UNIVERSIDAD POLITÉCNICA DE VALENCIA

Departamento de Ciencia Animal

Grupo de Investigación Acuicultura y Medio Ambiente
Laboratorio de Reproducción y Biotecnología Animal

"EMBRYOLOGICAL AND MICROMANIPULATION TECHNIQUES IN ZEBRAFISH (*Danio rerio*) AND PACIFIC OYSTER (*Crassostrea gigas*)"

TESIS DOCTORAL

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Los directores de la presente Tesis certifican que el tabajo se encuentra terminado y reúne todos los requsitos legales y académicos necesarios para su presentación.

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ABSTRACT

In this thesis there are various experimental studies. They are primarily conducted in zebrafish and in the Pacific oyster as experimental models, and pursue the development of relevant techniques in the field of biomedicine, toxicogenomics, environmental risk assessment and aquaculture.

In zebrafish, there have been developed and tested:

- Vitrification techniques of caudal fin tissue, blastomeres (in microvolumes) and of adult testicular tissue.
- The germ-line chimaerism technique at MBT stage of embryo development, with a previous penalization of recipient embryos by ultraviolet radiation.
- Chimaerism technique at larval stage (48-72 h) with previously criopreserved testicular cells as donors obtained from adult individuals.
- Nuclear transplantation technique using cell cultures from both adult somatic diploid cells and parthenogenetic haploid larvae as nuclei donors.
- Electroactivation technique of zebrafish oocytes (in ionic medium).

In Pacific oyster, there have been developed and tested:

- Assessment of seasonal evolution of gonadal maturation, gametes quality and fertilizability of Pacific oyster.
- Electrofusion technology of Pacific oyster zygotes obtained by in vitro fertilization.

Vitrification techniques of caudal fin tissue, blastomeres (in microvolumes) and of adult testicular tissue.

In this set of techniques, to highlight their different difficulty level as well as the results achieved. In this way, the vitrification of zebrafish caudal fin explants did not led to unexpected problems. In fact, efficacies achieved were in line with those achieved for the epthelial tissue cryobanking from five different mammalian species. This shows the versatility of this basic vitrification technique. In contrast, the blastomere cryopreservation has been a real challenge. The cell permeability patterns in aquatic species is very different from those in mammals. Therefore their final cryopreservation achievement came from an innovative approach: the blastomere vitrification in microvolumes (0.25µl) without their requirement of cryoprotectant permeation.

Regarding the testicular tissue cryopreservation, there were tested three procedures, two of conventional freezing and a vitrification one. This last was finally selected for its best results acomplished. Also, their vitrification procedure became more efficient when it was applied previous to the tissue trypsinization.

Germ-line chimaerism technique at MBT stage of embryo development, with a previous penalization of recipient embryos by ultraviolet radiation.

Regarding to the germ-line chimaerism at MBT stage various issues should be addressed. Firstly, the recipient embryo penalization with UV radiation is important but the not only requirement to chieve the best germ-line chimaerism results (50%). In fact, this penalization treatment must be completed with the embryo manipulation in a 300 mOsm/kg medium to avoid negative osmolar effects throughout the chimaerism procedure. Secondly, the importance of different embryo sensitivity to UV radiation according to the zebrafish strain used, being the *gold* strain more sensitive than the *wild*.

With respect to chimaerism using previously vitrified blastomeres in microvolumes, many problems must be solved in future. An example was the morphologic disruption caused by the blastomere volume increasment after thawing and the subsequent requirement of using micromanipulation pipettes of increased size.

Chimaerism technique at larval stage (48-72 h) with previously criopreserved testicular cells as donors, obtained from adult individuals.

This technique development was the logical consequence of a previous testicular tissue cryopreservation development indicated above. This technique is required to achieve the testicular cell integration, concretely spermatogonia, into the germ-line of recipient specimens. This procedure needed the use of 48-72 h age larvae, assuming that their immune system is not fully established and therefore it will not interfere with transplanted cells. Technical results on micromanipulation, larval survival and further development to adulthood were fully satisfactory. Despite these, no germ-line chimaerism was finally detected in any adult specimen. Among all reasons which would explain this, the more plausible by authors would be the possible dissynchrony between temporal patterns of spermatogonia development and those from testicular tissue, maybe faster than the first.

Nuclear transplantation technique using cell cultures from both adult somatic diploid cells and parthenogenetic haploid larvae as nuclei donors.

This group is perhaps the most original in techniques and the more forward looking from all provided in this thesis. Various items can be cited. Firstly, it was achieved a nuclear transplantation technique for non-activated oocytes. Moreover, none of the three nuclear transplantation (NT) developed techniques required the micropile detection as well as the previous recipient oocyte dechorionation. Secondly, it has been possible to compare the subsequent embryonic development effects among the three NT techniques assayed and with the fact that the oocyte was previously activated by either sperm fertilization (genetically inactivated or not) or by water stimuli. To this respect, the pipette puncture and water stimuli did not achieve further parthenogenetic development in any case when no cell nucleus was injected. This allows us to affirm the relevant participation of transplanted nucleus in the further embryo developmental ability. Thirdly, the recipient (egg) aging as a useful promoting factor of egg activation was rejected in contrast with the possitive results commonly observed in mammals.

In this section, the use of cell nuclei donors from gynogenetic haploid larvae cultures in nuclear transplant of non-enucleated oocytes acquires a special mention. This

technology would serve for genomic imprinting studies and even for a plausible alternative to the transgenic animal obtaining. The potentiality importance of this technique has only been evaluated preliminary in this thesis, although to date new experiments are being carried out.

Electroactivation technique of zebrafish oocytes (in ionic medium).

The most relevant aspect of the procedure is perhaps the easiness of using ionic media for electroactivation, especially when non-ionic media are the most commonly used. To other respect, the best electroactivation procedure (one DC square pulse for 20µs at 5.4V and applied at 0, 10 and 20min post-activation in system water) allowed the activation of 68% non-manipulated oocytes. Unfortunately, when the best electroactivation sequence (that cited above) was assayed in nuclear transplanted eggs, it produced their immediate lysis. These aspects will be the key of future works to allow the coupling of egg electroactivation and NT techniques.

Assessment of seasonal evolution of gonadal maturation, gametes quality and fertilizability of Pacific oyster.

This study do not refers the development of any micromanipulation technique. Despite this, it was essential for establishing the period of gametes availability throughout the year as well as their quality and allowed us to work, precisely, in the development of embryologic and micromanipulation techniques in this species. Results of this research were conclusive: only the period comprised from July to October assured the both gametes availability.

Electrofusion technology of Pacific oyster zygotes obtained by in vitro fertilization.

Based on results reached above, zygotic electrofusion assays were carried out in the period from July to October for their possible potentiality in tetraploid oyster obtaining. In this regard, many interesting results came to light. On the one hand, it was

observed that electric pulses (pursuing in this case the cell fusion) can be applied in both highly conductor (ionic) and high osmolarity media (in our case seawater, 1130 mOsm/kg). On the other hand, the fusion efficacy of Pacific oyster zygotes was high, and also they showed very low mortality rates as well as high normal developmental rates to D-larval stage (reached after 24 h culture post-electrofusion treatment). Also, it was observed that the electric treatment did not alter in a significant way the further cell cleavage patterns, assessed 25-30 min post-zygotic fusion. This would indicate the virtual damage absence on early embryos caused by the electric pulse.

