD) and cortical alveoli formation [1]. *In vitro* maturation (IVM) of early follicles provides an alternative for generating mature oocytes; and a successful protocol would help to understand the mechanism of regulating oogenesis and folliculogenesis. So far, only a few studies have assessed the ability of *in vitro* matured fish oocytes to develop into hatching embryos after fertilization [2-4]. However, these IVM protocols examined only the 17a,20b-dihydroxy-4-pregnen-3-one (DHP) as a maturation-inducing hormone.

As in other vertebrates, reproductive process in teleost is mainly regulated by the brain-pituitary-gonad axis. Carp pituitary extract (CPE) is widely used to induce spawning in fish farms, especially in developing countries. Studies have evaluated the efficiency of CPE to induce oocyte maturation *in vivo* [5] and *in vitro* in fish species [6]. CPE contains both gonadotropins FSH and LH; and gonadotropins receptors are located in the theca layer surrounding the follicle [7]. However, under inappropriate cultivation conditions, the follicle epithelial surface may be impermeable to gonadotropin hormones [8]. Therefore, medium composition, pH, osmolality and incubation period are crucial for successful gonadotropin responsiveness.

Pituitary gonadotropins, luteinizing hormone (LH) and follicle-stimulating hormone (FSH), are among the major regulators during oocyte maturation. A study in rainbow trout reported that follicular maturational competence acquisition was associated to an increased expression of FSH receptor [9]. Fish gonadotropins are not easily available; hence, hormones from mammalian sources have been suggested as the alternative in fish research [10]. Some researchers have examined the effect of human chorionic gonadotrophin (hCG) on IVM medium; they concluded that the hCG

treatment increased follicular diameter on early stage of zebrafish ovarian follicles [11] and promoted the maturation of zebrafish stage III oocytes [12]. Currently, there has been no reported usage of FSH or/and LH in IVM protocol of zebrafish follicles.

In this context, the present study aimed to assess the effects of CPE, FSH and LH on oocyte maturation and the ability of these mature oocytes to be fertilized and developed until hatching. *Danio rerio*, popularly known as zebrafish, was chosen for its ability to produce a large number of fully grown oocytes and its popularity as a vertebrate model organism for developmental studies.

2. Materials and Methods

2.1 Zebrafish maintenance and ethical statement

Mature zebrafish were kept in filtered and aerated 40 L fish tanks at 27±2°C (pH 7.2-7.4) with a light/dark cycle of 14/10 h. Fish were fed four times a day with Tetramin dry flake fish food (Tetra, Germany).

Experimental procedures and protocols described in this study were approved by the Ethics Committee of our Institution (Federal University of Rio Grande do Sul).

2.2 Collection of immature zebrafish oocytes at stage III

Ovaries were collected from adult females anaesthetized with a lethal dose of tricaine methane sulfonate (0.6 mg/mL) for 5 min and then decapitated. Ovaries were placed into 90% Leibovitz L-15 medium (pH 9.0) containing 20% fetal bovine serum (FBS) and 100 µg/mL gentamycin. This solution was isosmotic with ovarian fluid (0.29 Osm/kg).

Follicles were separated by gentle pipetting of the ovaries and immature oocytes at late stage (stage III) were collected. Only those follicles which displayed the

following characteristics were selected for culture: 1) 0.65–0.69 mm in diameter, 2) intrafollicular oocyte with dark ooplasm and distinct germinal vesicle (visible under transmitted light).

2.3 Experiment 1 - In vitro maturation time

The aim of Experiment 1 was to determine the length of maturation time.

IVM culture was an adaptation of a previous published protocol [3].

Stage III follicles were placed in wells of 12-well plates containing 4 mL of 90% L-15 medium supplemented with 100 µg/mL gentamycin, 20% FBS and one of the maturation induction treatments described below.

