

***In Vitro* Oocyte Maturation in a Hybrid Sturgeon, Bester: Changes in the Germinal Vesicle Breakdown and 17, 20 β - dihydroxy-4-pregnen-3-one Production**

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ABSTRACT

In order to determine the most effective hormones on the *in vitro* oocyte maturation (germinal vesicle breakdown - GVBD) in a hybrid sturgeon bester, ovarian follicles were incubated at the migratory nucleus stage in the presence of several steroids, gonadotropin preparations and forskolin. Occurrence of GVBD and 17,20 β -dihydroxy-4-pregnen-3-one (DHP) production were followed during an experimental period of one year of germinal vesicle (GV) migration. From all the steroids tested, three progesterone derivatives-17 α -hydroxyprogesterone (17 α OHP), 17, 20 β , 21-trihydroxy-4-pregnen-3-one (17, 20 β , 21-triol), and DHP-were the most potent steroid inducers of GVBD, followed closely by deoxycorticosterone (DOC). No GVBD responses were found in ovarian follicles in July when the GV was still central. The responsiveness increased gradually from then and reached its peak in November when the GV had migrated fully towards the oocyte periphery. Their potencies gradually declined later from December and the oocytes lost their ability to mature the next July when degeneration set in. DHP production by ovarian follicle during successive months of nucleus migration demonstrated a relatively similar pattern of GVBD frequencies. These results indicated again a relevant role for DHP on oocyte maturation in the bester and also suggested that GV localization along with *in vitro* oocyte maturation assay can be used as practical tools for selecting the appropriate individuals in exogenous induced spawning in bester.

Keywords: Bester, DHP, GVBD, *in vitro*, Maturation.

INTRODUCTION

Several *in vitro* investigations have revealed that various steroids can stimulate oocyte maturation in teleosts and its frequency is dependent on each steroid or its metabolites and the species under investigation (Goetz, 1983; Nagahama, 1987a). Among the steroids, 17,20 β -dihydroxy-4-pregnen-3-one (DHP) was the most effective steroid in oocyte maturation in the majority of fish examined (Jalabert, 1976; Nagahama *et al.*, 1983; Nagahama, 1987b; Nagahama,

1988). Reports regarding the most potent steroids on oocyte maturation in sturgeons are fragmentary. While DHP was reported as the most effective steroid in oocyte maturation *in vitro* in the white sturgeon *Acipenser transmontanus* (Lutes, 1985), data obtained for the stellate sturgeon *Acipenser stellatus* have established that its oocytes were progesterone sensitive (Dettlaff & Skoblina, 1969) or even cholesterol dependent (Skoblina *et al.*, 1981). No challenge has been made in the bester, a cultured hybrid sturgeon (*Huso huso* female X *Acipenser ruthenus* male), whose oocyte does not

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complete final maturation under cultured conditions and undergoes mass atresia after full migration of its germinal vesicle (GV) to the animal pole (Mojazi Amiri *et al.*, 1996a).

In sturgeons, GV requires a rather long period for complete migration during which oocyte sensitivity to the maturation-inducing hormone *in vivo* and *in vitro* will change (Dettlaff *et al.*, 1993). The GV polarization rate was used as a valid morphological criterion for selecting the appropriate female sturgeon in induced-spawning (Lutes *et al.*, 1987). No information was available concerning the bester in this regard.

In this detailed study, we first investigated the relative effectiveness of several steroids and non-steroidal substances, including mammalian gonadotropin, fish gonadotropin and forskolin on *in vitro* oocyte maturation in order to discover the most potent GVBD inducers. Later, differential changes in the sensitivities of GVBD and DHP production were surveyed during the long period of nucleus migration in order to determine whether maturation response or steroid production was dependent on GV localization.

MATERIALS AND METHODS

Experiment 1- *In Vitro* Oocyte Maturation

The fish used in this study was 13 or 14 year-old adult female bester held at Moiwa, Sapporo, Japan as previously described (Mojazi Amiri *et al.*, 1995a,b). Portions of ovaries with follicles at the migratory nucleus stage were biopsied through an incision 5 to 8 cm long on the lateral side of the abdomen. Follicles were isolated by dissection and five of them were incubated in each well of 24-well plastic tissue culture dishes (Falcon, Dincoin Park, NJ) containing 1 ml of Leibovitz-15 medium (L-15; Sigma, USA) with or without (control) various concentrations of ten steroids, human chorionic gonadotropin (HCG), forskolin and salmon pituitary extract (SPE). Hepes was added to the medium as a buffering agent at a concen-

tration of 5 mM (pH 7.5), penicillin at 70 mg/l and streptomycin at 100 mg/l. Three replicates were made for each dose of each preparation and control. Incubation was maintained for 40 hrs at 15 °C in a humidified incubator. After incubation, the medium was removed and oocytes were fixed with 1 ml of Bouin's added to each of the wells. Oocytes were cut along the animal-vegetal axis after 48 hrs of fixation, stained with some drops of hematoxylin added directly to the wells and the status of GVBD was observed after one or two hours. The state of oocyte maturation was recognized externally on the basis of the features of oocytes or GV status in cross sectional view internally.

