Comparative studies on in vitro sperm decondensation and pronucleus formation in egg extracts between gynogenetic and bisexual fish

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ABSTRACT

A cell-free system based upon the egg extracts from gynogenetic gibel carp (*Carassius auratus gibelio*) or bisexual red common carp (*Cyprinus carpio* red variety) was developed to investigate developmental behaviors of the demembranated sperm nuclei. Both red common carp and gibel carp sperm nuclei could decondense fully and form pronuclei in the red common carp egg extracts. Gibel carp sperm nuclei could also decondense fully and form pronuclei in the gibel carp egg extracts, but red common carp sperm nuclei could not decondense sufficiently in the same extracts. The significant differences of morphological changes were further confirmed by ultrastructural observation of transmission electron microscopy. The data further offer cytological evidence for gonochoristic reproduction in the gynogenetically reproducing gibel carp. In addition, the sperm nuclei in vitro decondensation is dependent on the pH in the extracts, and the decondensed efficiency is optimal at pH 7. However, no DNA replication was observed in the two kinds of egg extracts during the incubation period of the sperm nuclei. It is suggested that the egg extracts prepared from the gynogenetic gibel carp should be a valid in vitro system for studying molecular mechanism on gynogenesis and reproduction mode diversity in fish.

Key words: *decondensation, pronucleus formation, cell-free system, gynogenesis, gibel carp.*

INTRODUCTION

During maturation of most animal sperm cells, sperm-specific nuclear proteins replace somatic histones, and the chromatin becomes highly compacted in preparation for the sperm journey to the egg[1]. This nuclear condensation reverses rapidly following sperm-egg membrane fusion and sperm nuclear incorporation into the egg during fertilization. The sperm nuclear decondensation is obviously a critical early step in embryonic developmental process, necessary for duplication and ultimate transcription of the male genome[2]. Plenty of

information on the early events of sperm nuclei after fertilization, including swelling, chromatin decondensation, DNA synthesis, and nuclear envelope assembly, have been obtained from the studies in cell-free systems based on *Xenopus* eggs extracts [3-6], and cytosol extracts of *Drosophila* embryo[7], CHO cells[8], sea urchin eggs[9], and carrot cell[10].

Fishes, the biggest and most diverse community in vertebrates, are good experimental models for studies on cell and developmental biology by many favorable characteristics[11]. However, studies on the molecular mechanisms of fish sperm decondensation process has been difficult because it is hard to develop a nice cell-free system based on fish eggs for the performance of in vitro biochemical

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Received Dec-18-2002 Revised Mar-10-2003 Accepted Apr-11-2003

studies. Most of the studies on early developmental events of sperm nuclei after fertilization in fish were based on the methods of microinjection together with cytological and ultrastructural observation[12-14]. It is necessary to develop a nice cell-free system to study the sperm decondensation and pronucleus formation in fish.

It has been demonstrated in bisexual reproduction fish that sperm nucleus undergoes nuclear envelope breakdown, nucleus swelling, and pronuclear formation with a new nuclear envelope after fertilization[12, 15]. In contrast to common bisexual reproduction fish, within the fertilized eggs of gibel carp (*Carassius auratus gibelio*), the heterologous sperm nucleus keeps in condensation and is failed to form male pronucleus, while some of the homologous sperm nuclei can decondense and transform into male pronucleus. And there are about 5-20% males in natural populations of gibel carp[16]. Therefore, the gynogenetic gibel carp has been considered as a unique system for studying mechanism of oocyte maturation, sex determination, and early embryonic development[17-21]. However, how to explain the difference of early developmental behaviors between the homologous sperm and heterologous sperm in the gibel carp egg? What is the molecular mechanism by which the natural gynogenesis is regulated in gibel carp? In order to answer the two fascinating questions, an in vitro experimental system requires to be established. In this study, a cell-free system based upon the egg extracts from the gynogenetic gibel carp or bisexual red common carp is developed to investigate developmental behaviors of the demembranated sperm nuclei.

