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Electroactivation of zebrafish (*Danio rerio*) eggs

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Abstract

In zebrafish, initial egg activation by water after being discharged from the ovarian stroma is followed by normal cleavages only in inseminated eggs. When sperm (genetically active or not) is not used as activating agent, reproductive techniques (as either nuclear transplant or intracytoplasmic sperm injection are inefficient. In this work, three experiments of egg activation by electric stimuli were performed: In the first, six activation treatments were compared (Voltage × Pulses: 2.76 × 1; 2.76 × 2; 2.76 × 3 and 5.40 × 1; 5.40 × 2; 5.40 × 3). The group 5.40 × 3 showed the best results (32% activated). In the second experiment, an electrical treatment of 20 min was carried out. It consisted in a sequence of three equal electrical stimuli every 10 min (of 1 or 3 consecutive direct current square pulses for 20 μs each and applied at two voltage levels, 2.76 V or 5.4 V). It was observed that the number of pulses negatively affected the rates of damaged and lysed eggs. Moreover, only the 20 min treatment with the combination of 3 consecutive pulses at 2.76 V showed significant differences with their respective control group (43% *vs* 18% activated eggs, $p < 0.05$). In the third experiment, negative effects of egg ageing were observed. In conclusion, the best activation treatment for intact (non manipulated) zebrafish eggs concerns sequence B3. The electro-activation stimulus proposed here would be the only one available once the egg has already been activated by water.

Additional key words: egg activation; fish; nuclear transplant; oocyte.

Resumen

Electroactivación de oocitos de pez cebra (*Danio rerio*)

En pez cebra, una vez que los oocitos son descargados del estroma ovárico, su activación inicial por contacto en agua da lugar a divisiones normales únicamente cuando son fertilizados. Cuando no se utiliza semen (inactivado genéticamente o no), las técnicas reproductivas (como transplante nuclear o inyección espermática intracitoplasmática) son ineficientes. En el presente trabajo se desarrollaron tres experimentos de activación oocitaria por estímulos eléctricos: en el primero de ellos, se compararon seis tratamientos de activación (Voltaje × Pulsos: 2,76 × × 1; 2,76 × 2; 2,76 × 3 y 5,40 × 1; 5,40 × 2; 5,40 × 3). El grupo 5,4 × 3 mostró los mejores resultados (32% de activación). En el segundo experimento, se evaluó un tratamiento eléctrico de 20 min de duración. El tratamiento consistió en la aplicación de una secuencia de tres estímulos iguales cada 10 min (de 1 ó 3 pulsos de corriente continua y onda cuadrada de 20 μs de duración cada uno y evaluado a dos niveles de voltaje: 2,76 V y 5,4 V). En este trabajo se observó que el número de pulsos afectó negativamente a los oocitos, incrementándose los porcentajes de oocitos dañados y lisados. Además, la combinación de 20 min y 3 pulsos consecutivos de 2,76 V logró diferencias significativas con su grupo control (43% *vs* 18% oocitos activados, $p < 0,05$). En el tercer experimento, se observaron efectos negativos como consecuencia del envejecimiento oocitario. En conclusión, el mejor tratamiento de activación para oocitos de pez cebra intactos (no manipulados) fue la secuencia B3. El estímulo de activación eléctrica propuesto sería el único disponible una vez que los oocitos han sido activados por agua.

Palabras clave adicionales: activación oocitaria; oocito; peces; transplante nuclear.

Introduction

The zebrafish, has recently acquired a complex role in many emerging areas of knowledge, mainly in biomedical research (Nüsslein-Volhard and Dahm, 2002), becoming a new scientific tool in multitude of labs around the world. In this sense, several and varied are the topical areas where the zebrafish has established as a both efficient and capable vertebrate model for developing knowledge. As Streisinger (1986) pointed out, the reasons were their many useful biological characteristics.

In zebrafish, egg activation takes place after they are discharged from the ovarian stroma and come into

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contact with water (Sakai *et al.*, 1997), inducing an intracellular calcium wave discharge (Lee *et al.,* 1999). In consequence, activated eggs (oogenesis stage V and thus capable of being fertilized; Howley and Ho, 2000) undergo a programmed series of biological events briefly described by Lee *et al.* (1999): contraction of the egg surface, initial small separation of the chorion from the plasma membrane, cortical granule reaction, chorion elevation and ooplasmic segregation and followed by normal cleavages if fertilized. The spermatozoa can fertilize the eggs whether it is genetically active or not (Lee *et al.,* 1999; Nüsslein-Volhard and Dahm, 2002), and obtaining adult normal specimens or haploid larvae that survived for about five days (as it occurs with zebrafish androgenetic haploids; Ungar *et al.*, 1998) respectively. If sperm is not present an incomplete parthenogenetic development can be initiated, undergoing some abortive cleavages in the best of cases, but without reaching further development (Cardona-Costa *et al.*, 2009), or even the configuration of four well organized cells (Lee *et al.*, 1999).

