Insulin alone can lead to a withdrawal of meiotic arrest in the carp oocyte

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Meiotic arrest of oocyte in an Indian carp, *Labeo rohita* Ham. has been found for the first time to be withdrawn by insulin only. Addition of insulin to oocytes *in vitro* caused germinal vesicle breakdown (GVBD), one of the first visual markers to determine initiation of the final maturational process. Under the influence of insulin the germinal vesicle (GV) of the oocyte migrated towards the animal pole, reached the micropyle and then dissolved (GVBD). By using different concentrations of insulin i.e., 0·063, 0·63, 6·3 and 12·6 μM, optimum amount required was found to be 6·3 μM. Induction of GVBD by insulin could be blocked by cycloheximide (Chx), a translation inhibitor, while actinomycin D (AcD) had no effect suggesting non-involvement of transcriptional activity in this process. Addition of the maturation-inducing steroid 17*a*,20*b*-dihydroxy-4-pregnen-3-one (DHP) stimulated ($P < 0·01$) GVBD of carp oocytes and its combination with insulin showed an additive effect. Gonadotropin (GtH) caused GVBD but its effect was greatly augmented by insulin. Our results demonstrate that not only can insulin alone induce GVBD in carp oocytes, but it also augments the stimulatory effect of DHP or IGF-I or GtH on GVBD. This information will be important in hormonal manipulation during induced breeding of carp.

1. Introduction

Progress of germ cell meiotic maturation has to face a halt at the diplotene stage. Progestogens released from the somatic cells of ovarian follicles by the induction of gonadotropic hormone (GtH), withdraw this meiotic arrest in fully grown oocytes via the mediation of maturation promoting factor (MPF) (Schuetz 1967; Stith and Maller 1987; Nagahama 1987; Nagahama and Katsu 1996; Bandyopadhyay *et al* 1998). This progestogen in amphibia is progesterone. In fish it is mainly 17*a*,20*b*-dihydroxy-4-pregnen-3-one (DHP) and in rare cases 17*a*,20*b*,21-trihydroxy-4-pregnen-3-one is also involved (Schuetz 1967; Lin and Schuetz 1985; Nagahama *et al* 1983; Trant and Thomas 1988). Evidences obtained so far indicate that progesterone in amphibia and DHP in fish initially interact with the membrane receptor of oocyte to induce the cascade of cytosolic events including MPF formation which leads to the germinal vesicle breakdown (GVBD) (Masui and Clarke 1979; Maller and Krebs 1980; Nagahama and Katsu 1996). In mammals, IGF-I and II, transforming growth factor *b* (TGF*b*), epidermal growth factor (EGF) have been shown to be potent *in vitro* stimulators of oocyte maturation (Feng *et al* 1988; Das *et al* 1992; Reed *et al* 1993). There are conflicting reports in fish on the withdrawal of meiotic arrest by hormones. GVBD is the visual marker for determining the withdrawal of meiotic arrest. DHP induces meiotic maturation of oocyte in salmon and trout in *in vitro* incubation (Nagahama *et al* 1983; Nagahama and Katsu 1996). DHP alone has no effect on the final maturation of oocyte in red seabream while IGF-I was the most potent inducer

Keywords. GVBD; insulin; meiotic arrest

Abbreviations used: AcD, Actinomycin D; Chx, cycloheximide; DHP, 17*a*,20*b*-dihydroxy-4-pregnen-3-one; GnRH, gonadotropin releasing hormone; GtH, gonadotropic hormone; GV, germinal vesicle; GVBD, germinal vesicle breakdown; IGF-I, insulin-like growth factor-I; MEM, minimum essential medium; MPF, maturation promoting factor.