MATERIALS AND METHODS

Obtainment of eggs and preparation of egg extracts

The adult fishes, gynogenetic gibel carp (*Carassius auratus gibelio*) and bisexual red carp (*Cyprinus carpio*) red variety) were obtained from Guanqiao Experimental Station, Institute of Hydrobiology, Chinese Academy of Sciences. Eggs were obtained by two intraperitoneal injections with a mixture of HCG and LRH-A into the adult females. HCG and LRH-A were purchased from Shanghai Lizhu Dong Feng Biotechnology Co., LTD. The first injection was 600 IU HCG and 6 μ g LRH-A/kg fish weight. The second injection was 200 IU HCG and 2 μ g LRH-A/kg fish weight. The interval between two injections was 8 h. About 8 h after the second injection, eggs were obtained from the females. The eggs extracts were prepared essentially as described by Blow and Laskey with some modifications[22]. Briefly, eggs were rinsed 3 times

using 1/4 times MMR buffer (100 mM NaCl, 2 mM KCl, 1 mM MgCl₂, 2 mM CaCl₂, 0.1 mM EGTA, and 5 mM HEPES, pH 7.8), then dejellied in MMR buffer containing 2% cysteine (pH 7.8). The eggs were then rinsed 3 times, and the decondensing activity was induced by adding calcium ionophore A23187 (10 μ M in DMSO; Sigma Chemical Co.) to a final concentration of 100 nM for 15-20 min. After rinsing with MMR containing 2 mM MgCl₂, and 0.6 mM CaCl₂, the eggs were washed with lysate buffer (250 mM sucrose, 50 mM KCl, 2.5 mM MgCl₂, 10 mM HEPES, pH 7.4) and the lysate buffer was removed as much as possible by centrifugation at 500 g. The eggs were homogenized gently in a homogenizer in the presence of several protease inhibitors (aprotinin and leupeptin, final concentration 10 μ g/ml). The homogenates were subjected to centrifugation for 15 min at 9,000 g and were separated into three layers: a white lipid upper layer, a clear yellow middle layer and a viscous pellet. After removing the lipid layer, the yellow middle layer was collected with a chilled pipette and was clarified by re-centrifugation. The resulting cytoplasmic supernatant was recovered and either used freshly or frozen for future use.

Preparation of demembranated sperm nuclei

Demembranated sperm nuclei were prepared using a modification of the method described by Philpott et al[4]. Male fishes were injected with 500 IU HCG /kg fish weight. About 12 h later, the sperm were obtained from the injected male fishes and washed 4 times in buffer XN [50 mM HEPES-KOH (pH 7.0), 250 mM sucrose, 75 mM NaCl, 0.5 mM spermidine, 0.15 mM spermine]. Washed sperm were resuspended in 500 μ l of XN buffer. To demembranate sperm, 100 μ l of 2 mg/ml lysolecithin (Sigma) in buffer XN was added followed by incubation on ice for 10 min. Permeabilization was stopped by adding 1 ml of 3% bovine serum albumin (BSA) in buffer XN. Sperm nuclei were washed three times in buffer XN/0.4% BSA, and then finally resuspended in buffer XN/30% glycerol. Sperm nuclei were counted with a hemocytometer, and stored at -7°C.

In vitro decondensation and fluorescent microscopy

The eggs extracts were adjusted to pH 7.0 or other pH value with 50 mM Hepes (pH 7.8) and 0.1 N NaOH or HCl. The demembranated sperm was incubated at 23°C in 200 ml egg extracts containing an ATP regenerating system (2 mM ATP, 20 mM phosphocreatine, and 50 μ g/ml creatine kinase). Samples were mixed gently. 5 μ l aliquots were sampled at each time point, and mixed with 10 μ l solution containing 1 μ g/ml Hoechst 33258 and 1% formaldehyde. Aliquots were then viewed wet under a Leica DMIRB inverted microscope. Images were captured with a Leica DC100 image analyzer. Three fields from the same slide, containing 5-40 nuclei/field, were counted for determining the percentage of decondensation present at a given time.

Transmission electron microscopy

Samples for electron microscopy were fixed by overlaying with 800 μ l of cold 2.5% glutaraldehyde in PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 2 mM KHPO₄, pH 7.4), and kept at 4°C overnight. The samples were spun at 2,500 g for 5 min and the pellets washed three times with PBS. Pellets were postfixed for 3 h at 4°C using 1% OsO₄ in PBS, then further washed three times in

this buffer before block staining overnight in 2% uranyl acetate. The samples were dehydrated through ethanol and embedded in polymerized acrylic resin. Blocks were sectioned to give silver to pale gold sections that were taken onto collodion- and carbon-coated 600-mesh copper grids before double staining using uranyl acetate and Reynold's lead citrate (5 min each). The sections were viewed using a transmission electron microscope (JEM-1230) at 80 kV.

Flow cytometry analysis

For flow cytometric analysis, the demembranated sperm nuclei were prepared at different interval according to the method described by Blow and Watson[23]. The isolated nuclei were stained with 1 ml of a solution of 5 μ g/ml propidium iodide and 50 μ g/ml RNase. The above nuclei were analysed for DNA with the Coulter Epics Altra Flow Cytometer (Beckman Coulter Co., USA.). The laser was tuned to 488 nm line at a light power, which excites red fluorescence from the propidium iodide/DNA complex.

