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An agonist for membrane progestin receptor (mPR) induces oocyte maturation and ovulation in zebrafish *in vivo*

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Abstract

The maturation and ovulation of fish oocytes are well-characterized biological processes induced by progestins via coordination of nongenomic actions and genomic actions. Previously, we established a procedure that enables the induction of oocyte maturation and ovulation in live zebrafish by simple administration of the natural teleost maturation-inducing hormone 17 alpha,20 beta-dihydroxy-4-pregnen-3-one (17,20β-DHP) into the surrounding water. By this *in vivo* assay, the potencies of chemicals in inducing or preventing oocyte maturation and ovulation can be evaluated. The potencies of compounds in inducing ovulation of zebrafish oocytes also can be evaluated *in vivo* with improved *in vitro* assays. Here, we attempted to evaluate the effect of Org OD 02-0 (Org OD 02), a selective agonist for membrane progestin receptor (mPR), on fish oocyte maturation and ovulation with *in vitro* and *in vivo* assays. As reported previously, Org OD 02 triggered oocyte maturation *in vitro.* The same Org OD 02 triggered oocyte maturation within several hours *in vivo*. Surprisingly, Org OD 02 even induced ovulation both in *in vivo and in vitro*. Eggs from Org OD 02-induced ovulation could be fertilized by artificial insemination. The juveniles developed normally. These results indicated that Org OD 02 triggered physiological ovulation in live zebrafish. In summary, we have demonstrated the effect of Org OD 02 on fish oocyte maturation and ovulation *in vitro* and *in vivo*. The results suggested that Org OD 02 acted as an agonist not only of mPR but also of nuclear progesterone receptor (nPR).

Keywords: ovulation; oocyte maturation; Org OD 02; membrane progestin receptor; zebrafish

Introduction

Fish oocyte maturation is triggered by maturation-inducing steroids or substances (MIS) that induce the activation of M-phase-promoting factor (MPF) [1, 2]. During the course of oocyte maturation in fish, hydration is induced, and the oocyte becomes transparent. Ovulation, or extrusion from surrounding follicle cells, is induced immediately after oocyte maturation. Ovulated eggs remain in the female body until spawning. Spawned fish eggs instantly develop a fertilization membrane when they are released into the water regardless of whether fertilization has occurred. Both oocyte maturation and ovulation in freshwater fish are induced by the same MIS, 17 alpha, 20 beta-dihydroxy-4-pregnen-3-one (17,20β-DHP). Membrane progestin receptor (mPR) was identified in fish and is thought to mediate the MIS signal in the oocyte to induce oocyte maturation [3, 4]. Nuclear progesterone receptor (nPR) present in the cytoplasm has been proven to be a receptor for the induction of ovulation [5, 6]. Thus, it is thought that oocyte maturation and ovulation are induced by the same hormone but activated by two different pathways through mPR and nPR, respectively.

In vitro assays using fish oocytes dissected from ovaries have been used to study the actions of hormones and various chemical substances, including endocrine disruptors, on these biological processes [7-9]. We previously demonstrated highly specific binding of the natural MIS 17,20β-DHP to mPR. Additionally, a high degree of competition between diethylstilbestrol (DES) and 17,20β-DHP for mPR binding was demonstrated. The oocyte maturation-inducing activity of DES on goldfish and zebrafish oocytes *in vitro* is correlated with the agonistic activity of DES on mPRα [8]. Thus, the agonistic and antagonistic activity of substances on mPRα can be evaluated with *in vitro* assays using oocytes.

In vivo assays with living zebrafish have been used to study the actions of compounds on oocyte maturation and ovulation *in vivo* [10]. This technique has also been used for the selection of specifically expressed genes during ovulation (ovulation-inducing genes) [11, 12]. Because oocyte maturation is induced in almost the same period of time *in vivo* as *in vitro*, the actions of the applied compounds are thought to be due to direct effects on the receptors of oocytes. Thus, the agonistic and antagonistic activity of substances on mPR and nPR can be evaluated with *in vivo* assays using living zebrafish.