(Kagawa *et al* 1994a). Concentration of DHP receptors in the oocytes of spotted seatrout has been found to be increased by GtH treatment which could be the reason for the occurrence of GVBD via DHP mediation (Thomas and Patino 1991). IGF-I can cause meiotic maturation of striped bass oocyte (Weber and Sullivan 2000). In goldfish, DHP is active in withdrawing meiotic arrest, insulin has little meiotogenic effect and it can significantly enhance DHP effect (Lessman 1985). However, DHP can promote GVBD induction when oocytes of red seabream are prior-exposed to insulin-like growth factor-I (IGF-I) or HCG (Kagawa *et al* 1994b)

While DHP and IGF-I are found to be very common inducers of meiotic maturation of oocyte in number of teleostean fish, insulin involvement in this process still remains unclear. In this paper we report a distinct effect of insulin on the withdrawal of meiotic arrest in carp oocyte during *in vitro* incubation. Withdrawal of meiotic halt as determined by GVBD is clearly dose dependent and does not involve transcriptional process. Insulin can also augment the stimulatory effect of DHP or IGF-I or GtH on carp oocyte GVBD.

2. Materials and methods

2.1 *Animal*

Rohu (*Labeo rohita* Ham.) is a seasonally breeding freshwater Indian major carp. They normally breed once a year i.e. only during the monsoon (rainy season). The annual reproductive cycle of this fish may be divided into four phases: preparatory (January to March), prespawning (April and May), spawning (June, July and early August), postspawning (mid August to December). Carp belonging to spawning stage were used for all the experiments. Adult female (1·0–1·5 kg body wt.; 450–570 mm in body length) were acclimatized in large laboratory cement tank with continuous flow of water and aeration at 30 ± 2°C at least for three days prior to the experiment. They were fed *ad libitum* with commercial fish food (Shalimar fish food; Bird and fish food manufacturer, Mumbai).

2.2 *Hormones and chemicals*

The following hormones were used in different experiments: carp gonadotropin (cGtH) was purified by following the method of Banerjee *et al* (1989), DHP and IGF-I (human recombinant) were purchased from Sigma Chemical Co., St. Louis, MO, USA. Porcine insulin was a kind gift from Prof. Wakabayashi, Gumna University, Japan. Bovine insulin was purchased from Knoll Pharmaceuticals, India. Actinomycin D (AcD) and cycloheximide (Chx) were purchased from SRL, India.

DHP was dissolved in ethanol, 10 µl of which was transferred to minimum essential medium (MEM) to obtain a concentration of 1 µg/ml. IGF-I was dissolved in acetic acid (0·1 M) and further diluted with 0·01 M phosphate buffer saline (0·6%) pH 7·6.

2.3 In vitro *incubation of carp ovarian follicles*

Ovaries were dissected from the carp killed by decapitation and immediately placed in ice-cold oxygenated Earl's MEM obtained from GIBCO Laboratory, USA. The mesovarian 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 oxygenated ice-cold MEM. Ovarian follicles were very loosely attached to one another and strong pipetting of medium through the clusters can disperse them. Isolated follicles were pooled and screened to remove damaged follicles. Fully-grown follicles were then washed three times with MEM and about 100 of them were placed in a 5 ml sterile beaker containing 2 ml of MEM. The medium was supplemented with penicillin (100 U/ml) and streptomycin (100 µg/ml) and was gassed with 95% O_2/5% CO_2. Viability of ovarian follicles was detected to be about 95% as detected by using 0·1% trypan blue dye exclusion. Each experiment with either the same or different hormones was carried out with the follicles from one fish. Hence when "four experiments or four observations" are written, it indicates four different experiments, each one with ovarian follicles from one fish and conducted in duplicate. Incubation of ovarian follicles was performed at 28 ± 1°C with gentle shaking under an atmosphere of air. Follicles were initially incubated for 2 h and at 2 h hormones and other chemicals were added. Two hour preincubation time appears to be all right to waive the surgical shock as determined earlier (Datta *et al* 1999). Follicles were incubated at different time period or till 24 h at 28 ± 1°C in the absence (control) or presence of hormones. GVBD was determined at the end of incubation and expressed as a percentage of total number.

2.4 *Determination of withdrawal of meiotic arrest*

Maturational processes were assessed by immersing the ovarian follicles in a clearing solution containing ethanol/formalin/acetic acid; 6 : 3 : 1 (Levavi and Yaron 1986). This clearing solution was applied at the end of the incubation 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. Initiation of GV migration is

the first sign of meiotic arrest withdrawal, which finally lead to GVBD.