To other respect and because of the unavailability of specific installations and food required, treated embryos did not reach other key stages as “spat” or even the adulthood. At this moment, the ploidy assessment from obtained specimens at an individualized level is unavailable.

RESUMEN

En este trabajo de tesis, se presentan diversos estudios experimentales, desarrollados principalmente en pez cebra pero también en ostra del Pacífico, que persiguen la puesta a punto de técnicas relevantes para la utilización de estas dos especies en el campo de la biomedicina, la toxicogenómica y la acuicultura como modelo experimental.

En pez cebra, se han puesto a punto y testado:

- Técnicas de vitrificación de tejido de aleta caudal, de blastómeras (en microvolúmenes) y de tejido gonadal.
- Técnica de quimerismo de la línea germinal en estadio MBT, con una penalización previa por radiación ultravioleta de los embriones receptores.
- Técnica de quimerismo larvario (larvas de 48-72 h) utilizando como donantes células testiculares obtenidas de individuos adultos y previamente criopreservadas.
- Técnica de transplante nuclear utilizando como donantes núcleos de células somáticas adultas y larvarias.
- Técnica de electroactivación en medio iónico de oocitos de pez cebra.

In ostra del Pacífico, se han puesto a punto y testado:

- Evaluación de los cambios estacionales en la calidad oocitaria y espermática en ostra del Pacífico.
- Técnica de electrofusión cigótica de cigotos obtenidos por fecundación in vitro en ostra del Pacífico.

Técnicas de vitrificación de tejido de aleta caudal, de blastómeras (en microvolúmenes) y de tejido gonadal.

En relación con este grupo de técnicas, señalar el diferente nivel de dificultad de cada una de ellas e incluso de los resultados alcanzados. Así, la vitrificación de tejido de la aleta caudal no ha supuesto problema adicional respecto a la aplicación de dicha técnica en los últimos años a tejidos epiteliales de cinco especies de mamíferos, lo que da idea de la versatilidad en el uso de dicha técnica de vitrificación básica. Por contra, la criopreservación de blastómeras ha supuesto un verdadero reto, ya que los patrones de permeabilidad celular en esta especie acuática es muy diferente a la característica en mamíferos, lo que obliga en principio y siguiendo los métodos de vitrificación más convencionales (permeación intracelular de crioprotectores), a prolongar excesivamente los tiempos de permeación previos a la vitrificación. Ha sido resultado de un enfoque innovador el que se haya logrado la vitrificación de blastómeras aisladas con relativamente elevada viabilidad celular posterior, cuando la vitrificación se realizó en microvolúmenes (0.25µl) y sin pretender la permeación del crioprotector al interior de las células.

Por lo que se refiere a la criopreservación de tejido gonadal, se han testado tres procedimientos, dos de congelación y uno de vitrificación, de los que claramente éste último mejores resultados ha supuesto. Ciertamente, el procedimiento de vitrificación ha resultado mucho más eficiente cuando se ha aplicado sobre pequeños fragmentos de tejido testicular que cuando se aplicó después de su tripsinización.

Técnica de quimerismo de la línea germinal en estadio MBT, con una penalización previa por radiación ultravioleta de los embriones receptores.

Respecto a las técnicas de quimerismo de la línea germinal en estadio MBT, deben señalarse diversas cuestiones. En primer lugar, que el tratar los embriones receptores con radiación ultravioleta (UV), no es el único requisito para lograr los mejores resultados de quimerismo de la línea germinal, debiendo complementarse dicho efecto con la evitación del choque osmótico que supone la utilización de medios de 30 mOsm/kg para la micromanipulación. De otro lado, es relevante señalar la detección de

una sensibilidad diferencial al tratamiento por UV en función de la estirpe de pez cebra utilizada, siendo la estirpe *gold* más sensible que la *wild* a este respecto.

Por lo que se refiere a la técnica de quimerismo utilizando blastómeras vitrificadas en microvolúmenes, deberán asimismo resolverse en el futuro la desorganización morfológica del embrión receptor provocada por el aumento de tamaño tras su desvitrificación de las blastómeras y el consiguiente tamaño excesivo de las pipetas de micromanipulación.

Técnica de quimerismo larvario (larvas de 48-72 h) utilizando como donantes células testiculares obtenidas de individuos adultos y previamente criopreservadas.

Esta técnica es la consecuencia lógica de la criopreservación de tejido testicular indicada antes, puesto que se requiere para que dichas células testiculares, concretamente las espermatogónias, se integren en la línea germinal del individuo receptor. Se han utilizado larvas de 48-72 h asumiendo que en dicho estadio el sistema inmunitario no se ha establecido plenamente y, por tanto, no atacará las células transplantadas. Los resultados técnicos relativos a micromanipulación, supervivencias larvarias y desarrollo hasta adulto han sido plenamente satisfactorios, no así los de quimerismo de la línea germinal, ya que no se ha observado quimerismo de la línea germinal en ningún espécimen. De entre las razones que justificarían esta carencia, una resalta como más plausible a juicio de los autores, siendo ésta la posible disincronía entre las pautas temporales de desarrollo de las espermatogonias y aquellas propias del tejido testicular, al parecer más rápidas que lo primero.

Técnica de transplante nuclear utilizando como donantes núcleos de células somáticas adultas y larvarias.

Es este grupo de técnicas quizás más original y de mayor recorrido de cara al futuro de entre las aquí contempladas. Las aportaciones que a este respecto se han logrado con esta tesis podrían clasificarse en varios ítems. En primer lugar, el haber logrado el transplante nuclear sobre oocitos no activados sin necesidad de localizar el micropilo, siendo además que en ninguna de las tres alternativas de transplante nuclear

se ha requerido el decorionado de los oocitos receptores. En segundo lugar, haber podido comparar los efectos que sobre el desarrollo embrionario posterior y el alcance del mismo tienen en cualesquiera de estas alternativas de transplante nuclear el que el oocito haya sido activado mediante su fertilización con un espermatozoide no radiado, con uno radiado o tan solo por el efecto estimulante del contacto con el agua de sistema.

A este mismo respecto, señalar que la punción de la pipeta de transplante nuclear como la mera exposición a agua de sistema sin insertar núcleo celular alguno, no suponen en ningún caso el desarrollo posterior partenogenota, lo que nos permite afirmar el papel relevante que la presencia del núcleo donante ejerce sobre la capacidad de desarrollo embrionario. En tercer lugar se ha podido desestimar la posible utilidad del envejecimento oocitario como factor promotor de la eficacia de activación oocitaria, a diferencia, en este caso notable, de lo conocido en mamíferos.

Mención especial merece una aproximación al estudio del imprinting gamético e incluso de posible técnica alternativa para la obtención de animales transgénicos, que se deriva de la utilización como donante en transplante nuclear, sobre oocito no enucleado, de células obtenidas por cultivo de larvas ginogenotas haploides obtenidas por radiación ultravioleta de espermatozoides. La potencial importancia de esta técnica sólo ha podido ser evaluada muy preliminarmente en esta tesis, aunque en estos momentos ya se están desarrollando nuevos trabajos al respecto.