RESULTS

Similarity of morphological changes between red common carp sperm nuclei and gibel carp sperm nuclei in the egg extracts from red common carp

Incubated in the egg extracts from red common carp, both red common carp and gibel carp demembranated sperm nuclei underwent chromatin decondensation and similar morphological changes (Fig 1). The two kinds of sperm nuclei were highly condensed when added into the egg extracts from red common carp, and the gibel carp sperm nuclei are larger than the red common carp sperm nuclei (Fig 1A-a, B-a). At 30 min of incubation, 49.4% of the red carp sperm nuclei transformed into decondensed forms including forms of swollen conical, elongated bar, and swollen sphere (Fig 1A-c, Fig 2A). At 60 min after incubation, the decondensed nuclei increased to over 80%. At that time, these sperm nuclei had decondensed to form small pronuclei (Fig 1A-d), and the pronuclei further enlarged at 90 min (Fig 1A-e). Up to 120 min after incubation, the decondensed nuclei reached to 90.5% (Fig 1A-f, Fig 2A). Similarly, 44.0% of the demembranated sperm nuclei of gibel carp were decondensed at 30 min of incubation in the egg extract of red common carp. And they began to transform their shape from condensed small sphere to long bar or conical shape after 60 min. Moreover, the pronuclei were formed as similar to that of red carp sperm. The percentage of the decondensed sperm nuclei was 88.5% at 120 min of incubation (Fig 1B, Fig 2A).

Difference of morphological changes between red common carp sperm nuclei and gibel carp sperm nuclei in the egg extracts from gynogenetic gibel carp

When incubated in the egg extracts of gynogenetic gibel carp, the sperm nuclei of red carp were kept in condensed state or only underwent subtle swollen change during the incubation (Fig 3A), whereas the gibel carp sperm nuclei exhibited obviously morphological changes (Fig 3B). The typically decondensed changes and pronucleus formation (Fig 3B) were similar to that of red common carp sperm and gibel carp sperm in the egg extracts from red common carp. When introduced into the egg extract from gibel carp and observed immediately under fluorescent microscope, 83.8% of the demembranated gibel carp sperm nuclei displayed a condensed sphere. By 5 min of incubation, 46% of the sperm nuclei began to elongate, swell, and decondense. After incubation for 60 min, about 75% of the nuclei were decondensed, and the decondensed nuclei reached to 85.1% after incubation for 120 min (Fig 2B and Fig 3B). However, when incubated in the same egg extracts, most of red carp sperm nuclei did not show obviously morphological changes. No decondensed nucleus was observed during the whole incubation period (Fig 3A).

Pronucleus formation of gibel carp sperm nuclei in the egg extracts from gynogenetic gibel carp

Pronucleus formation was observed in both red common carp and gibel carp sperm nuclei after incubated in the egg extracts of red common carp for more than 60 min (Fig 1), and similar process of pronucleus formation was also revealed in gibel carp sperm nuclei after incubated in the egg extracts of gibel carp for about same time (Fig 3B). Under high magnification (1000 times), it was observed clearly that the chromatin of these decondensed sperm nuclei was loosed and with less density (Fig 4 a-d). Furthermore, we also observed that some of them transformed into decondensed swollen spheres whose chromatin was obviously loosed and the fluorescent intensity was also weakened, showing that in vitro male pronucleus formation was occurred in the above cell-free system (Fig 4 d), and that the pronucleus formation in vitro was similar to that in vivo observed previously in bisexual fish eggs[14]. However, no male pronucleus formation was ob-

Fig 1. Decondensation process of demembrated sperm nuclei in red common carp egg extracts (200 $\times$). **(A)** Red common carp sperm nuclei; **(B)** Gibel carp sperm nuclei. The sperm nucleus samples incubated at (a) 0 min, (b) 5 min, (c) 30 min, (d) 60 min, (e) 90 min, and (f) 120 min were collected and observed by fluorescent microscopy.

Fig 2. Percentage of decondensed sperm nuclei in red common carp egg extracts (A) and gibel carp egg extracts (B). 1. Red common carp sperm nuclei; 2. Gibel carp sperm nuclei.

served when red carp demembranated sperm nuclei were introduced into the eggs extract of gibel carp for 120 min (Fig 3A).

