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"Effect of Gametes Aging on their Activation and Fertilizability in Zebrafish (*Danio rerio*)"

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Effect of Gametes Aging on their Activation and Fertilizability in Zebrafish (*Danio rerio*)

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Abstract

The zebrafish represents an important model organism for biological research. In this context, in vitro collection and fertilization of zebrafish gametes are basic and widely used techniques for many topical research works. In this work, the fertilization ability and normal embryo development of gold-type zebrafish sperm and eggs were re-evaluated after being stored for different times at 8ºC in a modified medium (Hanks´ saline supplemented with 1.5 g BSA and 0.1 g ClNa; 320 mOsm, pH 7.4).

Results obtained indicated that the temporal limits usually recommended for zebrafish sperm to fertilize fresh eggs (2 h) can be extended for up to 24 h without significant differences compared with fresh sperm. In contrast with this, the rapid egg aging observed (even less than 1 h) recommends minimizing as far as possible the egg storage time prior to fertilization. These results suggest a possible strain difference in the fertilization response.

CONTENTS

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I. INTRODUCTION

In vitro collection and fertilization of zebrafish gametes are basic techniques for many topical research works. Main zebrafish laboratory guides recommend time limits to use gametes efficiently in the laboratory. These aging limits are 90 min (Westerfield, 2003) or 2 h (Nüsslein-Volhard and Dahm, 2002) for sperm stored in Hanks' buffered salt solution on ice, and 2 h (Sakai, 1997) for eggs stored in a modified medium at room temperature.

Our lab is focused on the development of cell nuclear transplant methods (NT) in zebrafish before, during and after egg activation by sperm fertilization and, for this reason, we followed the recommendations of Sakai (1997) (Hank's saline plus 0.5% BSA, 23 ºC) to the required egg activation delay. However, in our hands, the continuous detection of many spontaneous egg activations throughout each NT session at the temperature (23 ºC) recommended by Sakai led us to establish new egg storage conditions. In preliminary assays (results not published), we established the storage conditions that supposed, in our hands, the best results achieved in terms of rate reduction of spontaneous egg activation (Hank's saline plus 1.5% BSA and 0.1 g ClNa and temperature storage of 8 ºC). Under these storage conditions, it was also observed that sperm remained in optimal conservation state throughout each NT session, and thus facilitated the gamete mixing, manipulation and sperm use as an egg activating agent (by fertilization).

A casual observation in our lab showed that, on the one hand, both sperm and eggs from zebrafish gold strain remained in a non-activated state and showed normal activation signals after 24 h of storage at 8ºC. On the other hand, we also observed a normal rate of well developed embryos when fresh eggs were in vitro fertilized with 24

h aged sperm. In contrast with this, when 24 h aged eggs were fertilized with fresh sperm, initial activation signs followed by some abortive cleavages were also observed, but no further developments were finally achieved in any case. These observations indicated that the temporal limits usually recommended could perhaps be extended over some specific conditions. So, the aim pursued in this work was to re-evaluate the temporal limitations in zebrafish gametes for experimental use commonly established in this species (Westerfield, 2003; Nüsslein-Volhard and Dahm, 2002; Sakai, 1997; Ransom and Zon, 1999), particularly in NT protocols.

II. MATERIALS AND METHODS

a. Animal care and obtaining of gametes

Zebrafish (Danio *rerio*) specimens from the gold strain were maintained in 20 L aquariums under standard conditions (Westerfield, 2003) and taking into account temperature and light photoperiod. The female:male ratio was established at 2:1 per aquarium. Granular food was supplemented with recently defrosted chicken egg yolk and shrimp meat as recommended for egg production in zebrafish in substitution of live food (Simão *et al.*, 2007).

