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RESEARCH ARTICLE

INDUCTION OF GERMINAL VESICLE BREAK DOWN (GVBD) *IN VITRO* BY METABOLIC HORMONES IN FROG (*Euphlyctis cyanophlyctis*) OOCYTES

Katti Pancharatna*, Abhijit Ghadage and Manjunath G. Ghodageri

Department of Zoology, Karnatak University, Dharwad-580 003, India

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ABSTRACT

Present study is an attempt to understand role of metabolic hormones (insulin and thyroxine) in the induction of germinal vesicle break down (GVBD) in frog (*Euphlyctis cyanophlyctis*) oocytes using an *in vitro* system. Oocytes isolated manually from the ovaries of gravid females were exposed to various concentrations (n = 30) of insulin and thyroxine after equilibrating in culture medium for 16 hours. Oocytes exposed to culture medium alone served as controls. Three experiments were conducted to examine the effects of progesterone (1µM, 2µM and 3µM/ml), insulin (0.01µM, 0.1µM, 1µM and 10µM/ml) and thyroxine (0.01nM, 0.1nM and 1nM/ml) on induction of GVBD. In controls of all three experiments, 20-37% of oocytes underwent spontaneous GVBD at the end of 24 hours. Progesterone at all the concentrations tested elicited 100% GVBD at 24 hours. Insulin induced GVBD in a dose dependent manner i.e. 60%, 70%, 70% and 77% in 0.01µM, 0.1µM, 1µM and 10µM/ml concentrations, while, thyroxine elicited 40%, 64% and 56% GVBD in 0.01nM, 0.1nM and 1nM/ml doses respectively. Interestingly, the effects of either insulin or thyroxine were not potentiated in combination with progesterone suggesting involvement of diverse hormonal mechanisms in the induction of GVBD in frog oocytes.

INTRODUCTION

Oocyte maturation is the terminal and crucial stage of oogenesis that transforms an oocyte into a fertilizable egg (Masui and Clark, 1979). In the adult ovary, growing oocytes are arrested in the first meiotic phase (Prophase I) or the late G2 phase and resume meiosis usually in response to hormonal stimuli (Masui, 1985; Goncharov, 2002). Break down of oocyte nuclear membrane or germinal vesicle break down (GVBD) is considered as a hall mark for the initiation of oocyte maturation process. In an *in vitro* system, amphibian oocytes serve as excellent model to study the regulatory factors affecting or mechanisms controlling oocyte maturation owing to their availability in greater numbers, large size, requirement of relatively simpler culture system and ready response to hormones. Consequently, eggs of African clawed toad, *Xenopus laevis*, fowler's toad *Bufo fowleri*, leopard frog *Rana pipiens* and green frog *Rana esculenta* were intensely employed to understand hormonal mechanisms involved in the initiation of oocyte maturation (Schuetz, 1971; Masui and Clark, 1979; Masui, 1981; Lessman and Schuetz, 1981; Mulner and Ozon, 1981; Ramos *et al*., 2005; Browne, 2006). Effects of gonadotropins (Mulner and Ozon, 1981; Ramos *et al*., 2005) steroid hormones (Schuetz, 1971; Batten *et al*., 1988; Ferrel; 1999; Schmitt and Nebreda, 2002; Hammes, 2004), growth factors (Hirai *et al*., 1983), endocrine disrupter chemicals (Pickford and Morris, 1999; Fort *et al*., 2002), pH (Cooper and Fong, 2002) and hypergravity (Tazawa *et al*., 2005) in inducing or enhancing or attenuating the maturational competence amphibian oocyte *in vitro* have been worked out. Progesterone is known to be a potent inducer of GVBD in amphibian oocytes by activating maturational promoting factor (MPF) through a non- genomic mechanism (Bayaa *et al*., 2000; Morrison *et al*., 2000; Joesfsberg *et al*., 2007). As the general metabolic state is of the individual animal is extremely important in optimizing the normal reproductive processes in vertebrates, it may be of relevance to understand critically the role of metabolic hormones in fine tuning the processes oocyte development and maturation. The present work is an attempt to study the effects of insulin and thyroxine (individually and in combination with progesterone) in causing withdrawal of meiotic arrest and induction of GVBD in frog oocytes using an *in vitro* system. The results of such a study will also have implications in understanding the basic mechanisms underlying in the manifestations of various degrees of reproductive failures in insulin/thyroid hormone deficiency/disorder conditions in higher vertebrates including human.