Técnica de electroactivación en medio iónico de oocitos de pez cebra.

Respecto a esta técnica, lo más relevante que se deriva de estos estudios sea quizás la facilidad que supone el poder utilizar medios iónicos para electroactivación, máxime siendo que en prácticamente todos los experimentos de electroactivación recientes de los que se tiene conocimiento, el medio de electroactivación ha sido masivamente no conductor. De otro lado, el mejor protocolo de electroactivación (un pulso cuadrado de 5.4 V y 20µs de duración) permite hasta un 60 % de oocitos activados cuando estos no han sido previamente micromanipulados. Desafortunadamente cuando estas secuencias de pulsos se ensayaron sobre oocitos transplantados nuclearmente, la casi totalidad de ellos resultaron lisados. Esto obligará en el futuro a redefinir los pulsos eléctricos cuando se pretendan acoplar a la técnica de transplante nuclear.

Evaluación de los cambios estacionales en la calidad oocitaria y espermática en ostra del Pacífico.

Aunque este apartado no se refiere al desarrollo de técnica de micromanipulación alguna, resultaba imprescindible conocer los meses del año en los que se dispondría de gametos en cuantía y calidad suficiente para, precisamente, poder trabajar en técnicas embriológicas o de micromanipulación en esta especie. Los resultados han sido absolutamente concluyentes, de tal forma que sólo en los meses de Julio a Octubre, ambos inclusive cabe esperar que se disponga de gametos, mientras que en el resto del año tal disponibilidad resulta cuando menos incierta.

Técnica de electrofusión cigótica de cigotos obtenidos por fecundación in vitro en ostra del Pacífico.

En base a lo anterior, durante los meses señalados, se realizaron ensayos de electrofusión zigótica como paso previo a la posible obtención de ostras tetraploides. A este respecto, varios de los resultados obtenidos han resultado interesantes. De un lado, y con visos de poderse extender a otras especies marinas, se ha comprobado que la aplicación de pulsos eléctricos (en este caso de fusión celular) puede darse en medios altamente conductores y de elevada osmolaridad, en este caso agua de mar. De otro lado, la tasa de fusión celular en estadio zigótico ha sido altamente eficiente, con unas mortalidades sumamente reducidas y con elevadas tasas de desarrollo hasta larva de estadio D (24 h de cultivo post-fusión). También se ha podido comprobar que la fusión no altera de manera significativa los patrones de divisiones celulares posteriores, transcurridos 25-30 min de aplicación del pulso, lo que indicaría la práctica ausencia de daños ocasionados por el pulso sobre los embriones tempranos.

La no disponibilidad de las instalaciones y alimento requeridos para que el grado de desarrollo alcance estadios clave como “spat” e incluso adulto impide, por ahora, evaluar la ploidía de los especimenes así obtenidos.

RESUM

En aquest treball de tesi, se presenten diversos estudis experimentals, desenvolupats principalment en peix zebra però també en ostra del Pacífic, que persegueixen la posada a punt de tècniques rellevants per a la utilització d'aquestes dues espècies en el camp de la biomedicina, la toxicogenòmica i l'aqüicultura com a model experimental .

En peix zebra, s'han posat a punt i testat:

- Tècniques de vitrificació de teixit d'aleta cabal, de blastòmeres (en microvolums) i de teixit gonadal.
- Tècnica de quimerisme de la línia germinal en estadi MBT, amb una penalització prèvia per radiació ultraviolada dels embrions receptors.
- Tècnica de quimerisme larvari (larves de 48-72 h) utilitzant com a donants cèl.lules testiculars prèviament criopreservades obtingudes d'individus adults .
- Tècnica de trasplantament nuclear utilitzant com a donants nuclis de cèl·lules somàtiques adultes i larvàries.
- Tècnica d'electroactivació en medi iònic d'oòcits de peix zebra.

En ostra del Pacífic, s'han posat a punt i testat:

- Avaluació dels canvis estacionals en la qualitat oocitaria i espermàtica en ostra del Pacífic.
- Tècnica d'electrofusió zigòtica de zigots obtinguts per fecundació *in vitro* en ostra del Pacífic.

Tècniques de vitrificació de teixit d'aleta cabal, de blastòmeres (en microvolúms) i de teixit gonadal.

En relació amb aquest grup de tècniques, assenyalar el diferent nivell de dificultat de cada una d'elles i fins i tot dels resultats assolits. Així, la vitrificació de teixit de l'aleta cabal no ha suposat problema addicional respecte a l'aplicació d'aquesta tècnica en els últims anys a teixits epitelials de cinc espècies de mamífers, el que dóna idea de la versatilitat en l'ús d'aquesta tècnica de vitrificació bàsica. Per contra, la criopreservació de blastòmeres ha suposat un veritable repte, ja que els patrons de permeabilitat cel·lular en aquesta espècie aquàtica és molt diferent a la característica en mamífers, el que obliga en principi i seguint els mètodes de vitrificació més convencionals (permeació intracel•lular de crioprotectors), a prolongar excessivament els temps de permeació previs a la vitrificació. Un enfocament innovador ha permès aconseguir la vitrificació de blastòmeres aïllades amb relativament elevada viabilitat cel•lular posterior, quan la vitrificació es va realitzar en microvolums (0.25µl) i sense pretendre la permeació del crioprotector a l'interior de les cèl•lules .

Pel que fa a la criopreservació de teixit gonadal, s'han testat tres procediments, dos de congelació i un de vitrificació, dels que clarament aquest últim ha donat els millors resultats . Certament, el procediment de vitrificació ha resultat molt més eficient quan s'ha aplicat sobre petits fragments de teixit testicular que quan es va aplicar després de la seva tripsinització.

Tècnica de quimerisme de la línia germinal en estadi MBT, amb una penalització prèvia per radiació ultraviolada dels embrions receptors.

Pel que fa a les tècniques de quimerisme de la línia germinal en estadi MBT, s'han d'assenyalar diverses qüestions. En primer lloc, que el tractar els embrions receptors amb radiació ultraviolada (UV), no és l'únic requisit per aconseguir els millors resultats de quimerisme de la línia germinal. S'ha de complementar aquest efecte amb l'evitació del xoc osmòtic que suposa la utilització de medis de 30 mOsm/kg per a la micromanipulació. D'altra banda, és rellevant assenyalar la detecció d'una sensibilitat diferencial al tractament per UV en funció de la estirp de peix zebra utilitzada, sent l'estirp *gold* més sensible que la *wild* en referència a això.

Pel que fa a la tècnica de quimerisme utilitzant blastòmeres vitrificades en microvolums, hauran així mateix de resoldre's en el futur la desorganització morfològica de l'embrió receptor provocada per l'augment de mida de les blastòmeres després de la seva desvitrificació i el consegüent tamany excessiu de les pipetes de micromanipulació .

Tècnica de quimerisme larvari (larves de 48-72 h) utilitzant com a donants cèl.lules testiculars prèviament criopreservades obtingudes d'individus adults .

Aquesta tècnica és la conseqüència lògica de la criopreservació de teixit testicular abans indicada, ja que es requereix per a que aquestes cèl•lules testiculars, concretament les espermatogònies, s'integrin en la línia germinal de l'individu receptor.