In vitro ovulation-inducing assay was established by the improvement of conditions of medium [13]. By the technique, it was getting possible to evaluate the direct actions of chemicals on oocyte maturation and ovulation of zebrafish oocytes.

To investigate the effects of a selective agonist for membrane progestin receptor (mPR), Org OD 02, in *vitro* and *in vivo* assays were applied. We found that Org OD 02 could induce oocyte maturation *in vitro* and could even induce ovulation *in vivo*. These findings demonstrate that Org OD 02 acts on mPR and exerts nongenomic actions. These results suggested that Org OD 02 acts on both nPR and mPR as an agonist under physiological conditions.

Materials and methods

Materials. Zebrafish were raised and kept under standard laboratory conditions. The fish used for the experiments were maintained in a flow-through culture system maintained at 28.5 °C under a 14 hours light/10 hours dark cycle [14]. Org OD 02 was obtained from AXON Medchem BV (Groningen, Netherlands). 17,20β-DHP was purchased from Sigma Chemical Co. (St. Louis, MO). This study use of zebrafish, and

the experimental protocol for the use of animal was approved (approval no. 2019F-5) by the Institutional Ethics Committee of Shizuoka University, Japan.

In vitro Oocyte Maturation Assay. An *in vitro* zebrafish oocyte maturation assay was conducted as described previously [9]. Gravid females did not contain ovulated eggs were selected by trying to squeeze the eggs by pushing their abdomen at after the onset of light (9 am in our laboratory). Ovaries of zebrafish were isolated from sacrificed females and placed in fresh zebrafish Ringer's solution (116 mM NaCl, 2.9 mM KCl, 1.8 mM CaCl2, and 5 mM HEPES, pH 7.2) and washed with the same solution. The ovaries were dissected into ovarian fragments (each containing 2-10 oocytes) manually by using fine forceps. Fully grown immature oocytes were exposed *in vitro* by incubating the ovarian fragments in 4 ml of zebrafish Ringer's solution containing each experimental agent (diluted from a 1000-fold stock in ethanol) at 25.0 °C or room temperature with gentle agitation (40 rpm). To assess the maturation processes, germinal vesicles (GVs) were examined under a binocular microscope (SMZ645, Nikon, Tokyo, Japan). Germinal vesicle breakdown (GVBD) was assessed by scoring the oocytes that became transparent. % GVBD was determined in more than twenty oocytes of stage 3 in each experimental condition of each fish.

In vivo Oocyte Maturation and Ovulation Assay. An *in vivo* oocyte maturation and ovulation assay was conducted in zebrafish as described previously [10]. Gravid female zebrafish possessing full-grown immature oocytes were selected from a mixed group of 10-50 males and females held in a 20 cm x 25 cm square acryl case, which was 25 cm high and given continuous flow-through. Females confirmed to be fecund were selected by pre-spawning with 17,20β-DHP treatment. [15]. Pre-spawned females kept

for 7-10 days after spawning until females newly developed the full-grown immature oocytes. Females had not ovulated eggs were selected on the day of experiment by trying to squeeze the eggs by pushing the abdomen of fish at after onset of light (9 am in our laboratory). Selected females were transferred into a glass case containing 100 ml of water per fish. The fish were exposed to agents *in vivo* by adding each agent into the water (from a 10,000-fold stock in ethanol) at 28.5 °C. The treatments of fish were started 1-2 hours after onset of light (10-11 am in our laboratory). After four hours incubation, zebrafish ovaries were isolated from sacrificed females and placed in fresh zebrafish Ringer's solution (116 mM NaCl, 2.9 mM KCl, 1.8 mM CaCl2, and 5 mM HEPES, pH 7.2). The ovaries were dissected manually into ovarian fragments (each containing 1-10 oocytes) using fine forceps. The oocyte morphology was photographed under a binocular microscope (SZX12, Olympus, Japan). Germinal vesicle breakdown (GVBD) was assessed by scoring the oocytes that became transparent. Ovulation was assessed by scoring the oocytes that showed a clear fertilization membrane. The rates of GVBD and ovulation were determined in at least twenty oocytes in each experimental condition of each fish.