Carp pituitary extract (Danúbio®) was weighted, macerated and homogenized in L-15 medium. CPE concentration treatments were 16, 32, 48, 64 and 80 µg/mL.

FSH concentration was 0.5 µg/mL; LH concentration was 0.5 µg/mL; and finally the concentration of FSH combined with LH was 0.5 µg/mL each.

Ten follicles per well were cultured for 24 h at 27°C. The ability of stage III follicles to mature (stage V) was assessed every two hours. Follicles were considered mature when the germinal vesicle disappeared completely and oocytes became translucent (GBVD). Immature oocytes remained opaque and with their germinal vesicle intact.

2.4 Experiment 2 – Effects of carp pituitary extract (CPE) and pituitary gonadotropins (LH and FSH) on in vitro maturation and in vitro fertilization

The aim of Experiment 2 was to assess the effects of CPE, LH and FSH on *in vitro* maturation and *in vitro* fertilization of zebrafish stage III follicles.

IVM culture was the same previously described in Experiment 1.

95 According to the data obtained in Experiment 1, CPE treatments (16, 32, 48, 64
96 and 80 µg/mL) were cultured for 18 h and pituitary gonadotropin treatments (0.5 µg/mL
97 of FSH; 0.5 µg/mL of LH; and 0.5 µg/mL of FSH combined with 0.5 µg/mL of LH)
98 were cultured for 20 h.

99 Maturation rate was assessed as described in Experiment 1.

100 Follicular membrane of matured follicles at stage V was removed with a pair of
101 delicate forceps, and the oocytes were put into a culture dish containing 90% L-15
102 medium (pH 9.0). Two zebrafish males were euthanized by decapitation following
103 lethal dose of tricaine methane sulfonate (0.6 mg/mL) for 5 min. Testes were collected
104 from each male and placed in phosphate buffered saline (PBS), where sperm was
105 obtained by tearing the testes with the use of forceps and a needle. Immediately after
106 collection, sperm was activated by suspending 100 µL of sperm in 2 mL of 90% L-15
107 medium containing 10 defolliculated oocytes. After 1 min, inseminated oocytes were
108 washed three times in 90% L-15 medium, and incubated at 27°C in 2 mL 90% L-15
109 medium (pH 9.0). Oocytes were observed under a microscope 2 h following
110 insemination to assess cleavage rate and once again 72 h after insemination for hatching
111 rate. Cleavaged embryos were considered fertilized.

112 *2.5 Statistical analysis*

113 Experimental groups were composed by at least 100 oocytes in each group, and
114 the *in vitro* maturation and fertilization were done in triplicate. The distribution of the
115 data was evaluated and an abnormality was found even after transformation. The data
116 was analyzed using Chi-square and Kruskal-Wallis tests of the Statistical Analysis
117 System (SAS Institute version 9.0).

118 **3. Results**

3.1 Experiment 1 - In vitro maturation time

As shown in Figure 1, all CPE concentrations began oocyte maturation only 10 h after culture. The highest CPE maturation rate was observed in treatment 32 µg/mL at 18 h of culture (40.9±4.5%, Figure 1). However, there was no difference among 14 (34.5%), 16 (37.6%) and 18 h (40.9%) of IVM using 32 µg/mL of CPE. After 18 h of culture, all treatments presented oocyte degeneration. Oocyte degeneration was characterized by cellular content overflow, oocyte membrane deformity and rupture.