At the same artificial "dawn" time, those aquariums where fish showed reproductive behaviour were selected. Males and females were immediately separated to avoid natural breeding and were simultaneously anaesthetized with clove oil solution of 100 µl/l (Mylonas *et al.*, 2005; Grush *et al.*, 2004) in dechlorinated and decalcified tap water (system water) (Westerfield, 2003). All aseptic procedures and sterile instruments were used in subsequent steps. Sperm and eggs were in vivo extracted and in vitro fertilized taking into account the recommendations of Westerfield (2003) and Nüsslein-Volhard and Dahm (2002). In this way, sperm from at least 3 males (for each session) was recovered individually with glass capillaries (1 x 90 mm, Narishige Scientific Instrument Lab.), and immediately poured into a 1ml volume Eppendorf and diluted with 0.1 to 0.5 ml of modified Hanks´ buffered salt solution medium (100 ml of Hanks´ supplemented with 1.5 g BSA and 0.1 g ClNa; 320 mOsm, pH 7.4; and designed as CH), depending on initial whole sperm volume in order to make a "cloudy" suspension (Westerfield, 2003) (sperm concentration was adjusted to around 8.5 x 10^8 spz/ml, measured with a Thoma counting chamber). At this time, good quality eggs (translucent and yellowish appearance) recovered by gentle extrusion of the ovary were

maintained in CH. Then, eggs and sperm solution were immediately stored at 8ºC until use.

Before each fertilization assay, sperm motility was assessed by visual inspection at 200x magnification after adding water. Only the samples that showed optimal motility (80-100%) were used. Antibiotics were not added to sperm solution and eggs. Fertilization was performed in a 35mm Petri-dish (corning) after carefully mixing 15-20 eggs/plate and 50 µl of the sperm solution in a minimum volume of CH. Then, 3 to 5 system water drops were added to fertilize eggs (sperm concentration was around 2.8 x 10^8 at the fertilization stage). Finally, the plate was filled with system water after 5 min. Egg activation was assessed by the observation of chorion swelling and animal pole segregation (Lee *et al.*, 1999). Larval culture was carried out in 9 cm Petri-dish (15 larvae/plate) at 28.5ºC until the fifth day.

All chemical products and culture media were from Sigma-Aldrich.

b. Experimental design

Experiment 1 pursued the evaluation of fertilizability of fresh sperm (T0: control group) and after being stored at 8ºC for 24 h (T24), 48 h (T48), 72 h (T72) and 96 h (T96). Fresh eggs were used in all cases.

Experiment 2 aimed to assess the fertilizability of fresh eggs (T0: control group) and after being stored at 8ºC for 1 h (T1), 2 h (T2), 3 h (T3) and 4 h (T4). Sperm was also stored at 8ºC for the same time as the oocytes in each experimental group (from 0 to 4 h).

In these two experiments, egg and sperm fertilizability, embryo and larval development were assessed at mid-blastula transition (MBT) stage, 24 h and 5 d post-fertilization.

c. Statistical analysis

At least three replicates were done in all experimental groups. Results were analysed using the Chi-square test. When a single degree of freedom was involved, Yates' correction for continuity was performed.

III. RESULTS AND DISCUSSION

In relation with sperm fertilizability, results from Experiment 1 are presented in Table 1.

TABLE 1: Fertilization ability of sperm stored at 8ºC for different times (0 h, 24 h, 48 h, 72 h and 96 h) and further normal embryo development, using freshly obtained eggs.

	T0	**T24**	**T48**	**T72**	**T96**
Initial	267	298	62	26	272
MBT	131/267 (49.1%) [a]	120/298 (40.3%) [b]	7/62 (11.3%) [c]	3/26 (11.5%) [c]	0/272 (0%) [d]
Larvae 24h	89/267 (33.3%) [a]	88/298 (29.5%) [a]	3/62 (4.8%) [b]	0/26 (0%) [bc]	0/272 (0%) [c]
Larvae 5 d	55/267 (20.6%) [a]	81/298 (27.2%) [a]	3/62 (4.8%) [b]	0/26 (0%) [bc]	0/272 (0%) [c]

Columns with different superscripts are statistically different ($P<0.05$)

With respect to sperm characteristics, an optimal motility was observed in T0 group (control group; 80-100% of motility) and was also observed at 24 h, at 48 h and even, in many cases at 72 h. At 96 h, lower sperm motility was observed in samples (50-70% motility) as well as an obvious more limited duration of motility after being activated. Interestingly, sperm remained motile even at 120 h in some samples (about 10-20% motility), but with a clearly poor quality that made its evaluation irrelevant.

No significant differences in normal larval development at 5 d were observed between fresh (T0) and 24 h aged sperm (T24). In fact, the only significant difference observed at MBT stage in favour of T0 group disappeared at 24h post-fertilization.