S'han utilitzat larves de 48-72 h assumint que en aquest estadi el sistema immunitari no s'ha establert plenament i, per tant, no atacarà les cèl•lules trasplantades. Els resultats tècnics relatius a micromanipulació, supervivències larvàries i desenvolupament fins adult han estat plenament satisfactoris, no així els de quimerisme de la línia germinal, ja que no s'ha observat quimerisme de la línia germinal en cap espècimen. D'entre les raons que justificarien aquesta mancança, una és la que ressalta com més plausible a judici dels autors: la possible disincronia entre les pautes temporals de desenvolupament de les espermatogònies i aquelles pròpies del teixit testicular, que pel sembla són més ràpides que el primer.

Tècnica de trasplantament nuclear utilitzant com a donants nuclis de cèl•lules somàtiques adultes i larvàries.

Aquest grup de tècniques és potser més original i de major recorregut de cara al futur d'entre les aquí contemplades. Les aportacions referents a això que s'han aconseguit amb aquesta tesi podrien classificar-se en diversos ítems. En primer lloc, el fet d'haver aconseguit el trasplantament nuclear sobre oòcits no activats sense necessitat de localitzar el micròpil, sent a més que en cap de les tres alternatives de trasplantament nuclear s'ha requerit el decorionat dels oòcits receptors. En segon lloc, haver pogut comparar que els efectes sobre el desenvolupament embrionari posterior i l'abast del mateix tenen en qualsevol d'aquestes alternatives de trasplantament nuclear i que l'oòcit

hagi estat activat mitjançant la seva fertilització amb un espermatozoide no radiat, amb un radiat o tan només per l'efecte estimulant del contacte amb l'aigua del sistema. En aquest mateix sentit, s'ha d'assenyalar que la punció de la pipeta de trasplantament nuclear sense injectar nucli cel•lular algun així com l'exposició a aigua de sistema, no suposen en cap cas el desenvolupament posterior partenogenota, el que ens permet afirmar el paper rellevant que la presència del nucli donant exerceix sobre la capacitat de desenvolupament embrionari. En tercer lloc s'ha pogut desestimar la possible utilitat de l'envelliment oocitari com a factor promotor de l'eficàcia d'activació oocitària, a diferència, en aquest cas notable, del conegut en mamífers.

Es mereix menció especial a una aproximació a l'estudi del imprinting gamètic i fins i tot de la possible tècnica alternativa per a la obtenció d'animals transgènics, que es deriva de la utilització com a donant en trasplantament nuclear, sobre oòcit no enucleat, de cèl•lules obtingudes per cultiu de larves ginogenotes haploids obtingudes per radiació ultraviolada d'espermatozoides. La potencial importància d'aquesta tècnica només ha pogut ser avaluada molt preliminarment en aquesta tesi, encara que en aquests moments ja s'estan desenvolupant nous treballs al respecte.

Tècnica d'electroactivació en medi iònic d'oòcits de peix zebra.

Respecte a aquesta tècnica, el més rellevant que es deriva d'aquests estudis és potser la facilitat que suposa el poder utilitzar medis iònics per electroactivació, i amés, sent que en pràcticament tots els experiments d'electroactivació recents dels que es té coneixement, el mitjà d'electroactivació ha estat quasi sempre no conductor. D'altra banda, el millor protocol d'electroactivació (un pols cuadrat de 5.4 V i 20µs de durada) permet fins a un 60% d'oòcits activats quan aquests no han estat prèviament micromanipulats. Malauradament quan aquestes seqüències de polsos es van assajar sobre oòcits trasplantats nuclearment, la quasi totalitat d'ells van resultar llisats. Això obligarà en el futur a redefinir els polsos elèctrics quan es pretenguin acoblar a la tècnica de trasplantament nuclear.

Avaluació dels canvis estacionals en la qualitat oocitària i espermàtica en ostra del Pacífic.

Encara que aquest apartat no es refereix al desenvolupament de cap tècnica de micromanipulació, resultava imprescindible conèixer els mesos de l'any en què es disposaria de gàmetes a quantia i qualitat suficient per, precisament, poder treballar en tècniques embriològiques o de micromanipulació en aquesta espècie. Els resultats han estat absolutament concloents, de tal manera que només en els mesos de juliol a octubre, ambdós inclosos cal esperar que es disposi de gàmetes, mentre que a la resta de l'any tal disponibilitat resulta com a mínim incerta.

Tècnica d'electrofusió zigòtica de zigots obtinguts per fecundació *in vitro* en ostra del Pacífic.

En base a l'anterior, durant els mesos assenyalats, es van realitzar assaigs de electrofusió zigòtica com a pas previ a la possible obtenció d'ostres tetraploides. Referent a això, alguns dels resultats obtinguts han resultat interessants. D'una banda, i amb la possibles de poder-se estendre a altres espècies marines, s'ha comprovat que l'aplicació de polsos elèctrics (en aquest cas de fusió cel•lular) pot donar-se en mitjans altament conductors i d'elevada osmolaritat, en aquest cas aigua de mar. D'altra banda, la taxa de fusió cel•lular en estadi zigòtic ha estat altament eficient, amb unes mortalitats molt reduïdes i amb elevades taxes de desenvolupament fins a larva d'estadi D (24 h de cultiu post-fusió). També s'ha pogut comprovar que la fusió no altera de manera significativa els patrons de divisions cel•lulars posteriors, transcorreguts 25-30 min d'aplicació del pols, el que indicaria la pràctica absència de danys ocasionats per el pols sobre els embrions primerencs.

La no disponibilitat de les instal.lacions i l'aliment requerits per a que el grau de desenvolupament arribi a estadis clau com "spat" i fins i tot adult impedeix, per ara, avaluar la ploidía dels espècimens així obtinguts.

CONTENTS

CONTENTS

I. INTRODUCTION

I.1.

Zebrafish as experimental model

I.2.

Pacific oyster as experimental model

References

II. OBJECTIVES

III. EXPERIMENTS

III.1.

Cell Cryopreservation in zebrafish

III.2.

Chimaerism in zebrafish

III.3.

Nuclear transplant in zebrafish

III.4.

Zygotic electrofusion in P. oyster

References

CONCLUSIONS

I. Introduction.

The following text introduces the more three relevant scientific, and well differenced, areas of knowledge where the zebrafish (*Danio rerio*) and the Pacific oyster (*Crassostrea gigas*) play an important role as experimental models.

With the aim to avoid reiterative comments, this general introduction is complemented with those corresponding to every one of presented studies, in the section *III.Experiments*, in which particular aspects related to the content of each experiment are detailed.

I. 1. Zebrafish as experimental model.

Since 1986, when George Streisinger and colleagues introduced the zebrafish as a reliable and interesting animal model for genetic analysis of embryo development on vertebrates, several events were happened.

At the beginning of their discovering, this little fish was closely related to aquariophilia but, more recently it has acquired a more complex role in many emerging areas of knowledge. This fish is now a 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 model for developing knowledge. As Streisinger pointed out (1986), the reasons were their many useful biological characteristics.

There are three general areas of knowledge to be presented in this thesis related to the use of zebrafish: biomedicine, toxicogenomics/environmental risk assessment and aquaculture.

I.1.1. In biomedicine.