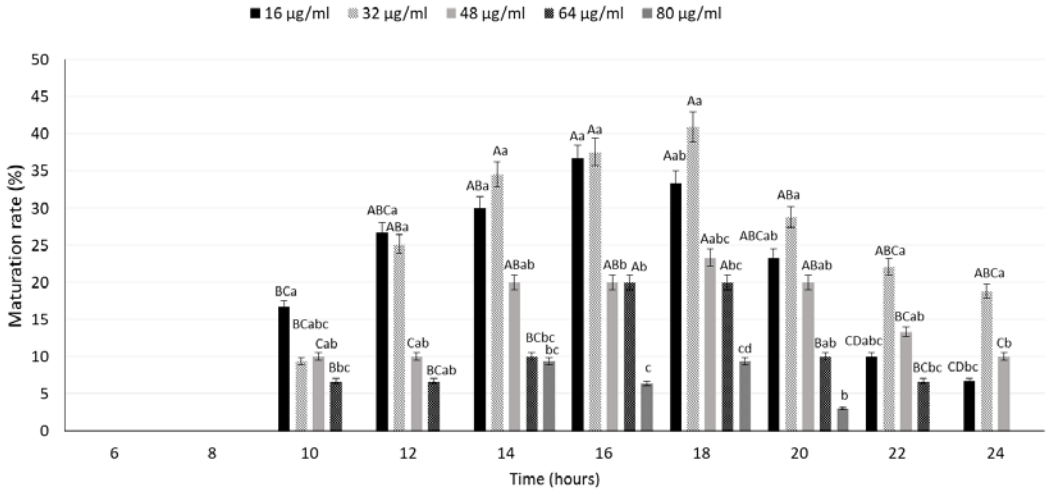

Fig. 1. Average maturation rate of oocytes exposed to different carp pituitary extract (CPE) concentrations (16, 32, 48, 64 and 80 µg/mL; n=100 oocytes per group) at 6, 8, 10, 12, 14, 16, 18, 20, 22 and 24 h of IVM at 27°C. Different lowercase letters indicate significant difference among treatments at the same time (hours) of culture (P<0.001). Different uppercase letters indicate significant difference among time (hours) of culture in the same treatment (P<0.001).

A significantly higher maturation rate (63.3±3.33%) was observed in oocytes that were cultured using the combination of FSH and LH at 14, 16, 18 and 20 h (Figure 2). This result indicated that FSH and LH act synergistically in stimulating the

maturation of stage III oocytes. Maturation process started after 8 h of culture, and the maturation rate started to decrease after 20 h (Figure 2).

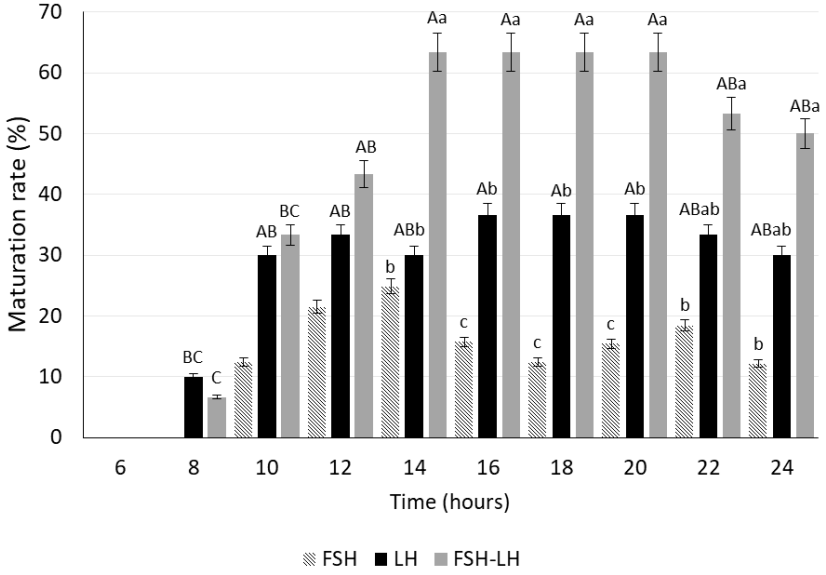

Fig. 2. Average maturation rate of oocytes exposed to pituitary gonadotropins treatments (0.5 µg/mL of FSH; 0.5 µg/mL of LH; and 0.5 µg/mL of FSH combined with 0.5 µg/mL of LH; n=100 oocytes per group) for 24 h of IVM at 27°C. Different lowercase letters indicate significant difference among treatments at the same time (hours) of culture (P<0.001). Different uppercase letters indicate significant difference among time (hours) of culture in the same treatment (P<0.001).