2.5 *Statistical analysis*

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

3. Results

Figure 1 shows the migration of GV in one oocyte in response to 6·3 μM insulin. It could be seen from figure 1a that GV was located at the centre of oocyte and on addition of insulin it started to migrate towards the micropyle at 12 h (figure 1b). GV reached the micropyle region of the animal pole at 15 h (figure 1c), and then dissolved i.e. GVBD occurred at 18 h (figure 1d). In control oocytes which were incubated *in vitro* without insulin GV remained at the centre at the end of 18 h without any sign of migration.

Figure 2 demonstrates the effect of four different concentrations of porcine insulin on oocyte GVBD at different time levels. Insulin (0·063 μM concentration) had practically no effect on GVBD while 0·63 μM induced significant increase of GVBD at 15 h ($P < 0·01$) which linearly increased till 21 h. Highest effect of insulin induced GVBD was observed with 6·3 μM concentration of insulin, the significant increase in GVBD% as compared with control ($P < 0·01$) could be detected at 12 h which linearly increased till 21 h. Insulin (12·6 μM concentration) did not produce any additional effect on GVBD over the 6·3 μM. In all the cases, insulin induced GVBD had the peak at 21 h which levelled off at 24 h. Comparison of bovine and porcine insulin on rohu GVBD showed porcine insulin to be more potent in comparison to bovine insulin. GVBD effect of both the insulins could be blocked by Chx while AcD had no effect (figure 3). IGF-I addition induced 76% GVBD with much less concentration as compared to insulin, though combination

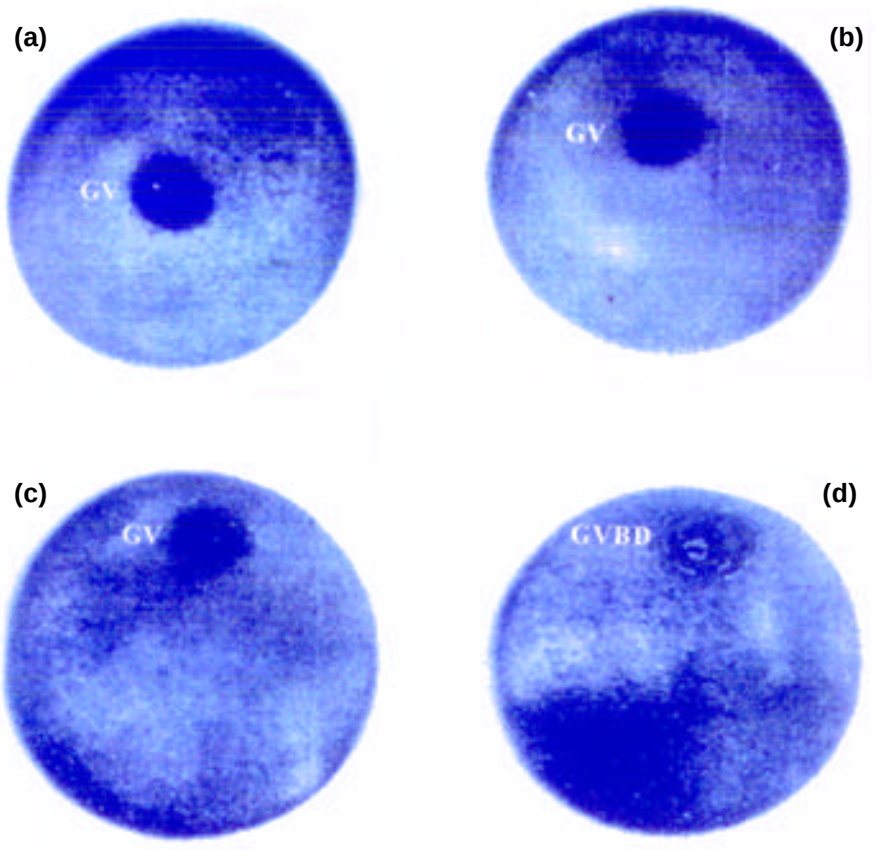

Figure 1. Image scan of carp oocyte GV migration to the micropyle region of animal pole. 6.3 μM dose of insulin was added to the incubation. (**a**) Without insulin (GV at the centre), (**b**) 12 h (initiation of GV migration), (**c**) 15 h (GV migrated towards the micropyle) and (**d**) 18 h (GV breakdown) after the addition of insulin.