In vitro Ovulation Assay. An *in vitro* zebrafish oocyte maturation and ovulation assay was conducted as described previously [13]. Females had not ovulated eggs were selected by trying to squeeze the eggs by pushing the abdomen of fish at after onset of light (9 am in our laboratory). Ovaries of zebrafish were isolated from sacrificed females and placed in fresh 90% L-15 medium pH 9.0 supplemented by 0.5% BSA and washed with the same solution. The intact follicles were isolated from ovarian fragments manually by using fine forceps. Fully grown immature oocytes were exposed *in vitro* by incubating in 4 ml of the medium containing each experimental

agent (diluted from a 1000-fold stock in ethanol) at room temperature with gentle agitation (40 rpm). To assess the ovulation processes, 4',6-diamidino-2-phenylindole (DAPI) was added into medium and blue fluorescent of follicle cell nucleus were examined under a fluorescent binocular microscope (SZX12, Olympus, Japan). Ovulation was assessed by scoring the oocytes that removed follicle cell layers. GVBD was also assessed by scoring the oocytes that became transparent. % Ovulation was determined in more than twenty oocytes in each experimental condition of each fish.

Fertilization. The fertilization ability of the ovulated eggs was assessed by *in vitro* insemination following standard methods [14]. The fertilization rate (%) was calculated by determining the percentage of embryos that developed to 4-cell or subsequent stages. The rate of fertilization was determined in at least twenty embryos.

Statistical Analysis. All experiments were repeated three times. One-way analysis of variance (ANOVA) was performed using GraphPad Prism (San Diego, CA). A P value < 0.05 was considered to indicate statistical significance.

Results

Externally Applied Org OD 02 Induces Oocyte Maturation and Ovulation in vivo.

As a first step, we confirmed the oocyte maturation-inducing activity of Org OD 02 on zebrafish oocytes in an *in vitro* assay. As reported previously, Org OD 02 induced zebrafish oocyte maturation (Fig. 1A) [16]. Oocytes became transparent after treatment with Org OD 02 or 17,20β-DHP but remained opaque after treatment with ethanol. To evaluate the effect of Org OD 02 on fish oocyte maturation and ovulation, we administered Org OD 02 directly into water in which we held live zebrafish. As

expected, externally applied Org OD 02 induced oocyte maturation *in vivo*. Oocyte maturation was induced *in vivo* when 17,20β-DHP was added into the water in a manner similar to that when the hormone was directly administered to oocytes *in vitro*. Figure 1B shows the morphology of the oocytes after the fish were treated for four hours with ethanol, 17,20β-DHP, or Org OD 02. The oocytes became transparent after administration of Org OD 02 or 17,20β-DHP but remained opaque after administration of ethanol (Fig. 1B). Surprisingly, Org OD 02 induced both oocyte maturation and ovulation. Eggs from fish treated with Org OD 02 or 17,20β-DHP formed fertilization membranes immediately after transfer into medium (Fig. 1B). For most fish, the ovulated oocytes (eggs) were activated immediately after contact with the water to form a fertilization membrane. Ovulation-inducing activity of Org OD 02 was also demonstrated *in vitro*. Org OD 02 induced ovulation of zebrafish oocytes by direct administration on to oocytes *in vitro* (Fig. 2). A dose of 0.01 μM Org OD 02 was sufficient to induce oocyte maturation *in vitro*, and the same concentration induced oocyte maturation and ovulation *in vivo* (Fig. 3). Furthermore the concentration to induce ovulation *in vitro* was also the same (Fig. 3C). Org OD 02 showed almost the same potency in inducing oocyte maturation and ovulation as 17,20β-DHP both *in vitro* and *in vivo.*