Corresponding author: pancharatnak@gmail.com

MATERIALS AND METHODS

Animals

Adult female *Euphlyctis cyanophlyctis* were collected from the vicinity of Karnatak University, Dharwad (15º 7'N 75º 3'E) in the month of May to July (breeding phase) and were maintained in glass aquaria under laboratory conditions (Temperature: 23 ± 1°C and Photophase: 11.30 – 12.30 hrs).

Preparation of oocytes for in vitro maturation assay

Ovaries were surgically removed from the gravid females under ether anesthesia, cut into small pieces and transferred to Medium A (Mulner and Ozon, 1981; Ozon *et al*., 1984). Fully grown follicles (1.2 to 1.3mm) diameter were separated, and isolated manually with the help of fine forceps and needles under a stereozoom. Isolated oocytes were selected randomly and allowed to equilibrate in Medium A for 16 hours at room temperature (22- 24°C) as a prerequisite for setting oocyte maturation (Mulner and Ozon, 1981; Sadler and Jacob, 2004; Sanchwez Toranzo *et al*., 2004).

Preparation of Culture Medium

Medium A (8.8mM NaCl; 1mM KCl; 0.33mM Ca $(NO_3)_2$; 0.41mM $CaCl_2$; 0.82mM $MgSO_4$; 2mM Tris (hydroxymethyl amino methane) adjusted to pH 7.2 was prepared in double distilled water (Mulner and Ozon, 1981) autoclaved for 20 minutes at 15Lb pressure before use.

Hormone Preparations

Progesterone (P)

Progesterone (Sigma-Aldrich, St Louis, Missouri, USA) was first dissolved in 0.01ml absolute ethanol and then made upto-required volume with medium A to prepare 1mM Stock Solution which was further diluted to get 1µM, 2µM and 3µM /ml progesterone.

Thyroxine

Thyroxine (Sigma-Aldrich, St Louis, Missouri, USA) was first dissolved in medium A to prepare required volume of 50µM/ml thyroxine (Stock Solution) which was further diluted to obtain 0.01nM/ml, 0.1nM/ml and 1nm/ml of Medium A.

Insulin

Insulin (Porcine, from Sigma-Aldrich, St Louis, Missouri, USA) was dissolved in Medium A to obtain 0.01µM, 0.1µM, 1µm and 10µM/ml concentrations.

Oocyte Maturation Assay (OMA)

All the glasswares used for the oocyte maturation assay were thoroughly cleaned autoclaved. Maturation of oocytes was studied in the batches of 10 oocytes in triplicate incubated in 1ml of medium in 24 well culture plates (Axygen Life Sciences, Union City, California, USA), at 23 ± 1°C with or without hormones. The rate of maturation was scored as percentage of Germinal Vesicle Breakdown (% GVBD) at every 4 hours for 24 hours.

Statistics

The data were analyzed using Two-way ANOVA followed by Duncan's multiple comparison test.