Surprisingly, despite the high motility rates of 48 h aged sperm, a lower fertilization rate was observed, with only 4.8% of initial eggs reaching the larval stage (5 d). Similar results occurred with the use of 72 h aged sperm, which led to development of 11% of embryos to MBT stage, but no subsequent larval development was observed. In T96 group, sperm completely lost its fertilization ability and was even unable to induce a parthenogenic-gynogenic haploid development in the eggs, which is possible when ultraviolet radiated sperm is used (Nüsslein-Volhard and Dahm, 2002).

In Experiment 2, in relation with egg obtaining, Westerfield (2003) pointed out that to ensure getting good eggs, they must be collected during the first 90 min after "dawn", but in our case, when the recovery time exceeded 30 min after dawn, eggs obtained were systematically of worse quality and degenerated in many cases.

In order to arrest zebrafish eggs from the wild strain in a non-activated state, Sakai (1997) used 0.5% BSA in Hanks' buffered salt solution. However, Sakai detected statistical differences between the group of 0.0% BSA (control group) and the group of 0.5% BSA, but not among all other groups with different BSA concentrations (from 0.01% to 4.0% of BSA). In our case, as our lab is focused on NT techniques, the percentage of BSA added to the CH medium to keep eggs inactivated was 1.5%, as Huang *et al.* (2003) recommended.

In relation to the storage temperature, in our case, eggs manifested spontaneous activation signs in CH when the storage temperature was higher than 8ºC, in contrast with results obtained by Sakai (1997), which were able to maintain zebrafish eggs from the wild strain at room temperature without spontaneous activation.

Results obtained in relation with fertilizability of aged eggs are presented in Table 2.

TABLE 2: Fertilization ability and embryo development using both eggs and sperm stored at 8ºC for different times (0 h, 1 h, 2 h, 3 h and 4 h).

	T0	**T1**	**T2**	**T3**	**T4**
Initial	267	189	272	49	18
MBT	131/267	44/189	5/272	0/49	0/18
	(49.1%) [a]	(23.3%) [b]	(1.8%) [c]	(0%)[c]	(0%)[c]
Larvae 24h	89/267	18/189	2/272	0/49	0/18
	(33.3%) [a]	(9.5%) [b]	(0.7%) [c]	(0%)[bc]	(0%)[bc]
Larvae 5 d	55/267	11/189	2/272	0/49	0/18
	(20.6%) [a]	(5.8%) [b]	(0.7%)[c]	(0%)[bc]	(0%)[bc]

Columns with different superscripts are statistically different ($P<0.05$)

Results obtained showed that in T0 group (control group) all eggs manifested activation signs, even when they were not fertilized, but the number of non-activated eggs increased during storage time (T1: 3/189; T2: 3/272; T3: 4/49; T4: 9/18; results not shown in Table 2). The low fertilization rates obtained in this and previous experiment (Control group: 20%) were probably as a consequence of the season (summer) and laboratory temperature daily variations (even higher than 30 ºC at night) that uncontrollably occurred in our lab during the experimental period. In fact, at that time, the natural fertilization rates in our zebrafish colony (evaluated in embryos collected by aquarium siphoning) were similar to those obtained *in vitro* (results not published). This was also indicated by other authors, where great variations were present between groups and collection days related with fertilization rates in zebrafish (Huang *et al.*, 2003).

In the case of T1 group, the rate of embryos that reached the MBT stage reduced to half when compared to the T0 group, and these differences increased gradually at 24 h and 5 d. With respect to T2 group, only very few embryos (1.8%) finally developed to MBT stage. In the case of T3 and T4 groups, no embryo development was detected. Although the efficient fertilization time limit proposed by Sakai (1997) was 1 h in wild zebrafish, the fertilization rate reached in the present work for the gold strain was lower. Perhaps a strain effect may explain such different results.

In conclusion, in nuclear transplant experiments, the extension of time for up to 24 h in the use of sperm as an activating or fertilizing agent is possible without significant reduction in activation or fertilization rate, but the rapid egg aging recommends minimizing as far as possible the egg storage time prior to fertilization or/and nuclear transplant, to even less than 1 hour and so, the information given in guides must be re-evaluated in each laboratory. In this respect, the effect of the zebrafish strain used in experiments must be also taken into account.

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