3.2 Experiment 2 – Effects of carp pituitary extract (CPE) and pituitary gonadotropins (LH and FSH) on in vitro maturation and in vitro fertilization

Table 1 shows maturation rate of oocytes exposed to different CPE concentrations (16, 32, 48, 64 and 80 µg/mL) after 18 h of culture; however, there was no significant difference among CPE treatments. There was no success in *in vitro* fertilization.

Table 1. Embryo development of zebrafish oocytes exposed to different concentrations of carp pituitary extract (CPE) during maturation.

Treatment	Number of Oocytes	Number of Matured Oocytes (% per Oocyte)[1]	Number of Cleaved Embryos (% per Oocyte)	Number of Live Larvae (% per Oocyte)	Percentage of Live Larvae / Cleaved
CPE 16 µg/mL	103	13 (12.8±4.0%)	0	0	0
CPE 32 µg/mL	111	27 (24.8±3.1%)	0	0	0
CPE 48 µg/mL	104	29 (27.0±2.8%)	0	0	0
CPE 64 µg/mL	100	23 (22.7±3.9%)	0	0	0
CPE 80 µg/mL	108	10 (9.6±2.6%)	0	0	0

[1]Not significant (P> 0.001)

As shown in Table 2, maturation rate in the treatment using the combination of FSH and LH was significantly higher (50.4±1.93%) when compared to the treatments using each hormone separately (FSH 15.7±1.46%, LH 31.8±1.88%). Only oocytes matured using the combination of FSH and LH were able to fertilize and hatch. Cleavage rate was 33.3±2.7% and hatching rate of live larvae was 20±0.4%.

Table 2. Embryo development of zebrafish oocytes exposed to pituitary gonadotropins (LH and FSH) during maturation.

Treatment	Number of Oocytes	Number of Matured Oocytes (% per Oocyte)[1]	Number of Cleaved Embryos (% per Oocyte)	Number of Live Larvae (% per Oocyte)	Percentage of Live Larvae / Cleaved
FSH	127	20 (15.75±1.46%)[c]	0	0	0
LH	129	41 (31.78±1.88%)[b]	0	0	0
FHS+LH	147	74 (50.40±1.93%)[a]	49 (33.3±2.7%)	29 (20.0±0.4%)	59.4

[1]Different letters indicate significant difference among treatments in the same column (P<0.001).

4. Discussion

This is the first study that assesses the effects of FSH and LH on *in vitro* maturation (IVM) of stage III zebrafish ovarian follicles. The results showed that stage III zebrafish ovarian follicles could be maturated *in vitro* for 20 h containing 0.5 µg/mL of FSH and 0.5 µg/mL of LH. In addition, FSH combined with LH was the only treatment that resulted in oocytes fertilized and hatched. However, the time required (20 h) for maturation using FSH and LH was longer when compared to results published using DHP (1 µg/mL), when stage III follicles matured into stage V in just 4.5 hours [3,4]. DHP is a specific hormone of fish in the maturation phase; therefore, a quicker action on oocyte maturation is expected. However, the cleavage (34%) and hatching (20%) rates obtained in the present study are lower than the cleavage (45%) and hatching (33%) rates obtained with DHP by Seki et al. (2008) [3]. Although the maturation conditions applied in our study were similar to those used by Seki et al. (2008) [3], the serum source used in it was different. Furthermore, these authors reported that the addition of 0.5% bovine serum albumin (BSA) to a maturation medium increased significantly cleavage and hatching rates to 70% and 63%, respectively. Culture media are usually supplemented with different types of serum, such as fetal bovine serum (FBS), estrous cow serum (ECS) or bovine serum albumin (BSA). Serum is an essential component of cell culture methodology, providing complex biological molecules, for example, hormones, growth factors, attachment factors, in addition to several low molecular weight nutrients. In the present study, maturation medium was supplemented with 20% of FBS unlike Seki et al. (2008) [3], whom supplemented the medium with 0.5% of BSA. A previous study demonstrated that FBS is a superior serum supplement compared to the often used 0.8% BSA for *in vitro* maturation (IVM) of bovine and hamster oocytes [13]. However, IVM medium containing FBS increased total lipid content and resulted in higher lipid accumulation in oocytes when compared