Results

Induction of GVBD and oocyte Maturation

Fully-grown isolated oocytes of *E. cyanophlyctis* underwent spontaneous GVBD at room temperature (22 to 24˚C) when placed in culture medium. The GVBD was evident visibly by the presence of a white spot in the dark pigmented area of the animal pole region of oocytes which was further ascertained by the absence of germinal vesicle in the heat fixed sectioned oocytes (Fig. 1a-b)

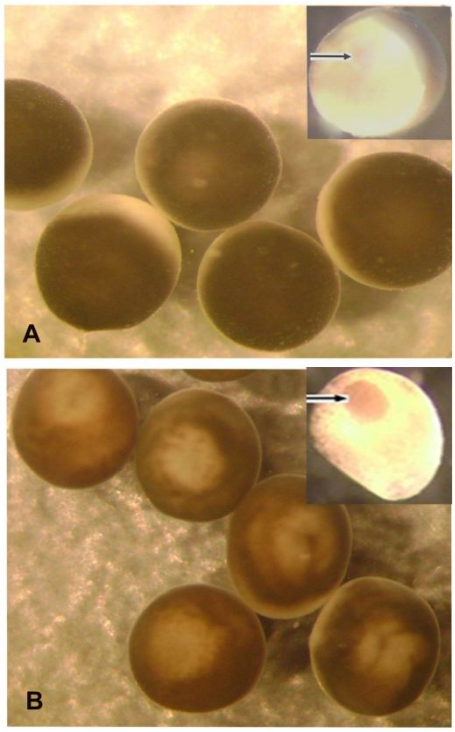


Fig. 1. Preovulatory oocytes (scale line = 250 µm). (A) Oocytes showing germinal Vesicle (A) and germinal vesicle break down (B). Sections of corresponding Heat-fixed oocytes (arrows) in the upper right squares. (Scale line = 500 µm)

Controls

Oocytes treated with the medium A for equilibration (16 hours) as a prerequisite for oocyte maturation assay, underwent spontaneous GVBD starting from 4 hours that reached 36.66% at 24 hours in different experiments (Figs. 2-4).

Effects of Progesterone

Exposure to progesterone enhanced the rate of GVBD by two to three folds when compared to controls (Fig.2). The GVBD was initiated from 4 hours onwards in all the three concentrations of progesterone that reached 100% at 24 hrs. The rate of GVBD remained comparable between 2µM/ml and 3µM /ml concentrations and was slightly lower in 1µM/ml concentration at corresponding time-intervals studied (Fig. 2).

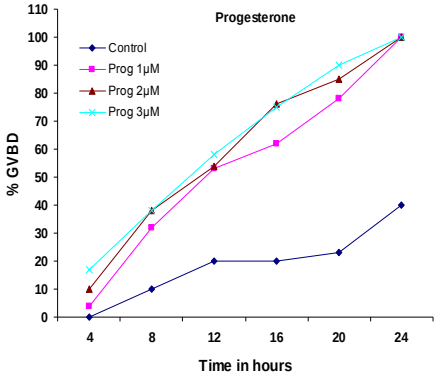


Fig. 2. Effects of graded concentrations of progesterone on oocyte maturation *in vitro* in *E. cyanophlyctis*

Effects of insulin and insulin+ progesterone

Oocytes exposed to insulin in concentrations of 0.01µM, 0.1µM, 1µM and 10µM/ml elicited 60%, 70%, 70% and 76.66% respectively at the end of the 24 hours (Fig. 3). In combination with 1µM/ml progesterone insulin elicited 63.33%, 63.33%, 60% and 73.33% GVBD in 0.01µM, 0.1µM, 1µM and 10µM/ml concentration respectively after 24 hours incubation (Fig. 3).