In biomedical research, animal models serve to study fundamental biological systems and diseases in a way that cannot be studied in humans (Wall and Shani, 2008). The zebrafish takes part in the understanding of several factors related with human health, being a cost-effective bridge between cell-based assays and mammalian whole-organism models (Brittijn *et al.*, 2009). In this context, we find the zebrafish in such interesting aspects as the inflammation and cicatrization processes (Martin and Feng, 2009) and also of infection, mainly because of its excellent optical accessibility at the embryonic and larval stages, when the innate immune system is already effective (Levraud *et al.*, 2009) and even, in adult zebrafish to analyze multiple disease states (Phelps *et al.*, 2009) or the interactions between host cells and bacteria (Brugman *et al.*, 2009). Also, the zebrafish takes part efficiently in the understanding of neurodegenerative diseases (Panula *et al.*, 2006; Senut *et al.*, 2009), skeletal and cartilage research (Chen *et al.*, 2009a; Witten and Huysseune, 2009), vascularization

(Montero-Balaguer *et al.*, 2009) and cardiovascularization processes (Santoro *et al.*, 2009) in contrast with difficulties arised in other available model organisms.

Current research also involves little known aspects about sleep function and mechanisms of sleep regulation (Zhdanova, 2006). The mechanisms of ageing to develop in future preventive and therapeutic strategies to prolong "helth span" (Kishi *et al.*, 2009) as well as the understanding of stress and affective disorders in humans (Egan *et al.*, 2009) are also of interest.

In line with this, there is indispensable the creation and study of specific zebrafish mutant lines (Talbot and Hopkins, 2000), and the genes knockdown with the use of morpholinos (Nasevicius and Ekker, 2000), or more recently using engineered zinc finger nucleases (Meng et al., 2008), to more effectively advance in many established research programs current in this field, as the *in vivo* study of forebrain GnRH neuronal development by the transgenic (Tg) GnRH3:EGFP zebrafish line (Abraham *et al.*, 2009).

As pointed out Eissen (1996), zebrafish has a number of features that facilitate recognizing and characterizing mutations. Development is external, making it relatively easy to identify and score mutants either for lethality or for specific and even quite subtle phenotypes. Embryos are optically clear so individual cells can be labelled and their development followed to learn how mutations affect embryonic cell fates. Individual or groups of cells can be transplanted to new locations to test autonomy of mutant genes. Also, these aspects have been reinforced after the complete zebrafish genome definition (Freeman *et al*., 2007).

One of main beneficed research programs by biomedical research advances has undoubtedly been the cancer research. Despite there is no currently better alternative to the use of animal models, at least if the target animal is human (Wall and Shani, 2008), several cancer models have been established by transgenic expression of human or mouse oncogenes in zebrafish (Ju *et al.*, 2009). The transparent zebrafish embryos invasion, circulation of tumour cells in blood vessels, migration and micrometastasis formation can be followed in real-time. These findings show that the zebrafish is a useful in vivo animal model for rapid analysis of invasion and metastatic behaviour of primary human tumour specimen (Marques *et al.*, 2009), leukaemias (Payne and Look,

2009) and, as a model to perform strategies for inhibiting tumor development (ie: in tumour angiogenesis, Harfouche *et al.*, 2009).

The regenerative medicine is a research area of great interest for solving in future topical human diseases. In this sense, an understanding of stem cell mechanisms, such as how they are established, their self-renewal properties, and their recruitment to produce new cells is therefore important (Hultman *et al.*, 2009). Into this topic area, there are many research programs, being the zebrafish a model for the regeneration of many organs (Moss *et al.*, 2009). So, we can find research groups working on in different related aspects as are the comprehension of appendix regenerative mechanisms, characterized by multiple biological structures involved (Lee *et al.*, 2009); the spinal muscular atrophy disease (Boon *et al.*, 2009), liver regeneration (Kan *et al.*, 2009) and the regeneration of adult zebrafish pancreatic beta-cells (Moss *et al.*, 2009) among others. Also, aspects of regeneration of some sensory organs structures are in the spotlight (retina regeneration by neurogenesis, Battista *et al.*, 2009; hair cells contained in the inner ear, Liang and Burgess, 2009).

I.1.2. In toxicogenomics/environmental risk assessment.

Always connected to biomedicine, we find the study and development of chemicals for biomedical applications such as cell-selective drug delivery and bioimaging (Nelson *et al.*, 2009). This interesting area is mainly focused in cancer research by the study of drug interactions for improving the efficacy and tolerability of cancer therapy and drug screening of antitumor metallodrugs (Klein *et al.*, 2009; Wang *et al.*, 2009). Some authors affirm that zebrafish embryos are also a reliable alternative for developmental toxicity studies to predict chemicals effects in mammals (ie: to distinguish teratogens from non-teratogens, Selderslaghs *et al.*, 2009) and allowing rapid toxicity screening (Fan *et al.*, 2009). Despite this, controversies appear when others point that zebrafish cannot replace mammalian models in the drug development pipeline (Brittijn *et al.*, 2009).

But the fact is that zebrafish is used in toxicology studies since the 1950s (Carvan *et al.*, 2007). In this sense, zebrafish has been used to analyze the effects of toxins and pollutants in the environment, and for creating biomonitors that emit alarm signals when a toxic compound is detected (Aleström *et al.*, 2006).

For that and, in combination with the establishment of transgenic sensor strains and the further development of existing and new automated imaging systems, the zebrafish embryos could be used as cost-effective and ethically acceptable animal models for drug screening as well as toxicity testing (de Castro *et al.*, 2009; Yang *et al.*, 2009). An example of this was the acceptance and mandatory use of the embryo toxicity test with zebrafish as whole effluent testing in Germany, Europe (approved in January 2005, Lammer *et al.*, 2009). To this respect, several studies carry out experiences with single chemicals or complex mixtures assessing their embryotoxic, neurotoxic or teratogenic damages (Chen *et al.*, 2009b; de Castro *et al.*, 2009; Froehlicher *et al.*, 2009; Kling and Förlin, 2009; Muller *et al.*, 2009; Selderslaghs *et al.*, 2009). Pesticides are also included in these assays (Dong *et al.*, 2009; Ma *et al.*, 2009; Shi *et al.*, 2009) as well as toxins produced from marine and freshwater microalgae, including those classified among the so-called "harmful algal blooms" (Berry *et al.*, 2007; Suyama *et al.*, 2009).

I.1.3. In aquaculture.