Org OD 02 Induces Natural Ovulation In vivo

To demonstrate that the ovulated oocytes arising from Org OD 02 treatment were normal, we conducted *in vitro* fertilization. Hormonal stimulation (with a surge of luteinizing hormone) for oocyte maturation and ovulation begins at night on the day before spawning, preparing the egg to be spawned after the onset of light [17]. We selected gravid females did not contain ovulated eggs by trying to squeeze the eggs by

pushing their abdomen at after the onset of light. Thus, the females used in this study should have been out of the natural spawning cycle, and the compounds added into the water could be used to induce oocyte maturation and ovulation artificially. The eggs ovulated following treatment with Org OD 02 were indeed fertilizable as same as eggs ovulated by 17,20β-DHP-treatment (Fig. 4). The fertilization rates of the ovulated eggs after Org OD 02 and 17,20β-DHP treatment were not significantly different. *In vitro* fertilized eggs developed normally and grown juveniles were fertile (data not shown). These results showed that Org OD 02-induced ovulation was identical to physiological ovulation and that Org OD 02 can be used as a new agent for artificially inducing ovulation in fish.

Discussion

We investigated the effect of a selective agonist for mPR, Org OD 02, on *in vitro* and *in vivo* oocyte maturation in zebrafish. Org OD 02 induced oocyte maturation *in vitro* and *in vivo*, as expected. Furthermore, we found that incubation with Org OD 02 induced ovulation *in vivo*. Eggs could be squeezed from zebrafish that had been treated with Org OD 02 for more than 3 hours. The squeezed eggs successfully developed a fertilization membrane immediately after contact with water. This is a natural characteristic of ovulated fish eggs, which are automatically activated by water contact and begin development without insemination. Fertilized eggs from Org OD 02-induced ovulation developed normally and were fertile (data not shown). The results showed that Org OD 02-induced ovulation was identical to physiological ovulation. The results of this study suggest that Org OD 02 might be applicable for induction of fish spawning. Since the method of injecting 17,20β-DHP is one of the usuful technique to induce fish spawning, the method of inducing spawning by injecting Org OD 02 should be tried.

Org OD O2 is being investigated for its action as a selective agonist of mPR [18]. It is used in experiments showing mPR involvement such as sperm motility activation [19], Ca^{2+} ion influx into T cells [20], and inhibition of breast cancer cell apoptosis [21]. Particulary it is used to indicate that progesterone promotes glioblastoma migration and proliferation, and that its action is mediated by mPR [22] [23]. Org OD O2 showed a similar action to progesterone, indicating that the target of this action was mediated by mPR but not nPR.

In this study, we demonstrated the oocyte maturation- and ovulation-inducing activity of Org OD 02 *in vitro* and *in vivo*. It was demonstrated that diethylstilbestrol (DES) possesses inducing activity of zebrafish oocyte maturation. Although, DES induces oocyte maturation but never induces ovulation *in vivo* [10]. The difference between these results is thought to be dependent on the difference in affinity for nPR. It has been reported that Org OD 02 possesses binding affinity for nPR (Ki=33.9 nM) [18]. In contrast, DES possesses significantly lower affinity for nPR (Ki=11 μM) [24]. This information could explains why Org OD 02, but not DES, could induce ovulation *in vivo*.

Our results demonstrated that Org OD 02 was a selective mPR ligand and that it induced oocyte maturation both *in vitro* and *in vivo*. However, Org OD 02 is also a ligand of nPR that induces ovulation under physiological conditions. Further research is needed to elucidate an mPR-specific ligand [25, 26].

In sum, we demonstrated the oocyte maturation- and ovulation-inducing activity of Org OD 02 *in vitro* and *in vivo*. Org OD 02 is known as a selective mPR ligand. Thus, it is expected to induce only oocyte maturation both *in vitro* and *in vivo*. However, our results demonstrated that Org OD 02 possess an activity to induce

ovulation under physiological conditions. These results suggested that Org OD 02 could be a ligand for both of mPR and nPR *in vivo*.