with insulin did not show additional stimulatory effect on oocyte maturation. DHP had significant stimulatory effect on carp oocyte GVBD but unlike IGF-I, its combination with insulin produced greater maturational effect. In both the cases of IGF-I and DHP, AcD showed no inhibition of GVBD stimulation. Chx on the other hand blocked this stimulation (table 1). Table 2 shows that GtH stimulation of carp oocyte GVBD could be significantly augmented in combination with insulin. AcD inhibited GtH augmentation of GVBD; when both GtH and insulin were present in the incubation it waived GtH stimulation but could not reduce insulin stimulatory effect on GVBD.

4. Discussion

This report clearly shows that insulin alone can cause withdrawal of meiotic arrest in carp oocyte in a dose dependent manner. More than 50% GVBD is induced by 6·3 μM dose, but 0·063 μM is incapable of producing any effect. Increasing insulin concentrations to 12·6 μM has no additional effect over 6·3 μM. This indicates selection of dose may be crucial in getting maturational effect of insulin. Except one report in red seabream (Kagawa *et al* 1994b), a daily spawning teleost, little or no activity was reported in goldfish (Lessman 1985) and striped bass (Weber and Sullivan 2000) respectively. Insulin has always been shown to have no individual effect on the withdrawal of meiotic arrest. However it can enhance the maturational effect of progestogens in amphibia and fish (Hirai *et al* 1983; Lessman 1985; Kagawa *et al* 1994b). Our report shows insulin alone can cause withdrawal of meiotic arrest in carp oocyte. We have incubated carp ovarian follicles with insulin *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 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). Such a clear effect of insulin on the withdrawal of meiotic arrest has not yet been demonstrated in any classes of vertebrate oocyte. Kagawa *et al* (1994b) used a single dose of insulin with one time intervel i.e. 24 h to demonstrate its maturational effect on seabream oocyte. Our experiment with Indian carp oocyte with various doses of insulin at different time levels provides better clarity, where 0·63 μM can be designated as ED 50 dose while 6·3 μM produces saturational effect. Kagawa *et al* (1994b) used bovine insulin and we have observed bovine insulin to be less competent in inducing of carp oocyte maturation as compared to porcine insulin. However, the choice of the type of insulin used, as also