As seen before, the zebrafish represents a versatile and well-characterized model with applications in many fields of study. In relation with aquaculture, the need for a well-characterized fish model has been satisfied by the zebrafish owing to the availability of functional genomics and molecular biology data to development of vaccines and therapies or marker-assisted breeding (Aleström *et al.*, 2006; Dahm and Geisler, 2006). In relation with this, already sequenced genes in zebrafish are useful to be compared with those characterized in farmed species (ie. two ghrelin receptor genes in channel catfish, *Ictalurus punctatus*; Small *et al.*, 2009) also, in transcription factors, where it is observed that liver X Receptor transcription factors of salmonids (Atlantic salmon, *Salmo salar*; rainbow trout, *Oncorhynchus mykiss*) are more closely with zebrafish than from medaka (Cruz-Garcia *et al.*, 2009). Other example could be the similar clock-gene expression in Atlantic salmon to that observed in zebrafish (Davie *et al.*, 2009). In relation with this, innovative technologies as transgenics have acquired much interest in their application to farmed fishes, due their potential to supply the ever growing demand of food products derived from aquatic resources (Wong and Van Eenennaam, 2008). In this sense, the feasibility to use the zebrafish in this research program (Eissen (1996, Talbot and Hopkins, 2000) is of great interest for further

transferring these technologies (as specific zebrafish mutant lines, Talbot and Hopkins, 2000; genes knockdown by morpholinos, Nasevicius and Ekker, 2000 or more recently using engineered zinc finger nucleases; Meng et al., 2008) to farmed species (ie. use of zebrafish U6 promoters to express shRNA in Nile tilapia and shrimp cell extracts; Boonanuntanasasarn *et al.*, 2009). But, in this case, the genetic reproductive containment of transgenic animals must be efficiently established (ie: gene knock-down technology by morpholino; Dahm and Geisler, 2006; Nasevicius and Ekker, 2000).

To other respect, research on nutrition and growth, stress, and disease resistance in the zebrafish can be expected to produce results applicable to aquacultural fish. For example improved husbandry and formulated feeds (Dahm and Geisler, 2006; Temple and langdon, 2009) or to comparing different anaesthetics in zebrafish juveniles and embryos (Macova *et al.*, 2008).

Other aspect to take into account is the economical importance of what zebrafish represents itself. Several companies have born around the world under the necessity of supplying zebrafish mutant lines to laboratories. Moreover there is also offered the sizing and training maintenance of zebrafish laboratory facilities. Also, collateral benefits appeared from unexpected ways as occurred with some mutant zebrafish lines that become economically important for aquariophilia.

In all these scientific areas, the development and improvement of micromanipulation and embryological techniques are indispensable for many research programs that use either gametic or embryonic biologic material, making their progress possible.

I.2. Pacific oyster as experimental model.

The Pacific oyster (*Crassostrea gigas*) is a protandrous hermaphrodite species, changing sex with an erratic and seasonal way (Pauley *et al.* 1988).

The commercial fishery for the Pacific oyster grew rapidly since its introduction from Japan to the western coast of the United States in 1903 (Glude and Chew 1982). Then, its adaptative ability of colonizing different environments around the world converted the P. oyster in the most cultured oyster around the world, now extended to all continents but Antarctica (Mann 1979) and with the largest production for an individual species (4.4 million tonnes; FAO, 2006).

Despite the P. oyster is mainly of interest for aquaculture, its intimate relation with humans has also promoted its use in the study of other different research areas, thus taking part in a greater or lesser extent in biomedical research and environmental risk assessment.

I.2.1. In biomedicine.

Bivalve mollusks are not widely used model organisms in human research. Despite this, oysters (and also mussels) play an important role into a specific area: the study of threats to human health from marine environment, including infectious diseases and harmful algal blooms (Hedgecock *et al.* 2005). The P. oyster is a shellfish filter-feeding animal that concentrates infectious agents and harmful algae. Because of this, the mollusk consumption, especially oysters, is thus an important mode of transmission of infectious disease to humans (Hedgecock *et al.* 2005), due they are traditionally consumed alive.

Last years, nonvertebrate model systems, as plants (*Arabidopsis thaliana*) and nematodes (*Caenorhabditis elegans*), were developed to study human infectious disease. Despite they were initially quite informative, recent studies achieved the successful identification of numerous virulence genes in human pathogens (Phelps and Neely, 2005). Their increasing interest is because of the ability to easily genetically manipulate the host (Darby *et al.*, 1999; Rahme *et al.*, 1995; Rahme *et al.*, 1997). To this respect, the creation of BAC (Bacterial Artificial Chromosome) libraries is now emerging in oysters and have been developed as part of an international effort to

develop tools and reagents that will advance the ability to conduct genetic and genomic research (Cunningham *et al.*, 2006). Their interest is in first instance for aquaculture production, but biomedical research could take benefit if useful aspects come to light regarding to the development of tools for nonvertebrate models, for example the evolution of molecular mechanisms of immune recognition and response to stress (Gueguen *et al.*, 2003; Tangy and Moraga, 2001) for the study of human infectious disease (Phelps and Neely, 2005).

I.2.2. In environmental risk assessment.

Aquatic toxicity tests play a crucial role in assessing the potential or actual impact of contaminants on the natural environment (da Cruz *et al.*, 2007). In these toxicity assays, is of interest the use of aquatic species with different feeding habits to evaluate the long term risk of environmental chemicals and metals impact (Bolognesi *et al.*, 2006; Lee *et al.*, 1996). In this context, oysters are of substantial economic and cultural significance in the ecology of the coastal marine environment (Hedgecock et al., 2005).

The oyster biological interactions with toxic agents can be measured at different stages of their live: in embryos (Beiras and His, 1995; Calabrese *et al.*, 1973), spat (Stachowski-Haberkorn *et al.*, 2008) and adult (Bolognesi *et al.*, 2006). Moreover, of much importance is also the development of chronic or sub-chronic tests, due they may have a higher ecological relevance when dealing with the embryonic development of a key species for the ecosystem at risk (da Cruz *et al.*, 2007; Muller *et al.*, 2009).

To this respect, the early-life-stages tests have evident ecological relevance because they reflect the possible reproductive impairment of natural populations (da Cruz *et al.*, 2007). In spat, the assessment of growth differences due to pesticide impacts can be measured by the shell area index increasment (Stachowski-Haberkorn *et al.*, 2008). In adult oysters, there can be evaluated concentrations of selected elements (Schuhmacher and Domingo, 1996), thus allowing the assessment of human health risks from exposure to toxic compounds (ie. Inorganic arsenic; Liu *et al.*, 2006). Also there are interesting developed models: the physiologically-based pharmacokinetic model, which will serve as a useful tool for predicting the kinetics of other persistent organic pollutants as well as, to dispose of a more refined ecological risk assessment by estimating dioxin concentrations in sensitive tissues such as the gonad (Wintermyer *et*

al., 2005). Moreover, this model permits the development of a gametogenesis protocol to serve also as a model for evaluating the toxic effects of chemicals on oogenesis and spermatogenesis (Wintermyer and Cooper, 2007).

For these reasons, oysters, especially the genus *Crassostrea*, have been commonly used for the determination and pollution risk prevention or in monitoring long term impacts (da Cruz *et al.*, 2007; His *et al.*, 1997; Woelke, 1972). Some examples are their increasing utilization in Brazil for risk prevention (da Cruz *et al.*, 2007); the long term genotoxic impact of Haven oil spill along the Ligurian coast in Italy (Bolognesi *et al.*, 2006); the ecotoxicological evaluation of industrial port of Venice in Italy (Libralato *et al.*, 2008); the bioaccumulation of chemicals through the marine food web in Sendai Bay, Japan (Okumura *et al.*, 2003); or the assessment of copper pollution status of the south-western area in Taiwan after the 1986 green oyster incident (Lee *et al.*, 1996).

I.2.3. In aquaculture.