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Figure legends

Fig. 1. Org OD 02 induced oocyte maturation and ovulation in zebrafish. (A) *In vitro* assay: three oocytes that showed typical morphological changes were selected and photographed after the oocytes were incubated with each compound (0.1% ethanol, 0.1 μM 17,20β-DHP or 0.1 μM Org OD 02) for two hours. The oocytes became transparent after treatment with 17,20β-DHP or Org OD 02 but remained opaque following treatment with ethanol. (B) *In vivo* assay: the morphology of oocytes and eggs from fish treated with 0.01% ethanol, 0.1 μM 17,20β-DHP or 0.1 μM Org OD 02 was photographed. After four hours of incubation, ovarian samples containing oocytes and eggs were excised from the females by dissection. The oocytes remained opaque following treatment with ethanol. A fertilization membrane developed around eggs whose ovulation was induced by 17,20β-DHP or Org OD 02 treatment, as indicated by the arrow.

Fig. 2. Org OD 02 induced ovulation of zebrafish oocytes *in vitro*. (A) *In vitro* assay of ovulation: three oocytes that showed typical morphological changes were selected and photographed after the oocytes were incubated with each compound (0.1% ethanol, 0.1 μM 17,20β-DHP or 0.1 μM Org OD 02) for four hours. Follicle cell layers glowing by

DAPI in blue were removed after treatment with 17,20β-DHP or Org OD 02 but remained following the treatment with ethanol. Follicle cell layer surrounding oocytes is indicated by the arrow. (B) The morphology of oocytes during ovulation was photographed after the induction of ovulation with Org OD 02. The edge of follicle cell layers during ovulation is indicated by the arrow.

Fig. 3. Dose dependency of Org OD 02 induced oocyte maturation and ovulation. (A) *In vitro* assay: ovarian fragments were dissected from sacrificed females and incubated with 0.1 μM 17,20β-DHP or the indicated concentrations of Org OD 02. Percentage of oocytes that induced germinal vesicle breakdown (%GVBD) was determined by scoring the oocytes that had become transparent. Each value represents the mean of data from nine different females. The vertical lines indicate the standard deviations. A one-way ANOVA was calculated on % GVBD. The analysis was significant, $F(5,48) = 151.9$, $p < 0.0001$. (B) *In vivo* assay: for each concentration, ovarian fragments were dissected from sacrificed females, and % GVBD (closed column) and % ovulation (open column) were determined by scoring the oocytes or eggs that had become transparent or formed fertilization membranes. Each value represents the mean of data from six to ten different females. The vertical lines indicate the standard deviations. A one-way ANOVA was calculated on % GVBD. The analysis was significant, $F(5,43) = 60.74$, $p < 0.0001$. A one-way ANOVA was calculated on % ovulation. The analysis was significant, $F(5,43) = 41.38$, $p < 0.0001$. (C) *In vitro* ovulation assay: oocytes were isolated from ovarian fragments and incubated with 0.1 μM 17,20β-DHP or the indicated concentrations of Org OD 02. % GVBD (closed column) and % ovulation (open column) were determined by scoring the oocytes or eggs that had become transparent and removed with follicular cell layers. Each value represents the mean of

data from three different females. The vertical lines indicate the standard deviations. A one-way ANOVA was calculated on % GVBD. The analysis was significant, $F(4,10) = 18.58$, $p = 0.0001$. A one-way ANOVA was calculated on % ovulation. The analysis was significant, $F(4,10) = 3.560$, $p = 0.047$. Values with different subscripts within the same criterion were significantly different ($p < 0.05$).