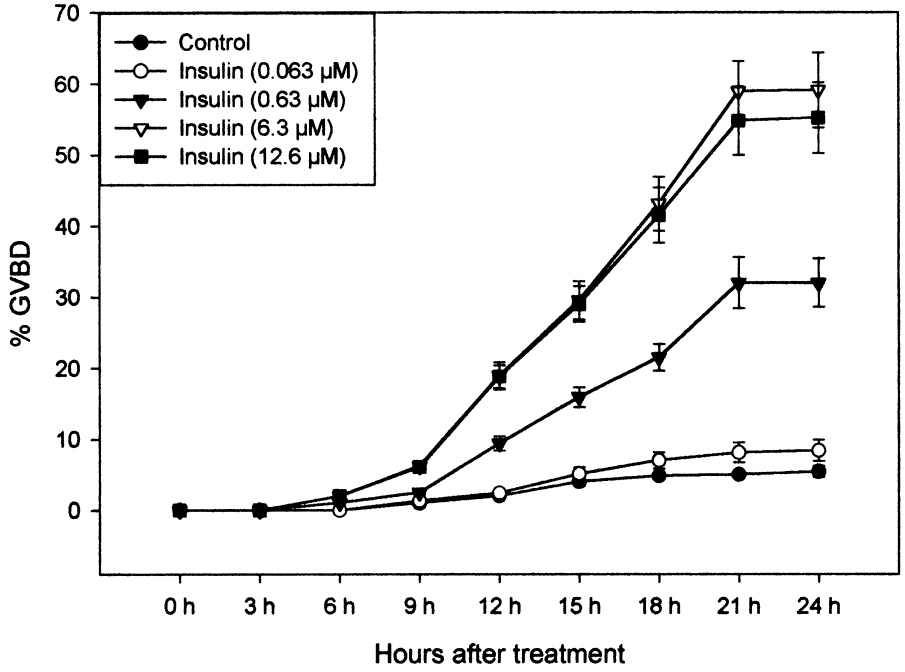

Figure 2. Induction of GVBD in carp oocyte by insulin with varied doses and time. Values are the mean ± SEM of 4 independent experiments.

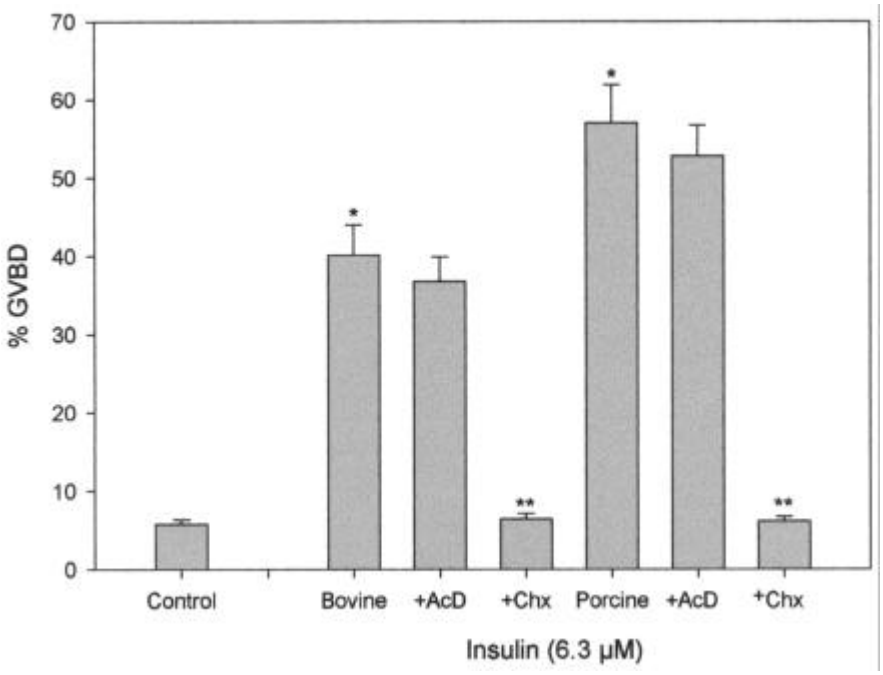

Figure 3. Effect of cycloheximide and actinomycin D (at a dose of 1 μg/ml) on bovine and porcine insulin induced% GVBD of oocytes. Values are the mean ± SEM of 4 independent experiments. *$P < 0.01$ in comparison to control. **$P < 0.01$ in comparison to bovine or porcine insulin.

Table 1. Effect of insulin in combination with DHP or IGF-I on carp oocyte GVBD.

	GVBD (%)	
Incubation[a]	Without insulin	Insulin 6·3 μM
Control	6·4 ± 0·65	56·4 ± 3·2
DHP	51·8 ± 3·1	94·4 ± 4·0*
IGF-I	76·4 ± 4·1	78·6 ± 2·0
DHP + AcD	46·5 ± 3·4	87·2 ± 4·5
IGF-I + AcD	73 ± 7·5	75 ± 3·2
DHP + Chx	6·8 ± 0·59	6·9 ± 0·62
IGF-I + Chx	7·0 ± 0·23	9·1 ± 0·6

[a]0·01 μg of DHP or 30 nM of IGF-I was added to the incubation of ovarian follicles alone or with insulin. AcD or Chx was added at a dose of 1 μg/ml where mentioned. Values are the mean ± SEM of four independent experiments.
*$P < 0·01$ in comparison to either DHP or insulin.