With the use of P. oyster there have established different types of techniques in relation with production systems (ie: telecaptation; Helm and Bourne, 2004) and breeding (oyster conditioning, gamete stripping, in vitro fertilization; Helm and Bourne, 2004) being some of such techniques of especial complexity (ie: triploidy induction, Helm and Bourne, 2004). In this last group, we can find a great variety of manipulation techniques (ie: embryo transfection, Buchanan *et al.*, 2001; microinjection of eggs, Cadoret *et al.*, 1997 and the obtaining of aneuploid, triploid, tetraploid and gynogenetic oysters, Eudeline *et al.*, 2000, Guo *et al.*, 1993, Guo and Allen, 1994). To this respect, the induction of triploidy has been the most popular one, being in use since decades ago (Nell, 2002).

Triploids are produced by preventing the egg undergoing meiosis so that it remains in the diploid (2n) state. When such an egg is fertilized by sperm in the 1n (haploid) stage the result is a triploid (Helm and Bourne, 2004). The interest in producing triploid oyster for aquaculture is mainly because of gamete output (functional reproductive sterility) and improved meat quality and growth (Dong *et al.*, 2005). Also, there was observed a better resistance to parasites and diseases as well as a reduction of mortalities in summer (Nell, 2002). There are many treatments capable of inducing triploidy: chemicals (Cytochalasin B, 6-dymethylaminopurine, Caffeine; Okumura,

2007); thermal shock (hot, cold; Okumura, 2007); pressure shock (Okumura, 2007) and by mating tetraploid (also obtained artificially) with diploid oysters (Guo *et al.*, 1996). Other applied technology in oyster is the cryopreservation. An example is the commercial availability of cryopreserved trochofore larvae of *Crassostrea gigas* for the first days feeding of fry larvae for commercial marine fish production. Their small size (50 µm) compared with rotifers or artemia is idoneous for this purpose. But, in this special case, trochofores must be penalized in development to avoid their further settle into the fish hatchery installations.

To other respect, P. oyster would not only provide a mollusk for comparative genomics but also a model species for a broad spectrum of genome level studies of shellfish biology (Hedgecock *et al.,* 2005). To this respect, it has detected a high genetic load in this species, which give support the dominance theory of heterosis and inbreeding depression and establishes the oyster as an animal model for understanding the genetic and physiological causes of this phenomenon (Launey and Hedgecock, 2001). Moreover, the creation of BAC (Bacterial Artificial Chromosome) libraries is now emerging in oysters to develop tools and reagents that will advance the ability to conduct genetic and genomic research (Cunningham *et al.*, 2006). In aquaculture, this advances will benefit the genetic selection improvement in breeding programs, even more, when the treats heritability in P. oyster are in general high (around 30%, López-Fanjul and Toro, 1990). An example is the creation of around 50 inbred strains to produce F1 hybrids for comparisons of growth and survival rates in hatchery and field trials, any of them in commercial production (Hedgecock *et al.*, 2005).

In all these scientific areas, the development and improvement of micromanipulation and embryological techniques will benefit the development of many research programs, being also useful for aquaculture, as it occurred in mammals for breeding programs (ie. embryo and sperm cryobanking, transgenesis).

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II. Objectives.

The objective of this thesis was the development of diverse embryological and micromanipulation techniques in zebrafish and Pacific oyster to be used as experimental models in different scientific areas. Specifically:

In zebrafish:

First general objective: Cryopreservation techniques.

- Vitrification of caudal fin explants.
- Vitrification of MBT embryo blastomeres.
- Cryopreservation of adult testicular tissue.

Second general objective: Chimaerism techniques.

- Chimaerism at MBT stage.
- Cell testicular transplantation at larval (48-72h) stage.

Third general objective: Nuclear transplant techniques.

- Nuclear transplant of adult somatic cells.
- Nuclear transplant of haploid somatic cells.

In Pacific oyster:

Fourth general objective: Zygotic electrofusion technology.

III. Experiments.

All experiments included here are presented as scientific papers. Many of them have been already published in both indexed and international scientific journals. The rest, are still in review process. With all of them are configured the different experimental parts of this thesis.

The findings reported are result of the Laboratory of Animal Reproduction and Biotechnology (LARB) team effort since 2006, being their contribution of different importance to the development of objectives, with the discussion of results being in consonance.

With the aimed of improving the contents presentation of this thesis, this section has been structured in four well differenced parts, and corresponding to the four different working areas where the doctorandus has been ascribed the experiments. According with this, each part is briefly introduced and the following information given:

- The linkage among the experimental works carried out.
- General results accomplished.
- The doctorandus participation level in terms of their responsibility in the settled works with which each part is configured.
- Experimental works. The paper citation (if published) or the manuscript reference number (if submitted to a journal) is included as a footnote on each study presented.

III.1. Cell Cryopreservation in zebrafish.

In the use of zebrafish as model for biomedicine, developmental biology research and in other related areas, there are some specific cellular types widely used and therefore acquiring much importance to be preserved.

Their interest reside mainly in the possibility of using these cellular types in somatic cloning by nuclear transplant (NT) or in the conservation of genetic endowment (mutations), artificial mutant lines, transgenic strains, etc. This is the case of somatic cells, being the epithelial fibroblasts of caudal fin the main tissue target used in fish for this purposes.

Early embryo blastomeres play a special paper in the generation of chimaeric fish. Thus meaning the further obtaining of viable gametes from these cells when they are inserted in a host embryo. Another cellular type with related characteristics of that (two) cited before are testicular cells, specifically spermatogonial cells. In fish, as well as it occurs in mammals, it is possible the spermatogonial cell incorporation into either developing or adult host testicular tissue in the way that further gamete production (sperm and eggs) is derived from transplanted cells. This interesting technique also permits their interespecific transplantation or in vitro culture, acquiring great interest in different scientific working areas. So, the availability of specific cryopreservation protocols for these cellular types is of much importance. Moreover, there are thousands of mutant fish lines in need of cryopreservation and stock centres are overwhelmed by the time it would take to do this. Also, many university laboratories are not well versed in cryo-techniques. The wonderful thing about efficient cryopreservation techniques should be they are fast and easy applicable to almost any research setting.

In this sense, the first of works entitled "**Vitrification of caudal fin explants from zebrafish adult specimens**" pursued the extension of a pre-defined cryopreservation technique in mammals to fish. The target tissue chosen for this purpose was the caudal fin. The cryopreservation technique employed, reached efficient rates in the cryopreservation of ear skin tissue of many economically important species of domestic mammals.

Results obtained in both substrate attachment and outgrowing of vitrified tissue pieces in zebrafish are high (63 % and 83 % respectively) using the best combination of cryoprotectant agents (20 % Dimethyl sulfoxide and 20 % Ethylen glycol prepared in

Hanks' buffered salt solution plus 20 % FBS). Moreover, fish fins achieve a complete regeneration after a partial amputation (in zebrafish at 6 days approximately), leading to the efficient further recovery of cell lines from very specific and important fish specimens without mutilating them.

- Throughout the development of this vitrification technique, the doctorandus participated in all steps, from animal anaesthesia, sterilization and fragmentation of fin samples, vitrification-warming procedures as well as tissue culture. He was also responsible of the manuscript preparation, especially the discussion section.

With respect to the development of a blastomere cryopreservation protocol, this was certainly a great challenge.

