Cryobiology xxx (xxxx) xxx–xxx

Contents lists available at ScienceDirect

Cryobiology

journal homepage: www.elsevier.com/locate/cryo

Chilling causes perivitelline granule formation in activated zebrafish oocytes

Fatemeh Jalali[a], Angela J. DiBenedetto[a], Jens O.M. Karlsson[b,*]

[a] Department of Biology, Villanova University, Villanova, PA 19085, USA
[b] Department of Mechanical Engineering, Villanova University, Villanova, PA 19085, USA

ARTICLE INFO

Keywords:
Chilling
Cryopreservation
Cryomicroscopy
Oocyte
Gamete
Teleost
Zebrafish
Danio rerio

ABSTRACT

Chilling sensitivity in oocytes of the zebrafish represents a potential obstacle to their successful cryopreservation. Here, we report the first cryomicroscopic observations of the response of zebrafish oocytes to chilling conditions. In activated stage V oocytes that had been exposed to hypothermic temperatures, we observed a latent effect of chilling, manifesting as a granular precipitate that appeared in the perivitelline fluid upon return to 28.5 °C. The granules were visible in unstained oocytes under transmitted light microscopy, and the resulting perivitelline turbidity increased in a dose-dependent manner with decreasing chilling temperature ($p < 0.001$), as well as with increasing time of hypothermic exposure ($p < 0.0001$). The change in appearance of the perivitelline space in oocytes that had been chilled and rewarmed became statistically significant after a 7-min exposure to 10 °C and after only 30 s at 1 °C ($p < 0.05$). Thus, even moderate chilling exposures can lead to detectable changes in activated zebrafish oocytes.

Economical germplasm banking technology is not yet available for maintenance of genetic strains of the zebrafish (*Danio rerio*), due to the lack of successful cryopreservation techniques for zebrafish embryos and oocytes. Chilling injury can represent a significant obstacle to cryopreservation, because preservation procedures typically require extended exposure to hypothermic temperatures before cryogenic stasis is achieved (for example, prolonged chilling may be inevitable during slow freezing procedures, or during exposure to vitrification solutions). Considering the known chilling sensitivity of zebrafish embryos [1,12] and immature oocytes [4,6,9,11], we instead turn our attention to the mature zebrafish oocyte, which is thought to be more resistant to chilling injury [4].

The report by Isayeva et al. [4] is the only previously published investigation of chilling sensitivity in mature (stage V) zebrafish oocytes. These authors found a 95% viability for stage V oocytes that had been exposed to 0 °C for 15 min, and only a slight reduction (to 88% viability) after a 60-min exposure to 0 °C, based on results from a trypan blue exclusion assay [4]. In contrast, we have now performed direct cryomicroscopic observations of stage V zebrafish oocytes subjected to chilling exposures less severe than those tested by Isayeva et al. [4], and found that oocyte appearance was sometimes profoundly altered after the hypothermic insult.

All zebrafish studies were performed using IACUC-approved protocols. Adult wildtype zebrafish (strain AB, 6–12 months) obtained from the Zebrafish International Resource Center (Eugene, OR), or received as a generous gift from the University of Pennsylvania (Philadelphia, PA), were maintained in a filtered, aerated housing system at a temperature of 28.5 °C, on a 14:10 h light/dark cycle, and were fed a combination diet of live brine shrimp and tropical fish flake food (Tetra, Blacksburg, VA) three times daily. Stage V oocytes were collected from ovulating females by gently squeezing the abdomen of the fish after anesthesia by immersion in 0.168 mg/ml tricaine (Sigma Aldrich, St. Louis, MO). Oocytes were maintained in vitro by incubation at 28.5 °C in Hank's balanced salt solution (Sigma Aldrich) supplemented with 0.5% (w/v) bovine serum albumin (Sigma Aldrich). The average time delay between oocyte collection and experiment was 42 ± 3 min, and the delay time did not have a significant effect on the outcome of chilling experiments ($p > 0.05$, ordinal logistic regression).