In mammals, oocyte activation is routinely induced or reinforced by electrical sequences associated to somatic cloning by nuclear transplant, NT (Onishi *et al.*, 2000; Okahara-Narita *et al.*, 2007), intracytoplasmic sperm injection, ICSI (Zhang *et al.*, 1997; Mansour *et al.*, 2009), or in the obtaining of parthenogenetic haploid/diploid embryos (Elsheikh *et al.*, 1995; Escribá and García-Ximénez, 2000). These set of techniques are nowadays of marked importance in regenerative medicine and/or assisted reproduction in vertebrate models and even, in humans (ICSI). To our knowledge, the use of egg electroactivation in fish nuclear transplant (NT) has only been carried out on medaka fish (Bubenshchikova *et al.*, 2007; Wakamatsu, 2008). In zebrafish, the NT of somatic nucleus is able to induce embryo development when activation is only promoted by water, usually reaching the MBT stage (Huang *et al.*, 2003; Pérez-Camps *et al.*, 2009; Siripattarapravat *et al.*, 2009b).

Little attention has been paid to what benefit could be provided by an additional artificial activation stimuli by electric pulses in the case of NT in zebrafish. In a same way it could be of interest its coupling to other techniques such as ICSI (Poleo *et al.*, 2001).

To this respect, the aim of the present work was to establish electrical parameters and conditions for an activation improvement of zebrafish eggs at 0 h, 1 h or 2 h after ovulation.

Material and methods

Animal care

Zebrafish (*Danio rerio*) specimens from the gold strain were maintained in 20 L aquariums under standard conditions (Westerfield, 2007). 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 as reported by Simão *et al.* (2007).

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

Eggs obtaining

The procedure carried out was described in detail by Cardona-Costa *et al.* (2009). Briefly, eggs were *in vivo* extracted at the artificial «dawn» time from those aquariums where fish displayed reproductive behaviour, from females previously anaesthetized with clove oil solution of 100 µL L^{-1} (Grush *et al.*, 2004; Mylonas *et al.*, 2005). Obtained eggs (from two females at least) were maintained inactivated until use at 8°C in a modified Hanks 'solution designed as CH (100 mL of Hanks' supplemented with 1.5 g BSA and 0.1 g NaCl; 310-320 mOsm, pH 7.4). Only eggs of good quality (translucent, granular and yellowish appearance; Westerfield, 2007) were used. Moreover, in our laboratory, a routinely egg quality assessment by fertilization (Cardona-Costa *et al.*, 2009) is carried out in each experimental session. When fertilization is lower than 70%, eggs are discarded.

Electroactivation

The electroactivation equipment consisted of an Electro Cell Manipulator (ECM 2001; BTX) complemented with a digital oscilloscope from Tektronix. The pulsing chamber used was model 453 from ECM.

The first step of this procedure consisted of the careful selection of 5 to 20 inactivated eggs contained in CH medium. With the aid of a Pasteur pipette, previously stretched and fire polished, a part of these eggs (per experimental group) were held in the pulsing chamber, containing system water as electroactivation medium. Immediately, the predefined direct current (DC) square pulse was applied. This pulse was syste-

matically checked through the oscilloscope. Finally, electropulsed eggs were incubated in Petri dishes (Corning) with system water at 28.5°C.

In each experiment (see experimental design), control groups were only activated by water.

Assessments of damage/lysis, and functional activation were assessed at one hour after the treatment ending (see experimental design).

Efficiency of electroactivation treatments was evaluated by the eggs rate that showed, at least, one abortive cleavage (Lee *et al.*, 1999).

Experimental design

Preliminary assays

As an initial approach, the electrical conditions reported by Wakamatsu (2008) for medaka egg activation (a double electrical DC pulse of 8.0 V cm^{-1} for 20 µs, voltage applied with our employed fusion chamber: 2.76 V) were tested. In addition, different voltages were also tested [2.76 V, 5.4 V, 8.3 V, 11.4 V and 13.8 V; corresponding to 2.76 (×1), 2.76 (×2), 2.76 (×3), 2.76 (×4) and 2.76 (×5)].