Viewed externally clear black and white rings were seen on the surface of the immature or premature follicles, while the rings were deformed and their color changed in matured ones. Viewed internally, GV was at the center or on the periphery near the animal pole in immature or premature follicles while it had disappeared in matured oocytes (Figure1).

Experiment 2- Changes in GVBD Response and DHP Production

Ovarian follicles from at least three different adult female fish at the migratory nucleus stage were biopsied continuously at one or two month intervals during the whole period of nucleus migration to the outermost oocyte periphery of the animal pole (from July 1994 to July 1995). These follicles were then incubated with or without (control) various concentrations of the three most effective steroids in GVBD used in experiment 1 and changes in the GVBD response and DHP production were noted. Three replicates were made for each dose of the preparation and for the controls in all trials. Incubations were conducted for 40 hrs at 15°C and the media were removed later and frozen at -40 °C until radioimmunoassay for DHP analysis based on the protocol designed by Young *et al.*, (1983). The status of GVBD was determined by the method pre-

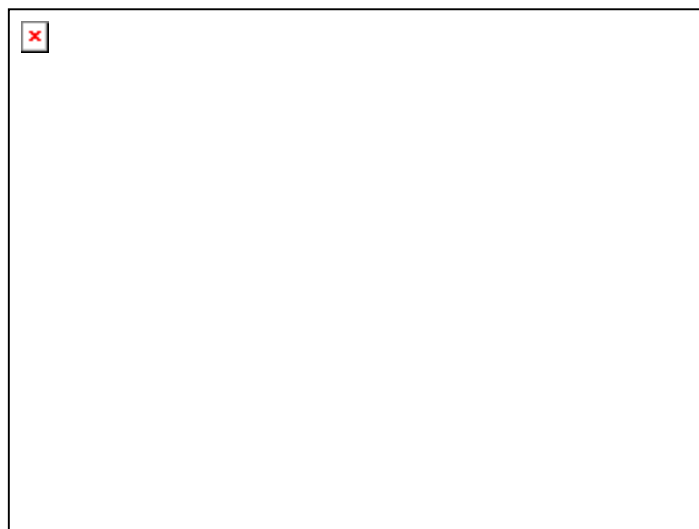


Figure 1. Changes in external (a) and cross-sectional view (b) of oocytes during 17, 20- dihydroxy-4-pregnen-3-one-induced maturation.

viously described.

Statistical Analysis

One way analysis of variance (ANOVA) followed by Duncan's multiple range test were employed to identify the significant differences in GVBD frequencies and DHP levels.

RESULTS

Experiment 1

All the steroids (except estradiol-17 β , E₂ and Testosterone, T) were 100% effective in GVBD at the concentration of 1000 ng/ml (Table 1). Of the 10 steroids tested at a concentration of 100 ng/ml, 17 α -hydroxy progesterone (17 α OHP) was the most effective steroid followed by 17, 20 β , 21-trihydroxy-4-pregnen-3-one (17, 20 β , 21-triol), DHP, Deoxycorticosterone (DOC) and progesterone at 100, 85, 76, 67 and 51% of GVBD responses respectively. 17,20 α -dihydroxy-4-pregnen-3-one, pregnenolone, and 17 α OH pregnenolone exhibited lower

GVBD at 20, 10, 4%. DHP was able to induce 13% of GVBD and 17, 20 β , 21-triol, 10% at lower concentrations of 10 and 1 ng/ml. Minimally active steroids were T and 17 β -estradiol (E₂), neither of which were able to induce 100% of GVBD at any dose levels tested. Ovarian follicles did not show any response to the gonadotropin preparations such as human chorionic gonadotropin (HCG) and salmon pituitary extract (SPE) in any dose or trial tested. Forskolin was also unable to induce GVBD at any dose levels. None of the L-15 controls underwent GVBD during the incubation period.