189 to media with BSA [14]. Furthermore, Del Collado et al. (2016) [14] showed that
190 lowering the FBS concentration to 5% reduces lipid accumulation in embryos without
191 adversely affecting oocyte maturation and embryo development. Therefore, we suggest
192 that the addition of low serum concentrations, such as 0.5% BSA or 5% FBS, instead of
193 20% FBS, to the IVM medium containing FSH and LH should be tested in order to
194 improve maturation, cleavage and hatching rates.

195 The proposal to use CPE was due to its easy handling and storage, since it does
196 not need refrigeration and its dosage is readily calculated by the gonad weight. A
197 previous study using pituitary extract on IVM of *Dicentrarchus labrax* oocytes obtained
198 a maturation rate of 89% [6]. In the present study, the best maturation rate using CPE
199 was 40.9% (32 μg/ mL at 18 h). The difference between the studies could be due to
200 variation among pituitary extracts. Therefore, the disadvantage of using CPE is the large
201 difference in pituitary samples from the same batch, since they are obtained from
202 different donors. Thus, the hormone concentration may vary among batches. Our data
203 showed that the pituitary concentration used in IVM was dose-dependent, as reported by
204 Sorbera et al. (1999) [6].

205 As in mammals, cytoplasmic maturation may occur asynchronously with nuclear
206 maturation. The oocyte requires a longer cytoplasmic maturation for the acquisition of
207 competence to be fertilized and hatch *in vitro*. In teleost, there are two different
208 gonadotropins FSH and LH [15]. FSH level is high during oocyte growth and LH level
209 is high in the maturation stage [16, 10]. Since fish gonadotropins are not easily
210 available, hormones from mammalian sources have been proposed to fish maturation
211 [11]. In order to evaluate the performance of hormones in maturation, the fertilization
212 and hatching rates were determined in Experiment 2. Only oocytes cultured for 20 h
213 using the combination of FSH (0.5 μg/mL) and LH (0.5 μg/mL) were able to fertilize

and hatch. According to Kwok et al. (2005) [10] both FSH and LH receptors were abundantly expressed in zebrafish gonads. However, these authors also showed that bovine FSH stimulated both zebrafish FSH and LH receptors over the high concentration range (1–4 μg/mL), whereas bovine LH was effective in stimulating LH receptor at low concentrations (0.005–5 μg/mL). Therefore, we believe that increasing the FSH concentration from 0.5 to 1 μg/mL or higher could improve the maturation rates of zebrafish oocytes. It is necessary to investigate other concentrations of FSH and LH and the interaction between them.

The results obtained from the present study showed that FSH combined with LH is effective on IVM of zebrafish oocyte and suggests that these hormones could be applied to IVM of other teleost. Furthermore, the developed protocol may be used as a tool for understanding the mechanism of oogenesis and folliculogenesis regulation in fish. Further studies are needed to design and improve maturation and fertilization medium for *in vitro* culture of fish oocytes, since this is an area of study little explored in fish species.

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Author's contributions

Conception and design of the study: LAS, LSM, TZ, DPS. Acquisition of data: LAS, LSM. Analysis and interpretation of data: LAS, LSM, RBR, LRE. Drafting the

article or revising it critically for important intellectual content: LAS, LSM, TZ, RBR, DPS. All authors read and approved the final version to be submitted.

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