Oocytes were observed during chilling exposure and subsequent rewarming by cryomicroscopy. Cryomicroscopy specimens were prepared by mounting 1–5 oocytes in supplemented Hank's buffer, sandwiched between two glass coverslips using a silicone rubber spacer, and loaded into the temperature-controlled microscope stage (BCS196, Linkam Scientific Instruments, Surrey, UK) at an initial temperature of 28.5 °C. Oocytes were imaged at 5× or 20× magnification under brightfield illumination, and each experiment was documented in a 20-min video acquired at 50 frames/s using a digital camera (Phantom v7.1, Vision Research, Wayne, NJ). A detailed description of the cryomicroscopy system was published previously [5].

Even though the collected oocytes were stored in a buffer formulation that had been designed to block oocyte activation for up to 5 h [8,10], a large fraction (> 70%) of video recordings were found to

* Corresponding author. Department of Mechanical Engineering, Villanova University, 800 Lancaster Avenue, Villanova, PA 19085-1681, USA.
E-mail address: karlsson@alum.mit.edu (J.O.M. Karlsson).

https://doi.org/10.1016/j.cryobiol.2018.01.012
Received 11 July 2017; Received in revised form 24 January 2018; Accepted 28 January 2018

show some degree of separation (on the order of 10 μm or more) between the chorion and the oolemma even prior to cooling, indicating that the oocytes were experiencing early stages of activation at the start of the cryomicroscopy experiments. This may be explained in part by the ability of mechanical stimuli (such as those that may occur when mounting oocytes for microscopy) to trigger activation of zebrafish oocytes [3].

In initial trials to check for evidence of chilling injury, oocytes were subjected to a multistep cooling profile (cooling from 28.5 °C to 1 °C at 60 °C/min, from 1 °C to −0.5 °C at 6 °C/min, then holding isothermally for 30 s before cooling at 0.5 °C/min to a final holding temperature of −0.9 °C). At select time points in the temperature profile, oocytes were warmed rapidly (60 °C/min) to 28.5 °C, and held at this temperature for 3–19 min. Video was acquired from the start of the initial cooling ramp, until the end of the experiment. In activated oocytes that had been cooled to −0.9 °C, the accumulation of a granular substance in the perivitelline fluid (in the space between the oolemma and the elevated chorion) could be seen during the warming ramp and the subsequent isothermal hold at 28.5 °C (see Supplementary Video S1). In contrast, no such perivitelline granules were observed in activated oocytes that had been rewarmed prior to reaching subzero temperatures (see Supplementary Video S2).

Supplementary video related to this article can be found at http://dx.doi.org/10.1016/j.cryobiol.2018.01.012.

Systematic measurement of the number of perivitelline particles was not practical, due to various confounding factors (including sample drift, dynamic changes in the size and shape of the perivitelline volume, as well as movement of particles into and out of focus). Instead, we defined a semiquantitative ordinal scoring system to grade the severity of perivitelline turbidity in each video. Grade 1 was defined to describe oocytes with no granules in the perivitelline space, as well as oocytes with negligible levels of perivitelline granule formation (fewer than $\sim 10^{-4}$ particles/μm^2). Grade 2 encompassed formation of moderate numbers (fewer than $\sim 10^{-2}$ particles/μm^2) of mostly small granules, whereas Grade 3 encompassed formation of numerous granules of various sizes, producing a mottled appearance of the perivitelline space. Micrographs depicting oocyte appearance representative of each of the defined turbidity grades are shown in Fig. 1.

The kinetics of darkening of the perivitelline space was examined in a few videos representative of each of the defined turbidity grades. As shown in Fig. 2, the amount of light transmitted through the perivitelline space after granule formation decreased with increasing turbidity grade, as expected. The perivitelline darkening reaction started during the warming ramp, and the turbidity increased gradually over a time interval of 30–60 s, as perivitelline particles became more numerous. Although the average gray-level of the perivitelline area tended to become somewhat lighter after a few minutes, granules remained visible in chilled oocytes for the duration of the observations (which lasted up to 19 min).