Experiment A

In line with results obtained in the preliminary assays, the following electrical parameters were finally tested: 1, 2 or 3 consecutive DC pulses for 20 µs each at room temperature, in system water as electroactivation (ionic) medium. Electric field intensity was established at two levels (E_1 = 8 V cm^{-1} *vs* E_2 = 16 V cm^{-1}; voltages with the fusion chamber employed were 2.76 V and 5.40 V respectively). In this way, a total of 6 experimental groups were established in this first experiment (Voltage × Pulses: 2.76 × 1; 2.76 × 2; 2.76 × 3 and 5.40 × 1; 5.40 × 2; 5.40 × 3). Eggs were manipulated during the first 30 min after ovarian extraction. A general control group (Control A) of activated eggs in system water, but without pulsing, was established in parallel with the experimental groups.

Experiment B

Siripattarapravat *et al.* (2009a) established an elapsed time of 30 min (at 28°C culture) between egg activation and the first mitotic cleavage in zebrafish. Experiment B pursued the maintaining of the reduced MPF levels, due to the activation stimulus, but which rise after 10 min of such activation stimulus when this activation process is only promoted by the water stimulus (incomplete parthenogenetic activation). In this way, an electric treatment extended to 20 min was tested, where 1 or 3 consecutive DC pulses for 20 µs each were applied at 0 min, 10 min and after 20 min of their initial activation in water (pulsing medium). Two voltage levels (2.76 V and 5.4 V) were used [(Voltage × Pulses) × 3 sequences: B1 = (2.76 × 1) × 3; B2 = (2.76 × 3) × 3 and B3 = (5.4 × 1) × 3; B4 = (5.4 × 3) × 3].

Eggs were manipulated during the first 30 min after their ovarian extraction. A general control group (Control B) of activated eggs in system water, but without pulsing, was established in parallel with the experimental groups.

Experiment C

Because zebrafish egg ageing is incompatible with fecundation but still tolerates (for longer) the activation stimulus, this third experiment pursued the assessment of the better/worse response of egg activation in aged eggs previously stored at 8°C in CH medium (non activating medium; Cardona-Costa *et al.*, 2009) for different times.

The better combinations from among all experimental groups tested were selected for their best and similar egg activation rates by electric pulses, and used in this experiment. So, the experimental groups were carried out as a result of joining the different egg ageing times tested (0 h, 1 h and 2 h post-ovarian extraction) and the best electric treatments obtained previously (B2 and B3, see results). In addition, three control groups of eggs activated in system water at 0 h, 1 h and 2 h after their ovarian extraction, but without pulsing, were established in parallel with their respective experimental groups.

Statistical analysis

In all experimental groups from each experiment (A, B and C), a minimum of three replicates were done in alternative daily sessions. Results were analysed using the Chi-square test. When a single degree of freedom was involved, Yates' correction for continuity was performed.

Table 1. Percentage of activated eggs under 1, 2 or 3 consecutive direct current square pulses for 20 μs stimulus at two voltage levels (2.76 V and 5.4 V). In Control A, eggs were only activated in system water

	Control A	Voltage × Pulses					
		2.76 × 1	2.76 × 2	2.76 × 3	5.4 × 1	5.4 × 2	5.4 × 3
No. initial	65	81	80	63	92	96	60
No. activated (%)	14^{a} (9)	10^{a} (8)	17^{ab} (14)	19^{ab} (12)	21^{ab} (19)	19^{ab} (18)	32^{b} (19)

Data with different superscripts are statistically different ($p < 0.05$).

Results

Preliminary assays

Results showed that voltages of 8.3 V and higher were not suitable for electroactivation assays. The reason was the high degree of internal damage caused in eggs, observed as a non-clear delimited region between the animal and vegetal egg poles, once the ooplasmic segregation ended. Moreover, from 11.4 V onwards, lysis of egg plasma membrane was also observed (8.3 V: 4 eggs damaged from 8; 11.4 V: 5 eggs lysed from 9; 13.8 V: 2 eggs lysed from 3; results not shown).

Experiment A

Table 1 shows the differences in the percentage of activated eggs only raised levels of significance between the 5.4 × 3 group when compared with Control A (only activated in system water) and the 2.76 × 1 group.

It must be noted the low activation rates achieved in all experimental groups assayed, even the group 5.4 × 3 in which the most intense electrical stimuli was applied and only yield a 32% egg activation. The almost no lysis observation in experimental groups would indicate that a voltage increase could be tested, at least to 8.3V as the upper limit, due its detrimental effects observed previously in preliminary assays.