Fig. 4. Externally applied Org OD 02 induced natural spawning. (A) Fertilization rates were compared between eggs extruded from females treated with 17,20β-DHP *in vivo* and those extruded from females treated with Org OD 02 *in vivo*. Fish were treated with the indicated concentration of 17,20β-DHP or Org OD 02. Artificial fertilization of the extruded eggs was conducted using sperm obtained from the males. The fertilization rate was calculated by counting the number of fertilized eggs. Each value represents the mean of data from six to ten different females. The vertical lines indicate the standard deviations. A one-way ANOVA was calculated on % Fertilization. The analysis was significant, $F(5,12) = 46.53$, $p < 0.0001$.Values with different subscripts within the same criterion were significantly different ($p < 0.05$). (B) Fishes developed normally from eggs ovulated by Org OD 02-treatment. A group of two-month-old juveniles that developed from the eggs whose ovulation was induced by Org OD 02 was photographed.

Figure 1

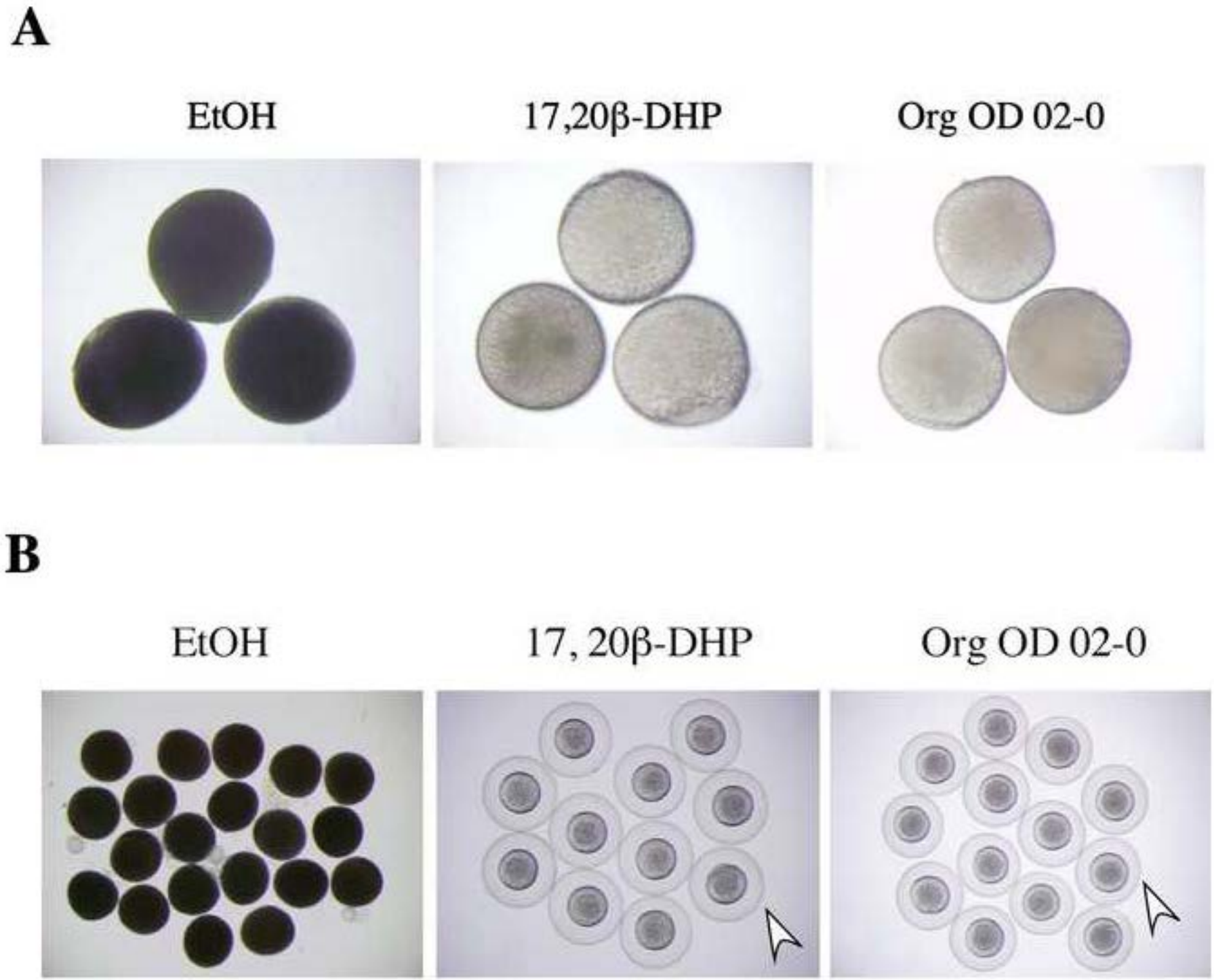

Figure 2

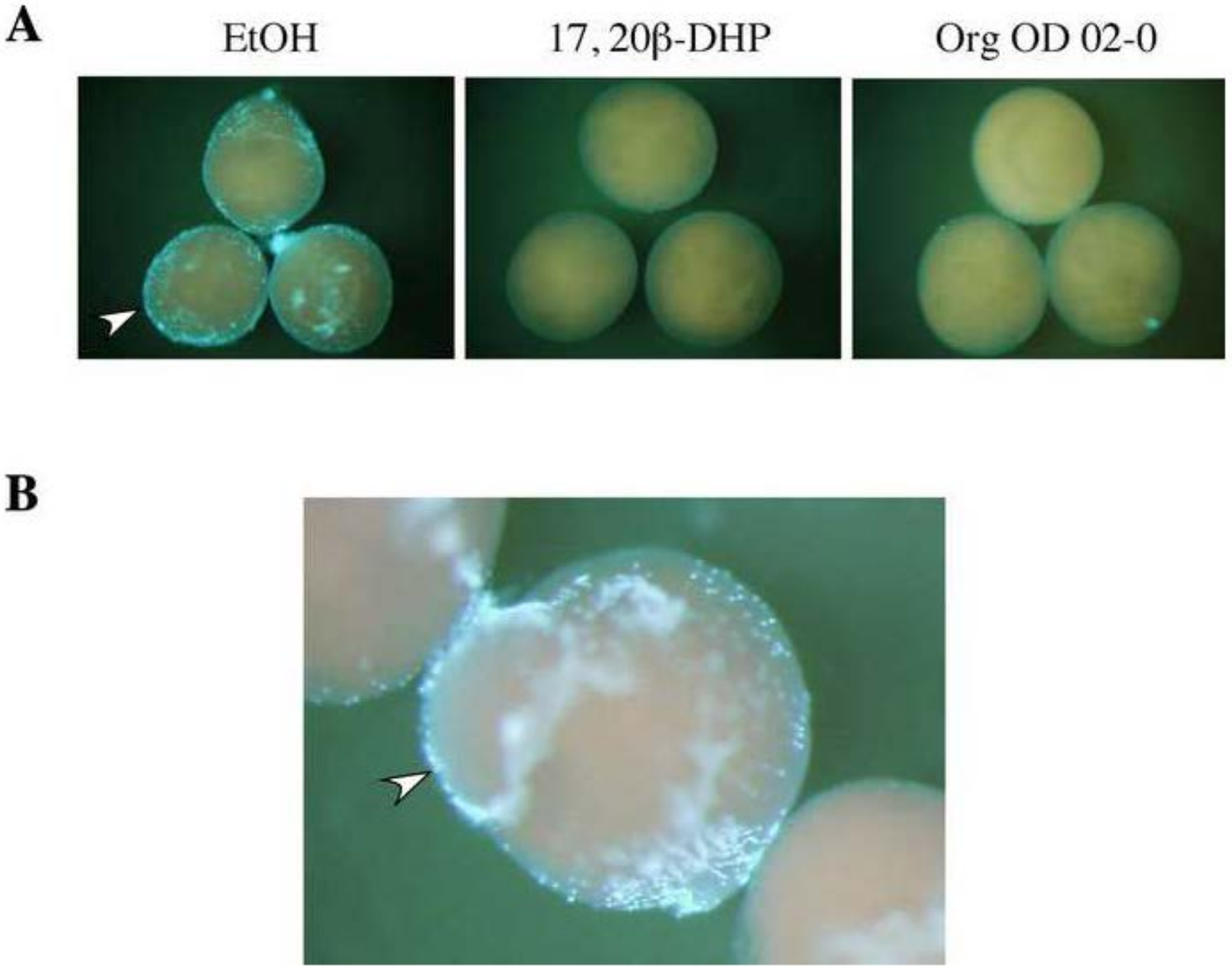

Figure 3

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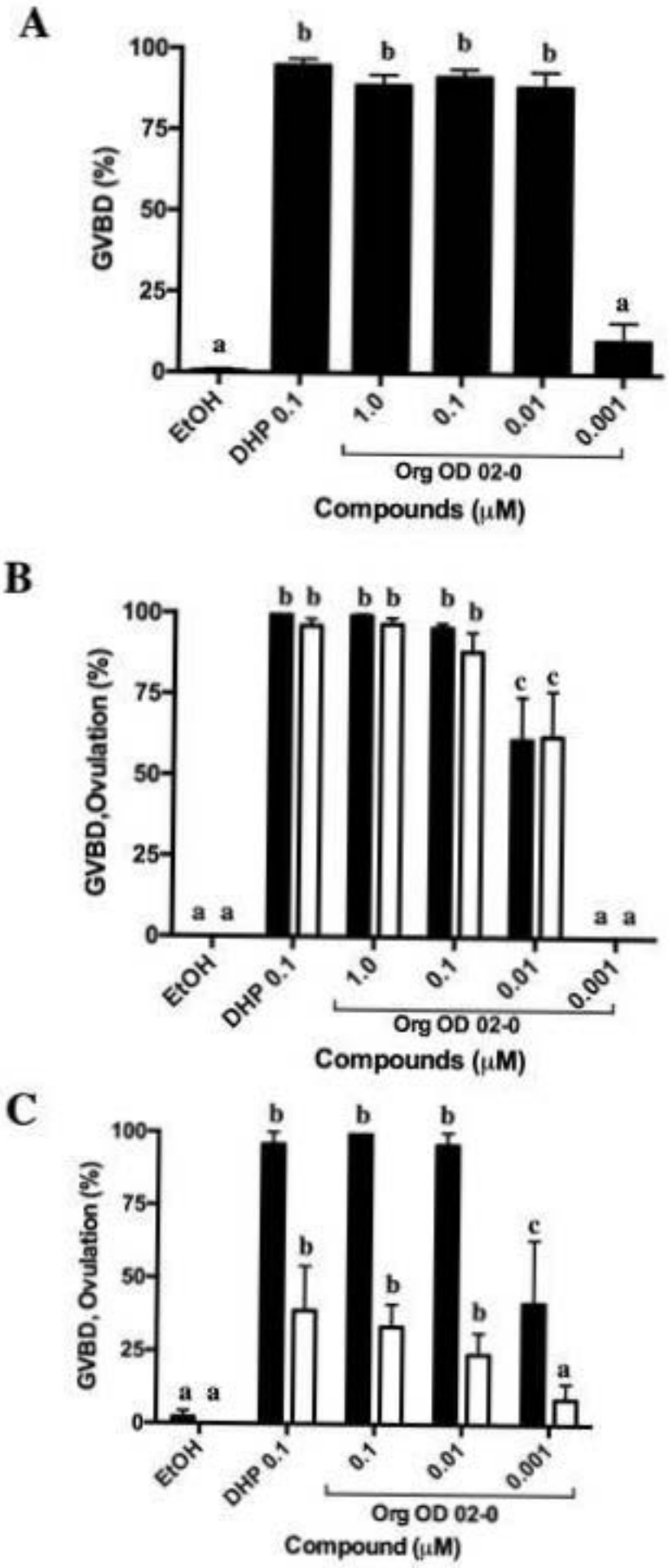

Figure 4