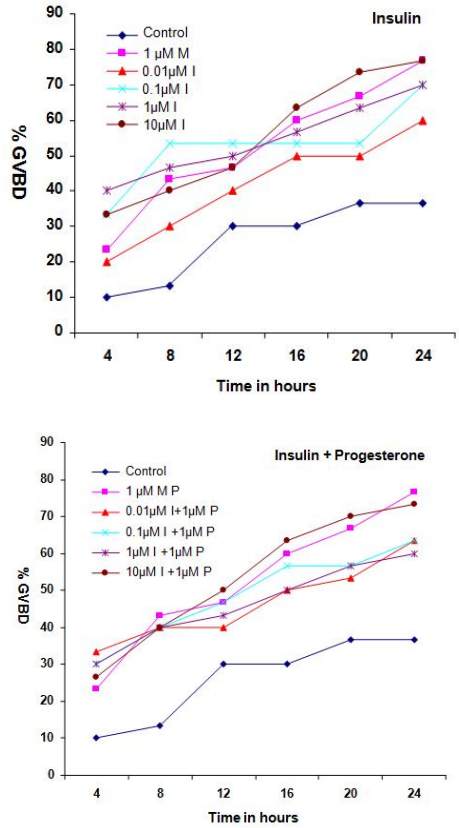
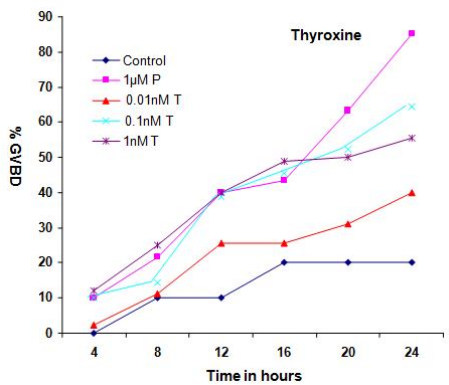


Fig. 3. Effects of graded concentrations of insulin and insulin + progesterone on oocyte maturation *in vitro* in *e.cyanophlyctis*

Effects of thyroxine and thyroxine + progesterone

Oocytes exposed to thyroxine individually in concentrations of 0.01nM, 0.1nM and 1nM/ml exhibited 40%, 64.44% and 55.56% GVBD respectively after 24 hours (Fig. 4). When oocytes were exposed to same concentration of thyroxine in combination with 1µM/ml progesterone elicited 40%, 33.33% and 23.33% GVBD after 24 hours (Fig. 4). The results remained highly consistent between the treatment groups at all time intervals (Fig. 4).



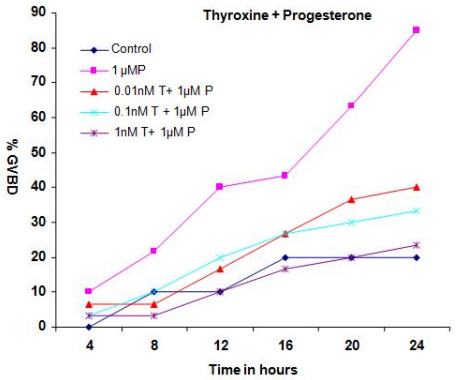


Fig. 4. Effects of graded concentrations of thyroxine and thyroxine + progesterone on oocyte maturation *in vitro* in *E.cyanophlyctis*

DISCUSSION

Production of mature oocytes and steroid hormones are the two basic functions of a vertebrate ovary (Tokarz, 1978; Jones, 1978). Maturation of a fully-grown oocyte is a complex process that includes final meiotic cell division to prepare the female gamete for fertilization (Masui, 1981). A wide range of hormones (steroids and nonsteroids), growth factors, ions, and non physiological reagents, physical factors are known to trigger oocyte maturation and the topic has been addressed/reviewed excellently from time to time (Schuetz, 1971; Bravo *et al*., 1978; Masui and Clark, 1979; Wallace and Misulovin, 1980; Masui, 1981; Lessman and Schuetz, 1981; Mulner and Ozon, 1981, Belle *et al*., 1984; Ishikawa *et al*., 1989; Deshpande *et al*., 1995; Zelarayan *et al*., 1995; Duesbery and Masui, 1996; Morrison *et al*., 2000; Bagowski *et al*., 20001; Schmitt and Nebrada, 2002; Cooper and Fong, 2002; Jessus and Ozon, 2004; Hammes, 2004; Tazawa *et al*., 2005; Browne *et al*., 2006). A large body of literature has been accumulated on the oocyte maturation of amphibians especially on the African clawed toad, *Xenopus laevis* (Wasserman *et al*., 1980; Mulner and Ozon, 1981; Deshpande and Koide, 1982; Godeau *et al*., 1985; Pickford and Morris, 1999; Cooper and Fong, 2002; Fort *et al*., 2002; Romo *et al*., 2002; Schmitt and Nebrada, 2002; Salder and Jacobs, 2004; White *et al*., 2005; Tajawa *et al*., 2005; LaChapelle *et al*., 2007).