To determine the effects of temperature and exposure time on perivitelline granule formation, we conducted a full factorial experiment, in which samples containing up to 5 oocytes each were rapidly cooled (at a rate of 60 °C/min) to one of two chilling temperatures (1 °C or 10 °C), and held for various time intervals (0, 0.5, or 7 min) before rewarming (at 60 °C/min) to 28.5 °C. The severity of granule formation was assessed by scoring the appearance of a representative video frame acquired during the isothermal hold 28.5 °C, following each hypothermic exposure. The appearance of control oocytes was graded after a 20-min hold at 28.5 °C. Out of a total of 117 oocytes used in these experiments, a few (3%) were omitted from analysis because they were found to have been mechanically damaged (ruptured) during the process of sample mounting. Among the remaining 113 oocytes, 23 (20%) did not exhibit any separation of chorion and oolemma by the end of the video recording, thus precluding evaluation of the perivitelline space. Thus, a total of 90 intact, activated oocytes were graded using the semiquantitative scoring system defined in Fig. 1.

As shown in Fig. 3, the average severity of granule formation seen upon rewarming from chilling exposure was dose-dependent, increasing with decreasing chilling temperature and with increasing exposure time. No granule formation was evident in oocytes that had been rewarmed immediately (0 min hold) upon reaching 10 °C, nor in the majority (13/19 = 68%) of oocytes rewarmed immediately from 1 °C, indicating that the mechanism that triggers granule formation is not instantaneous. Because the severity data were neither normal nor homoscedastic, a two-way Brunner-Dette-Munk analysis was conducted, showing that there was a statistically significant effect of both temperature ($p < 0.001$) and exposure time ($p < 0.0001$), but that the interaction between temperature and time was not significant ($p > 0.1$). Pairwise comparisons using the Games-Howell post hoc test revealed that the difference in mean granule formation severity between oocytes held at 10 °C and 1 °C became statistically significant after a 7-min exposure ($p < 0.05$). Moreover, the perivitelline turbidity grade following chilling was significantly different from controls after 7 min at 10 °C, and after only 0.5 min at 1 °C ($p < 0.05$). The dose-dependence of perivitelline granule formation is consistent with previous observations of dose-dependent chilling injury in stage V zebrafish oocytes, reported by Isayeva et al. [4]. These authors found that the membrane integrity of stage V oocytes chilled to 0 °C or −5 °C tended to decrease with reduced temperature and with increased hypothermic exposure time [4].

Although the identity of the granular material observed in our experiments has not been determined, the following behaviors were evident under high magnification (see Supplementary Video S3). First, the granules appeared to move freely under the influence of perivitelline fluid convection and Brownian impulses. Second, the granule size tended to increase with time, whereas the number of perivitelline particles appeared to initially increase and subsequently decrease, consistent with an aggregation process and/or an Ostwald ripening mechanism. Although the majority of particles appeared to form in situ within the perivitelline space, it is possible that the substance may have originated from the oolemma in a form initially not detectable by light microscopy, and subsequently grown to visible dimensions. The concentration of granules was generally higher near the oolemma and lower near the chorion, consistent with origination at the cell membrane. It is in principle possible that granulogenesis involves some exogenous substance transported from the buffer into the perivitelline space, but the fact that we never observed granule formation outside the chorion implies that at least one constituent required for granule formation must originate from the oocyte itself. Moreover, our dose-response data indicate that accumulation of this endogenous substrate in the perivitelline space increases for more severe chilling exposures. Therefore, the formation of perivitelline granules can be interpreted as a biomarker for the chilling-induced transformation of some endogenous ovular component.

Supplementary video related to this article can be found at http://dx.doi.org/10.1016/j.cryobiol.2018.01.012.

Previously published experiments indicate that the membrane integrity of stage V oocytes exposed to 0 °C for 15 min (95 ± 3%) is indistinguishable from controls when assayed after rewarming to 22 °C [4]. This suggests either that damage to the oolemma is not required for granule formation, or alternatively, that if perivitelline granules are caused by material that has leaked from the oocyte during warming, then the loss of membrane integrity must be transient and occur at temperatures lower than 22 °C. Because the oocytes in our samples were at least partially activated, it is possible that the observed granular precipitation was caused by interference of hypothermic temperatures with the cortical granule reaction. Cortical granule cores are normally globular and transparent in appearance when released at room temperature [2], but it is possible that chilling causes some physical transformation of this exudate. Moreover, cortical granule exocytosis is normally accompanied by complex membrane reorganization processes [2], which may degenerate at hypothermic temperatures. Further work