Experiment 2

Exogenously added steroids could not stimulate any level of GVBD in any of the trials during July when the GV was located at the center towards the oocyte's periphery (Figure 2). GVBD responses increased significantly ($p < 0.01$) in October with levels of 100, 100, 75, 50 and 25% in the presence of five steroids (17 α OHP, 17, 20 β , 21-triol, DHP, DOC and progesterone, respectively) and reached its peak in November at the end of GV migration. GVBD rates decreased

**Table 1.** Relative effectiveness of several steroids and gonadotropin preparations on GVBD.

Treatment	Concentration (ng/ml)			
	1000	100	10	1
17 α - OH progesterone	100*	100	0	0
17 α , 20, β 21- tri OH-4-pregnen-3-one	100	85	10	0
17,20 β -di OH-4-pregnen-3-one	100	76	13	0
Deoxycorticosterone	100	67	0	0
Progesterone	100	51	0	0
17 α ,20 α - di OH 4-pregnen-3-one	100	40	7	7
Pregnenolone	100	30	0	0
17 α - OH pregnenolone	100	13	0	0
Testosterone	100	0	0	0
Estradiol-17 β	0	0	0	0
Concentration (IU/ml)				
HCG	1000	500	200	100
	45	13	0	0
Concentration (μ M/ml)				
Forskolin	100	10	1	0.1
	0	0	0	0
Concentration (pieces/ml)				
Samon pititary Extract	100	10	1	0.1
	0	0	0	0
L-15	0	0	0	0

gradually from that point ($P < 0.05$) and oocytes lost their ability to be matured in the presence of progesterone in December, for DOC in March and for the other steroids in July of the next year when degeneration started.

DHP levels in the media were high in July in the presence of all precursors used except pregnenolone (45-55 ng/ml) (Figure 3). Production increased slightly in October-November (55-65 ng/ml) ($p < 0.05$) but continued without significant differences from December to May of the next year ($p > 0.05$). In July, at the beginning of oocyte degeneration, production decreased significantly in the presence of all steroids ($p < 0.01$; 33 ng/ml for 17 α OHP and 2-4 ng/ml for the others). DHP production in all control trials was low or non-detectable (less than 50 pg/ml).

DISCUSSION

The results of experiment 1 demonstrated that progestagens are the most potent GVBD inducers followed by corticosteroids. Similar

findings were reported in another cultured sturgeon *A. transmontanus* (Lutes, 1985). DHP was one of the three most effective progestagens for oocyte maturation and was partially effective even at the lower concentration of 10 ng/ml. Progestagens in general and DHP in particular have been established as the *in vitro* inducers of GVBD in a variety of teleost species including rainbow trout *Oncorhynchus mykiss* (Jalabert, 1976), amago salmon *Oncorhynchus rhodurus* (Young *et al.*, 1982), Ayu *Plecoglossus altivelis*, goldfish *Carassius auratus* (Nagahama *et al.*, 1983), red sea bream *Pagrus major* (Adachi *et al.*, 1988), Zebrafish *Brachydanio rerio* (Selman *et al.*, 1994).

17 α OHP, 17, 20 β , 21-triol and DHP at the lower concentrations of 10 and 1 ng/ml have caused only 10% GVBD in the bester, while these steroids at the same concentrations induced 100% of GVBD in white sturgeon *A. transmontanus* (Lutes, 1985). DOC also was able to generate 100% of GVBD in this species at the lower concentration of 62 ng/ml, ten times less than used for oocyte maturation in the bester. On the other hand, progesterone used as a maturation inducing

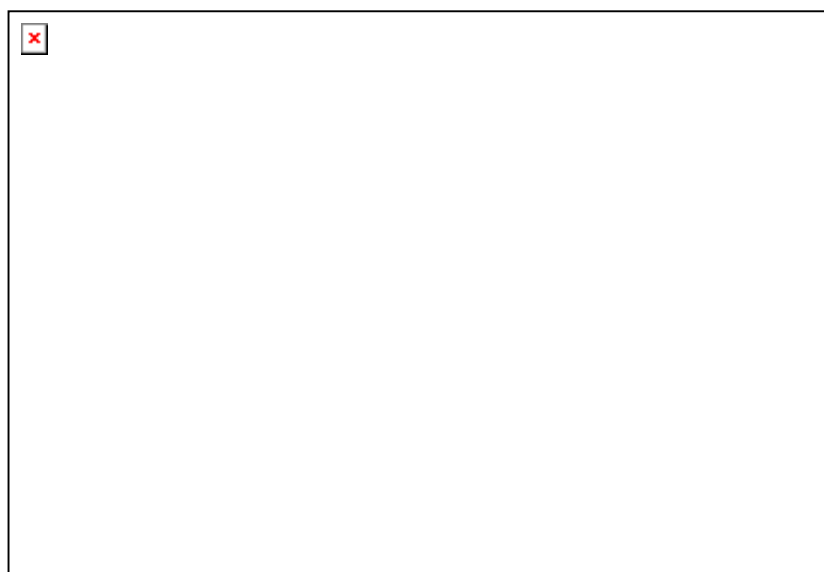


Figure 2. Developmental changes in the GVBD response of oocytes during germinal vesicle migration.