However, it was also expected to extend this activation treatment throughout the zygotic phase. Because it was predictable that an increment of activation rates would be observed, higher voltage levels were discarded as they could excessively affect the eggs. In this sense, in the following experiment there were evaluated four electrical treatments by joining the two voltage levels previously used and the major and minor number of pulses.

Experiment B

In this experiment, the electrical treatment was extended to a sequence of three pulses stimuli distributed equally for 20 min (one pulse every 10 min) to maintain the MPF levels low until completion of the zygotic stage. The stimuli applied were adjusted to the two most extreme combinations of voltage and number of pulses (2.76 × 1 *vs* 5.4 × 3) established in experiment A (Table 2).

As a general comment, to point out that the expected increase in egg activation was accomplished accomplished when treatments were extended throughout the zygotic phase. Also, it was observed that the number of pulses applied negatively affected the percentage of

Table 2. Percentage of activated eggs and damage-lysis rates as result of applying an electrical treatment of 20 min duration, consisting of a sequence of three equal electrical stimuli (of 1 or 3 DC square pulses) for 20 μs at two voltage levels (2.76 V and 5.4 V). In Control B, eggs were only activated by system water

	Control B	(Voltage × Pulses) × 3 sequences			
		B1 (2.76 × 1) × 3	B2 (2.76 × 3) × 3	B3 (5.4 × 1) × 3	B4 (5.4 × 3) × 3
No. initial	62	44	58	44	30
No. damaged/lysed (%)	0^{a} (0)	0^{a} (0)	17^{b} (10)	2^{a} (1)	100^{c} (30)
No. activated (%)	18^{b} (11)	18^{b} (8)	43^{c} (25)	36^{bc} (16)	0^{a} (0)

Between columns, data with different letters differ statistically ($p < 0.05$).

damaged and lysed eggs significantly. In this sense, the combination of voltage and number of pulses in the B4 group produced a multiplicative effect that damaged or lysed all treated eggs. In relation to egg activation rates, the B2 group was the only one that showed positive and significant differences with Control B, reaching a percentage of 43% activated eggs. On the other hand, groups B1 and B3 did not differ significantly from the control, although differences were relevant in the case of group B3 due to the high percentage reached (36%, Table 2). Although it is obvious that a comparison is not correct, it is important to highlight the higher activation rates achieved in this experiment B (43%) in comparison with experiment A (32%).

Experiment C

Results are presented in Table 3. In this experiment, with respect to the damaged/lysed eggs, differences were not observed among the three control groups of different egg ageing times, as well as among their activation rates. However, when the most intense treatment (B3) was applied, the eggs were considerably damaged (35%) when a determined egg aging was surpassed.

The activation efficacy of B2 and B3 treatments was lower as egg age increased. Despite this, group B3 maintained the activation rates in 1 h aged eggs but declined significantly at 2 h. Whatever the case, results obtained in this third experiment confirm that B2 and B3 treatments are susceptible to be used for the artificial activation reinforcement of zebrafish eggs, reaching activation rates for up to 66-68% in the case of B3 group.

Discussion

The definition of an egg electroactivation procedure involves the combination of several and different electrical parameters and conditions, mainly the electric field intensity and the number of pulses applied.

Whatever the case, all of them pursue a rational similarity with the sperm-activating stimuli aimed at achieving a better activation response in eggs. In this respect, the selection of an appropriate activation medium is important. In mammals, the use of non-ionic media in electroactivation processes is common (Chang *et al.*, 1992) although the ionic media are also efficient (Elsheikh *et al.*, 1995; Rickords and White, 1992). In contrast with this, to our knowledge the only information available in fish refers to Bubenshchikova *et al.* (2007) and Wakamatsu (2008). In those cases, an ionic medium was used (a balanced salt solution medium designed for medaka) for the electroactivation of medaka fish eggs. Unfortunately, no further bibliography was found. So, initially, an ionic medium (system water in our case) was also used as the starting point of our electroactivation assays in zebrafish eggs.