Table 2. Influence of insulin on GtH mediated final maturation of carp oocyte.

	GVBD(%)	
Incubation[a]	Without Insulin	Insulin 6·3 μM
Control	5·4 ± 0·51	58·4 ± 3·2
cGtH	62·3 ± 4·2	87·8 ± 4·5*
cGtH + AcD	7·1 ± 0·68	52·9 ± 4·0
cGtH + Chx	6·3 ± 0·59	7·2 ± 0·73

[a]cGtH (0·1 μg/ml) was added to the incubation of ovarian follicles in the absence or presence of insulin. AcD or Chx was added at a dose of 1 μg/ml. Values are the mean ± SEM of four independent experiments.
*$P < 0·01$ as compared to GtH or insulin.

the dosage depends on the species. For example, in goldfish insulin alone is not a potent inducer of meiotic maturation but it plays an important role in meiotic reinitiation by enhancing progestogen activity (Lessman 1985). In the case of this carp, addition of insulin into the *in vitro* incubation induces meiotic maturation. How insulin does this is not clear, it may either directly act on oocyte membrane as has been indicated by Hirai *et al* (1983) in the case of *Xenopus* oocyte or may also act through follicular cells by producing mediators. Based on the available information, it is still not possible to suggest the pathway of insulin action in the oocyte but our observations have set the foundation for such work with insulin in the future.

Both DHP and IGF-I are known to be potent stimulators of meiotic maturational event in fish (Nagahama *et al* 1983; Nagahama and Katsu 1996; Patino and Kagawa 1999; Weber and Sullivan 2000). Their combination sometime results in summation effect on GVBD (Kagawa *et al* 1994b). The DHP mechanism of action is fairly well

known from the work with amphibian and piscine oocytes. It binds to the oocyte membrane receptor, causes the formation of MPF, a dimer protein complex of cdc 2 kinase and cyclin B. MPF then effects GVBD (Hirai *et al* 1992; Nagahama and Katsu 1996). How IGF-I potentiates GVBD is not known; its binding to piscine ovarian follicular cell membrane may increase DHP production in granulosa cells (Maestro *et al* 1995). IGF-I is also a highly potent stimulator of meiotic reinitiation in Indian carp oocyte, it acts in much lower doses and produces far greater effect than insulin. This is expected as IGF-I and insulin have two different classes of receptors in fish ovary; the number and affinity of IGF-I receptors are far higher as compared to insulin receptor (Maestro *et al* 1997). But our objective is different, we intend to observe the lone effect of insulin for its use in aquaculture practice. In the field, induced breeding of economically important fish would be practically impossible by combining synthetic gonadotropin releasing hormone (GnRH) with IGF-I as the cost of IGF-I is exorbitantly high. Insulin on the other hand, is not that expensive and easily available. GnRH binding to pituitary gonadotropin cell stimulates GtH release and GtH in turn induces the production of DHP from ovarian follicular cells, a highly potent agent for meiotic arrest withdrawal. We have found GtH maturation activity in carp oocyte is significantly augmented by insulin. This has an impact on aquaculture practice where hormonal induction of ovulation depends on meiotic arrest withdrawal. Insulin and GtH have clear differences with respect to the response to transcription and translation blockers, the former responding to translation blockers while GtH activity can be blocked by both. This indicates a difference in their pathway of action, which also favours their combined enhanced effect on carp oocyte meiotic reinitiation. Insulin has never been seriously considered as a potent agent for oocyte meiotic arrest withdrawal, our demonstration on its individual and combined effect with GtH will attract its use in aquaculture practice especially in the induced breeding of Indian carp.

Acknowledgements

The authors gratefully acknowledge the financial support for this work from the Indian Council of Agriculture Research, New Delhi (Project No. 4(19)-96-ASR-I). The authors are also grateful to Prof. Wakabayashi, Gumna University, Japan for porcine insulin.

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MS received 28 May 2001; accepted 30 July 2001

Corresponding editor: HYUK B KWON