hormone *in vitro* in other sturgeon species at 5 to 10 $\mu\text{g/ml}$ was many times higher than we used for bester (Dettlaff *et al.*, 1993). These data suggested that different species of sturgeons may show different reactions in GVBD or steroid metabolize *in vitro* to the presence of the same precursors. The similar ovarian response to exogenous 17, 20 β , 21-triol and DHP over a wide range of concentrations may suggest a minor conversion

of DHP to 17, 20 β , 21-triol. However, the *in vitro* production of DHP in bester maturing oocytes from exogenous pregnenolone (Mojazi Amiri *et al.*, 1999a), or *in vivo* DHP production in maturing fish after LH-RHa (Mojazi Amiri *et al.*, 1999b) is maximal. Therefore, a major role for DHP during final oocyte maturation in bester is assumed.

T caused a relatively lower rate of GVBD compared with other steroids even at the



Figur 3. Developmental changes in 17 α , 20 β -dihydroxy-4-pregnen-3-one production of oocytes during germinal vesicle migration.



higher concentration of 1000 ng/ml, whereas E₂ was always inactive in GVBD induction. No information was available on the effectiveness of androgens and estrogens on GVBD in sturgeons but they usually have low potency in oocyte maturation in teleost (Donaldson & Hunter, 1983; Selman *et al.*, 1994).

Oocyte did not respond to SPE or HCG, surprisingly in the light of their abilities for the *in vitro* oocyte maturation in teleosts (Wallac and Selman., 1980; Young *et al.*, 1982). Gonadotropic luteinizing hormone (LH), HCG and even pituitary fraction from stellate sturgeon *A. stellatus* failed to induce GVBD in white sturgeon *A. transmontanus* (Lutes, 1985). Recent efforts towards the *in vivo* induction of oocyte maturation and ovulation in the bester using luteinizing hormone releasing hormone analogue (LH-RHa) have been successful and 50% of treated individuals spawned (Mojazi Amiri *et al.*, 1999b). Gonadotropin also led to maturation and ovulation in many sturgeons *in vivo* (Doroshov *et al.*, 1993). The lack of any effects of SPE in this experiment may be related to the low dosage of the preparation we used (0.01 pieces of pituitary) or because it was not a homoplastic gonadotropin material. Failure of HCG in the induction of GVBD may have been due to the lack of stimulation potency of HCG in the secretion of maturation-inducing hormone (MIH) confirmed by our previous study (Mojazi Amiri *et al.*, 1999a). Forskolin did not stimulate *in vitro* oocyte maturation in the bester. As in many teleost reported, forskolin causes GVBD via an increase of cyclic AMP in follicular layers, which in turn stimulates MIH synthesis (Iwamatsu *et al.*, 1987; Nagahama 1987a; Tan *et al.*, 1986). Failure of forskolin in the induction of GVBD in this experiment may be related to the lack of sufficient MIH secreted in only a few incubated follicles compared to the large number of follicles present in the ovaries as reported earlier (Mojazi Amiri *et al.*, 1996a).

The responsiveness of ovarian follicles was low when the nucleus was still central,

and increased gradually concomitantly with GV migration towards the oocyte periphery and reached its peak when GV had fully advanced to the animal pole cortex. GVBD frequencies decreased relatively later and oocytes finally lost their ability to be mature when degeneration started. These results indicated that frequencies of GVBD are correlated with the advancement of nucleus migration and suggested that GV localization can be used as a practical tool to distinguish suitable individuals for exogenous induced-maturation. The oocyte polarization rate was used as a factor to predict ovulatory successes in white sturgeon *A. transmontanus*, (Lutes *et al.*, 1987) or in other sturgeon species (Dettlaff *et al.*, 1983). In many teleost species however, frequencies of GVBD were dependent to the position of the germinal vesicle in the oocyte prior to incubation (Geotz and Theofan, 1979; Nagahama, 1983; Scott, 1987).