Recent studies by Siripattarapravat *et al.* (2009a) reported that no significant differences were found in the initial dropping of MPF activity in zebrafish eggs activated by water either in presence of absence of sperm. Nevertheless, they observed that MPF levels from 10 min to 30 min post-fertilization/activation were different between both activation procedures. Indeed, MPF values in eggs activated parthenogenetically by water increased slightly from 10 min post-activation, which coincided with the extrusion of second polar body, and showed a slightly greater peak

Table 3. Percentage of activated eggs and damage-lysis rates in eggs previously aged for different times (0 h, 1 h and 2 h) as a result of applying two different electrical treatments of 20 min duration and consisting of a sequence of three equal electrical stimuli at two voltage levels (B2: 2.76 V × 3 Pulses; B3: 5.4 V × 1 Pulse). In Controls, eggs were stored in CH medium at 8°C and activated in system water

	Egg ageing time								
	0 h			**1 h**			**2 h**		
	Control C_0	**B2**	**B3**	**Control C_1h**	**B2**	**B3**	**Control C_2h**	**B2**	**B3**
Initial (No.)	76	92	57	101	110	62	142	132	66
Non activated (%)	92 (70)	40 (37)	23 (13)	88 (89)	70 (77)	27 (17)	91 (129)	86 (113)	47 (31)
Damaged/lysed (%)	0^{ac} (0)	4^{ab} (4)	9^{b} (5)	2^{abc} (2)	3^{abc} (3)	7^{ab} (4)	0^{c} (0)	2^{abc} (3)	35^{d} (23)
Activated (%)	8^{a} (6)	56^{c} (51)	68^{c} (39)	10^{a} (10)	27^{b} (30)	66^{c} (41)	9^{a} (13)	12^{a} (16)	18^{ab} (12)

Between columns, data with different letters differ statistically ($p < 0.05$).

difference at 20 min post-activation, in contrast with those eggs activated by fertilization. It is known in mammals that egg MPF activity can be maintained at low levels by the application of electrical pulses at different time intervals after their first artificial activation, as normally occurs after normal egg activation by fertilization (Escribá and García-Ximénez, 1999). The treatment tested in the second experiment pursued an artificial maintenance of MPF activity at low levels by means of the temporal distribution of electric pulses throughout the post-fertilization period up to the corresponding time of the first mitotic cleavage. Results obtained in the second experiment indicated the general improvement upon the activation rates achieved in the first experiment. Here, the B2: $(2.76 \times 3) \times 3$ and B3 $(5.4 \times 1) \times 3$ treatments were established as the most efficient.

The third experiment evaluated the activation ability of eggs stored (aged) for up to 2 h. Under our storage conditions (Cardona-Costa *et al.*, 2009), this was the maximum time in which eggs were still able to be fertilized.

The results obtained in this last experiment showed a clear advantage in the activation capability of fresh eggs compared with those partially aged (2 h). This result is contrary to that observed in mammals, where oocyte ageing improves their activation rates, whether normal or parthenogenetic, and is explained as a greater feasibility of aged oocytes to maintain reduced their MPF levels (Escribá and García-Ximénez, 1999). However, in this case the subsequent embryo development can also be penalized (Kaufman, 1981; Stice and Robl, 1988; Collas and Robl, 1990; Tanaka and Kanagawa, 1997). It is possible that the degree of ageing tested in present experiment was somewhat excessive.

In general, in this work we observed a positive relation in the use of either higher voltage or number of pulses (higher stimulation degree) and the percentage of activated eggs. So, it can be proposed that the best activation treatment for intact (non manipulated) zebrafish eggs concerns sequence B3: an electrical pulse sequence of 20 min, where 1 pulse of 20 μs of 5.4 V was applied at 0 min post-activation in system water, at 10 min and finally at 20 min (3 pulses in total). This treatment was able to activate 66% of fresh and aged (for 1 h) eggs.

None of the activation stimuli proposed here induced parthenogenetic development of eggs until the second mitotic cleavage, with four well organized cells (Lee *et al.*, 1999). This fact represents a notable difference compared with mammals (Elsheikh *et al.*, 1995; Escribá and García-Ximénez, 2000), although in this species the intensity and duration of electrical stimuli are also essential for the oocyte activation and parthenogenetic developmental rates. Until now, in zebrafish, these events (parthenogenetic development) have only been possible via genetically inactive sperm (Nüssslein-Volhard and Dahm, 2002). However, the activation stimulus proposed here could serve for improving the embryo developmental rates in procedures which involve nuclear injection techniques in zebrafish such as NT (Huang *et al.*, 2003; Siripattarapravat *et al.*, 2009b; Pérez-Camps *et al.*, 2009) or ICSI (Poleo *et al.*, 2001) as occurs in mammals (Macháty, 2006). Moreover, in fish, the electrical (and/or chemical) stimuli would be the only ones available in these procedures once the egg has already been activated by water.

In conclusion, it can be proposed that the best activation treatment for intact (non manipulated) zebrafish eggs concerns sequence B3, and that the electroactivation stimulus proposed here would be the only one available once the egg has already been activated by water.

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