Present investigation is an attempt to study the *in vitro* induction of oocyte maturation and the factors controlling it in Indian skipper frog, *E. cyanophlyctis* that has been an excellent model for research in reproductive biology, development and toxicology. In the present study, relative efficacy of metabolic hormones thyroxine and insulin were tested in various concentrations individually and in combination with progesterone for the induction of GVBD and oocyte maturation in *E. cyanophlyctis.* GVBD was readily induced in the oocytes at room temperature when they were exposed to culture medium with or without hormones. As a prerequisite for oocyte maturation assay, when the isolated, defolliculated oocytes were exposed to Medium A for equilibration, nearly 20% to 36.66% of them showed the visible signs of GVBD indicating mere presence of ions in solution i.e. exposure to mere culture medium triggered GVBD in oocytes which were fully grown. Involvement of calcium ions in progesterone induced maturation of oocytes is

already reported for *Xenopus laevis* (Wasserman *et al*., 1986; Duesbery and Masui, 1996). The spontaneous GVBD and oocyte maturation is also reported earlier in the oocyte of *Xenopus laevis* and *Bufo arenarum* (Wallace and Misulovin, 1980; Zelareryan *et al*., 1995). That the hormone progesterone is an inducer of GVBD has been reported earlier for this species (Ghodageri and Pancharatna 2012) and for other anuran amphibians, *Bufo fowleri*, *X. laevis*, and *Rana pipiens* (Schuetz, 1971; Bagowski *et al*., 2001; Browne *et al*., 2006). The cytoplasmic regulation of the production of maturation promoting factor (MPF) through a membrane receptor elucidating a nongenomic mechanism for the progesterone mediated GVBD is well established for amphibians(Duesberry *et al* 1998; Masui, 2001; Bagowsky *et al*., 2001; Bayaa *et al*., 2000; Morrison *et al*., 2000; Joesfsberg *et al*., 2007). African clawed toad, *X. laevis* is the most intensively studied model system for studies on oocyte meiotic maturation. In the present experiments, insulin alone induced GVBD in 60-77% of oocytes and 60 – 73% when combined with progesterone indicating insulin also induces oocyte maturation in fully grown preovulatory oocytes. Meiotic maturation of oocytes can be initiated by insulin was reported in *X. laevis* by El Etr *et al*. (1979).

Insulin is able to induce GVBD in oocytes incompetent to mature spontaneously and enhances spontaneous and progesterone induced oocyte maturation in B. *arenarum* (Toranzo *et al*., 2004). Thyroid hormone (T3) induced GVBD at a lower rate (40–65%) compared to that induced by insulin and progesterone. There is no report on the effects of thyroid hormone on oocyte maturation of amphibians and obviously this is the first study to understand the involvement of thyroid hormone in oocyte maturation. However, thyroid hormone is known to induce *in vitro* GVBD in mouse (Cecconi *et al*., 2007). In the present study oocytes exposed to thyroxine in combination with progesterone showed much lower rate of GVBD (23 – 40%) after 24 hours incubation as compared to those elicited by thyroid hormone alone indicating effects of thyroxine were not potentiated in presence of progesterone. The results remained highly consistent between the treatment groups at all time intervals. In conclusion, the results reveal metabolic hormones (insulin and thyroxine) positively influence GVBD but do not seem to synergize with that of progesterone in inducing GVBD. The mechanisms operative during the differential induction of GVBD need to be further elucidated.

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