GVBD responses decreased relatively from November onward, while GV was at the same location as in the previous month. This result may indicate that prediction of ovulatory success on the basis of GV localization is not fully reliable. In contrast, *in vitro* oocyte maturation assay can provide more accurate prediction in this case matter. Practically, small portions of biopsied ovarian follicles from postvitellogenic individuals can be incubated with one of the three most effective steroids (e.g. DHP) and the individuals with the highest GVBD selected. This same procedure has been used with a higher concentration of progesterone (5-10 µg/ml) to distinguish fish suitable for induced oocyte maturation in white sturgeon *A. transmontanus* (Lutes, 1985) and stellate sturgeon *A. stellatus* (Dettlaff *et al.*, 1993). Indeed, both GV position and the final oocyte maturation assay can be used to achieve maximal egg production.

DHP production demonstrated a relatively similar pattern of GVBD frequencies during whole period of experiments, except in July at the initiation of GV migration. Recently we showed that ovarian follicles can produce higher levels of DHP even at the terti-

ary yolk stage (Mojazi Amiri *et al.*, 1999a). The fact that in this experiment DHP production was relatively higher at the initiation of GV migration may be related to this finding. DHP production increased from October when GVBD responses elevated and declined in the next July concomitantly with the drop of GVBD, confirming a direct correlation between GVBD frequencies and DHP production levels. Results from our previous study indicated a surge in DHP production at the migratory nucleus stage and our *in vivo* experiment also showed that serum DHP levels in LH-RHa-induced ovulated female besters was ten times higher (2 ng/ml) compared with nonovulated individuals (Mojazi Amiri *et al.*, 1999b). Taken together these results may indicate again DHP as a relevant steroid in oocyte maturation in the bester.

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رسیدگی کامل تخمک ماهی دو رگه خاویاری بستر به طریقه *In Vitro*: تغییر در میزان شکسته شدن هسته زاینده و تولید هورمون DHP

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چکیده

جهت تشخیص موثرترین هورمون تاثیرگذار بر رسیدگی کامل جنسی تخمک (شکسته شدن هسته "GVBD" Germinal Vesicle Breakdown) ماهی خاویاری بستر (هیبرید بین فیل ماهی ماده و استرلیاد نر)، تخمک‌های ماهی مزبور در مرحله مهاجرت هسته به طرف میکروپیل در حضور تعداد زیادی از هورمونهای استروئیدی، ترکیبات گنادوتروپین و ماده‌ای به نام فورسکولین (Forskolin) به طریقه *In vitro* به میزان ۴۰ ساعت انکوبه گردید. چگونگی حصول پدیده شکسته شدن هسته تخمک (GVBD) و تولید هورمون DHP- $17-20\beta$ در طول یکسال زمان مهاجرت هسته از مرکز تخمک تا میکروپیل مورد بررسی قرار گرفت. از تمام هورمون‌های استروئیدی استفاده شده، هورمونهای با مشتقات پروژسترونی از جمله DHP و $17, 20\beta, 21$ -trihydroxy-4-pregnen-3-one ($17, 20\beta, 21$ -triol) و 17α -hydroxyprogesterone (17α OHP) از موثرترین هورمون‌های القاء کننده پدیده GVBD بوده اند. و هورمون deoxycorticosterone (DOC) در مرحله بعدی از نظر تاثیر قرار داشته است. هیچ عکس‌العملی از نظر حصول شکسته شدن هسته تخمک در ماه جولای زمانی که هنوز هسته در مرکز تخمک قرار داشت پدیدار نشد. این عکس‌العمل به تدریج پس از آن زمان افزایش یافته و در ماه نوامبر موقعی که هسته مهاجرت خود به طرف سوراخ میکروپیل را کامل کرده بود به حداکثر خود رسید. این پتانسیل از ماه دسامبر به تدریج کاهش یافته و تخمک توانائی خود را به القاء رسیدگی کامل جنسی توسط هورمون در ماه جولای سال بعد موقعی که دژنره شدن تخمک آغاز شد از دست داد. تولید هورمون DHP توسط تخمک در طول ماههای مهاجرت هسته به طرف سوراخ میکروپیل نیز روندی همچون روند حصول GVBD را نشان داد. همراه با دیگر دانسته‌ها که از مطالعات قبلی‌مان در رابطه با چگونگی تولید هورمونهای جنسی توسط تخمک ماهی بستر بدست آمد، این مطالعه بار دیگر نقش تاثیرگذار هورمون DHP را در رسیدگی کامل جنسی تخمک ماهی بستر نشان داد. این مطالعه همچنین ثابت نمود که جای قرار گرفتن هسته همراه با آزمایشات رسیدگی کامل تخمک به طریقه *In vitro* می‌تواند به عنوان شاخص‌هایی برای تعیین چگونگی رسیدگی کامل جنسی و همچنین به عنوان یک ابزار عملی برای یافتن مولدین مناسب جهت القاء تکثیر مصنوعی بکار گرفته شود