Ultrastructural changes of red common carp sperm nuclei and gibel carp sperm nuclei in the egg extracts from gynogenetic gibel carp

In order to further investigate the structural and morphological differences of gibel carp or red common carp sperm nuclei following incubation in the egg extracts from gynogenetic gibel carp, transmission electron microscopy were applied to ultrastructural investigation. As shown in Fig 5, the gibel carp sperm nuclei obviously enlarged, and the chromatin dispersed after one-hour incubation in the egg extracts from gibel carp (Fig 5A-b, 5B-b). However, no obvious change in size, morphology or chromatin decondensation was observed when the demembranated red carp sperm nuclei were introduced into the same cell-free system and incubated

for the same time (Fig 5A-d, 5B-d).

DNA content analysis on different decondensation sperm nuclei by flow cytometry

DNA contents of the two kinds of sperm nuclei incubated for different times in the two egg extracts were analyzed with flow cytometer to investigate whether DNA replication was occurred during sperm nuclei decondensation in vitro. As show in Fig 6, no change in the DNA contents was observed in all the samples during incubation of 300 min, suggesting that no DNA replication was performed in the two kinds of egg extracts during the incubation period of the sperm nuclei.

Effect of pH on sperm decondensation

In some animal species, fertilization often results in an increase of cytoplasmic pH from approximately 6.3 to 7.2 in vivo[24, 25]. In order to optimize pH in the cell-free system, we tested the effects of pH on the decondensation of demembranated sperm nuclei. As shown in Fig 7, the decondensed efficiency is optimal at pH 7 whether the extracts are from red common carp eggs or from gibel carp eggs.

DISCUSSION

As shown in the present study, a cell-free system has been established on the basis of the egg extracts from bisexual red common carp and gynogenetic gibel carp, and significant differences of morphological changes are revealed by fluorescent microscopy and transmission electron microscopy between demembranated red common carp sperm nuclei and gibel carp sperm nuclei in the egg extracts from gynogenetic gibel carp. The red carp sperm nuclei are kept in condensed state in egg extracts from the gynogenetic gibel carp (Fig 3A), whereas the gibel carp sperm nuclei in the gibel carp extracts undergo obviously morphological changes, such as full decondensation and pronucleus formation (Fig 3B) that are similar to that of red common carp sperm and gibel carp sperm in the egg extracts from bisexual red common carp (Fig 1). The data further offer cytological evidence for gonochoristic reproduction in the gynogenetically reproducing gibel carp. Consistent with the Previous studies on molecular markers[21, 26] and karyotypic diversity[27], the two kinds of differently developmental behaviors be-

Fig 3. Decondensation process of demembrated sperm nuclei in gibel carp egg extracts (200 $\times$). (A) Red common carp sperm nuclei; (B) Gibel carp sperm nuclei. The sperm nucleus Samples incubated at (a) 0 min, (b) 5 min, (c) 30 min, (d) 60 min, (e) 90 min, and (f) 120 min were collected and observed by fluorescent microscopy.

Fig 4. Several typical pronucleus morphology (a-arc, b-conical, c-long bar, d-swollen sphere) observed in the incubation group of gibel carp sperm nuclei in the gibel carp egg extracts (1000 $\times$).

tween red common carp sperm nuclei and gibel carp sperm nuclei in the egg extracts from gynogenetic gibel carp should be the response mechanism of two reproductive development modes, i.e., gynogenetic and gonochoristic reproduction. Thus, the cell-free system prepared from the gibel carp eggs can mimic the developmental processes occurred in fertilization in vivo, and further confirms that the gibel carp possesses two reproductive modes.

Previous investigations in many bisexual species had demonstrated that the sperm decondensation in cell-free system was not species-specific[28-30]. And some heterologous sperm nuclei with far relationship to the species from which the extract were made could be decondensed in the *Xenopus laevis* egg extract or cell-free extract of carrot[10, 31]. We observed that both the red carp and gibel carp sperm nuclei incubated in the red carp egg extracts decondensed and swelled obviously, and underwent pronucleus formation (Fig 1). However, no red carp sperm nuclei could decondense fully after incubation for 90 min or even 120 min in the gibel carp egg extracts, whereas more than 80% of gibel carp sperm nuclei underwent decondensation and pronucleus formation at the same time point in the same egg extracts (Fig 2B, Fig 3A and 3B). This phenom-

enon implies that different sperm nuclei incubated in the gibel carp egg extracts undergo different developmental process.

After fertilization in vivo in bisexual fish, the membrane of egg was fused with sperm membrane. And a series of physiological changes including the activation of some factors required for the collapse of sperm nuclear membrane were occurred after the fusion, which was a prerequisite for the decondensation of sperm nucleus in vivo. Different from observation in vivo, it was observed that both intact red carp sperm nucleus and gibel carp sperm nucleus could not be decondensed in the above two kinds of egg extracts (data was not shown), which indicated that the collapse of sperm nuclear membrane was required for the decondensation of sperm nucleus in vitro. We speculated that the above difference was resulted from the inactivation of some factors required for the collapse of sperm nuclear membrane.

The formation of male pronucleus is a prerequisite for the fusion of male pronucleus and female pronucleus. In the gibel carp egg extracts, the demembranated gibel carp sperm nuclei were able to form male pronuclei with loose chromatin and weak fluorescent intensity after 120 min of incubation, whereas no male pronucleus formation was observed when the demembranated red common carp sperm nuclei were introduced into the same system at the same time point. In contrast, both red common carp and gibel carp sperm nuclei were able to transform into male pronuclei in the red common carp egg extracts after 120 min incubation. The difference of male pronucleus formation between gibel carp and red common carp sperm nuclei in the gibel carp egg extracts is likely related to the mechanism underlying different reproduction modes. Therefore, we propose that some regulative factors might exist in the gibel carp egg cytoplasm. A series of molecular and biochemical studies on identification of the candidate factors have been initiated in our laboratory[32-36], and the studies will help us to elucidate the molecular basis underlying the reproduction modes.

In the cell-free system of *Xenopus* eggs, 70%-100% of *Xenopus* sperm nuclei had been demonstrated to replicate completely under optimal conditions[22]. DNA replication initiation of sperm nu-

Fig 5. Electron micrographs (A, 8,000 $\times$; B, 20,000 $\times$) of condensed and decondensed sperm nuclei. (a) the condensed gibel carp sperm nuclei before incubation, (b) the decondensed gibel carp sperm nuclei incubated in the gibel carp egg extracts for 60 min, (c) the condensed red common carp sperm nuclei before incubation, (d) the condensed red common carp sperm nuclei incubated in the gibel carp egg extracts for 60 min.

Fig 6. DNA content detection of sperm nuclei in egg extracts. (A-F) gibel carp sperm nuclei in gibel carp egg extracts, (G-L) red common carp sperm nuclei in gibel carp egg extracts, (M-R) red common carp sperm nuclei in red common carp egg extracts, (S-X) gibel carp sperm nuclei in red common carp egg extracts. Samples were collected at 0 h (A, G, M, S), 1 h (B, H, N, T), 2 h (C, I, O, U), 3 h (D, J, P, V), 4 h (E, K, Q, W), and 5 h (F, L, R, X), and detected with flow cytometry as described in materials and methods.

Fig 7. Effect of pH on sperm nuclei decondensation in cell-free system. (A) Gibel carp sperm nuclei incubated in gibel carp egg extracts. (B) Red common carp sperm nuclei incubated in red common carp egg extracts.

clei was also observed in a human cell-free system [37]. However, no DNA replication was observed during the incubation period of the sperm nuclei in the two kinds of egg extracts. Sheehan et al had isolated the *Xenopus* egg extract into different fractions required for chromatin decondensation and DNA replication respectively[38], indicating that chromatin decondensation and DNA replication were two different processes and that they required different factors. Moreover, some factors related to the initiation or activation of DNA replication had been identified[39]. The reason that no DNA replication was observed in the fish cell-free system might be that the factors required for initiating the DNA replication lacked or inactivated in the extracts. The formation of male pronucleus was judged by some morphological changes which were occurred after sperm nucleus was introduced into the cell-free extract in previous studies[40]. Therefore, we considered that the male pronucleus was formed after some morphological transformations of the sperm nucleus were happened in the fish cell-free system in spite

of the absence of DNA replication.

In some species, fertilization often results in an increase of cytoplasmic pH from approximately 6.3 to 7.2 *in vivo*[24, 25]. And the optimal pH for sperm nucleus decondensation in cell-free system from sea urchin eggs is 7.0[9]. As shown in Fig 7, response of our system was also optimal at pH 7.0, confirming that pH could play important effect on sperm nucleus decondensation in cell-free system.

Although no DNA replication is observed in the cell-free system reported here, it can mimic the process of sperm nuclei decondensation after fertilization *in vivo*. It will allow experiments with immunodepletion or biochemical studies otherwise difficult or impossible to perform *in vivo*. Such experiments should facilitate to elucidate molecular mechanisms underlying reproductive mode diversity of gibel carp. Therefore, the egg extract should be a valid *in vitro* system for studying the mechanism.

ACKNOWLEDGMENTS

This work was supported by grants from the National Natural Science Foundation of China (No. 30130240 and 30070379), the Project of Chinese Academy of Sciences (KSCX2-SW-303) and the Frontier Science Projects Program of the Institute of Hydrobiology, Chinese Academy of Sciences (No. 220309).

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