First trials pursued the cryopreservation of embryo blastomeres by vitrification in plastic straws (0.25 ml) due that the entire embryo cryopreservation still represents a front barrier very difficult to overcome, concretely in fish species, because their embryo size is relatively big. Under our carried out conditions, it was concluded that the either vitrification of entire blastoderms or isolated blastomeres in plastic straws (0.25 ml) was not a reliable option. So it was developed a new and original vitrification technique in microvolumes to accomplish our objective. This technique is detailed in the manuscript "**Vitrification of zebrafish embryo blastomeres in microvolumes**". Briefly, it consisted in the blastomere (both grouped and isolated) impregnation with the vitrifying solution (5 M Dimethyl sulfoxide prepared in Hanks' buffered salt solution plus 20 % FBS) without cryoprotectant cell permeation. Then, microdrops of 0.25µl containing blastomeres were held on to a nylon filament (loop; 0.15mm diameter) and immediately submerged in liquid nitrogen (blastomeres were exposed to cryoprotectants for a maximum of 25 sec prior vitrification). The procedure established here reached a 93.4% blastomere survival and allowed the rescue of as much as 20% of the total blastomeres from each zebrafish blastula embryo. Also, it was developed a loop container which consisted in a conventional screw cap plastic container of 30 ml (with the body drilled to facilitate the entry of liquid nitrogen). This container allowed a suitable cryobanking management of loops (maximum load capacity of 20 loops).

Further to the blastomere vitrification achievement, the possibility to use these cells as donor cells in chimaerism experiments was also assessed and presented in the manuscript "**Can vitrified zebrafish blastomeres be used to obtain germ-line**

chimaeras?". Unfortunately, to this respect, several difficulties arose. They were mainly related to a major increase volume of thawed blastomeres, a major medium volume injected in host embryos and a more bore sized micropipettes (60-70 µm) required to insert treated blastomeres and which could cause embryo disorganization.

The scanty number of chimaeras that reached adulthood (4 %) was not enough to detect the thawed blastomeres ability on further, integration and participation in the host germ-line. In fact, neither somatic nor germ-line chimaerism was observed. These aspects will be the basis of future studies related to improve chimaerism using vitrified blastomeres, being the increment of effectives number an important key point to be considered.

- The procedure of blastomere vitrification technique in microvolumes exclusively came from the development of a doctorandus idea. In their extension to chimaerism, it took part the experienced professor Francisco-Simão, although the solution of reported problems throughout the technique re-adaptation, from conventional chimaerism to the characteristics of a new cellular type (vitrified/thawed blastomeres), were responsibility of doctorandus.

According to the objectives described in the conservation of important cellular types, different cryopreservation methods were assayed in order to establish a testicular cell cryopreservation procedure in zebrafish. The way of how this objective was finally accomplished is described in the manuscript "**Effects on cell viability of three zebrafish testicular cell or tissue cryopreservation methods**". Among three procedures assayed, the most efficient was vitrification of testicular tissue pieces in plastic straws (0.25 ml) with the subsequent elimination of connective tissue after warming, using 20% Dimethyl sulphoxide and 20% Ethylen glycol solution in Hanks' buffered salt solution plus 20 % FBS as vitrifying solution. Cell survival rates were, in this case, 94% and the appearance of pseudopodia in warmed cells indicated their spermatogonial condition.

As said before, nuclear transplant and chimaerism are available techniques to use in the recovery of genetic endowment. But these techniques require: in the case of NT the insertion of a cell nucleus into non-activated/fertilized eggs or in early zygotes and, in chimaerism, a specified number of inserted donor cells into early embryos at blastula stage. However, a different cell insertion technique is required for testicular

cells. Spermatogonial cells transplantation is achieved in many fish species, although major larval sizes have been used than the zebrafish. In our case, after the establishment of an efficient testicular cell vitrification method, we developed an efficient testicular cell transplantation technique from a technical point of view to insert these cells in the germ ridges area of 48-72 h aged larvae, in order to assess their germ-line contribution. This technique was carefully described in the manuscript entitled "**Testicular cell transplant in zebrafish (*Danio rerio*) using cryopreserved adult testicular cells as donors**". Useful innovations were therefore described, especially those related to larvae immobilization along the whole injection process as well as the manual cell injection of thawed cells and also, in the correct deposition place. Unfortunately, and despite high survival and normality rates of transplanted larvae achieved (176 injected larvae in 12 transplantation sessions) results of germ-line chimaerism were null, possibly due to some asynchrony between the gonadal colonisation ability of spermatogonia and the embryo rhythm of gonadal development.

- The development of these two techniques for adult testicular cells (vitrification/thawing and their further transplantation to host larvae) was the result of the contribution of the two first authors, participating the doctorandus to a lesser extent.

STUDY 1. Vitrification of caudal fin explants from zebrafish adult specimens.*

Abstract

No data on vitrification of tissue samples are available in fishes. Three vitrification solutions were compared: V1: 20% ethylene glycol and 20% dimethyl sulphoxide; V2: 25% propylene glycol and 20% dimethyl sulphoxide, and; V3: 20% propylene glycol and 13% methanol, all three prepared in Hanks' buffered salt solution plus 20% FBS, following the same one step vitrification procedure developed in mammals. Caudal fin tissue pieces were vitrified into 0.25 ml plastic straws in 30s and stored in liquid nitrogen for 3 days minimum, warmed (10s in nitrogen vapour and 5s in a 25ºC water bath) and cultured (L-15 plus 20% FBS at 28.5ºC). At the third day of culture, both attachment and outgrowing rates were recorded. V3 led to the worst results (8% of attachment rate). V1 and V2 allow higher attachment rates (V1: 63% vs V2: 50%. $P < 0.05$) but not significantly different outgrowing rates (83%-94%). Vitrification of caudal fin pieces is advantageous in fish biodiversity conservation, particularly in the wild, due to the simplicity of procedure and equipment.

Keywords: Cryopreservation, vitrification, cryoprotectants, zebrafish, tissue culture, biodiversity.

* This paper has been published in the journal "*CryoLetters*" with the following reference: Cardona-Costa J, Roig J, Pérez-Camps M and García-Ximénez F. (2006). Vitrification of caudal fin explants from zebrafish adult specimens. CryoLetters 27(5): 329-332.

INTRODUCTION

Tissue and cell sample cryopreservation from any endangered species, either domestic or wild, have been proposed by McLaren (2000) as part of biodiversity conservation strategy independently of whether somatic cloning technology is available or not at present. Somatic cells should be also considered for fish genome cryobanking of valuable or endangered fish (Mauger *et al.*, 2006). At present, cloning technology is available in fishes (Huang *et al.*, 2003). Moreover, cells cultured from fin explants were recently used for cloning (Ju *et al.*, 2003; Liu *et al.*, 2002).

Cryopreservation by conventional procedures of in vitro cultured fibroblasts does not involve special difficulties in fishes (Wang *et al.*, 2003; Zhang and Rawson, 2002). However, little attention was paid to the cryopreservation of the explants themselves and to the ability of fin explants to unfailingly outgrow somatic cells (Mauger *et al.*, 2006).

In rabbit and pig, we have shown that vitrification is an adequate procedure to cryopreserve epithelial tissue samples (Silvestre *et al.*, 2002), including post-mortem (Silvestre *et al.*, 2003). These results have become widespread recently in the main economically important species of domestic mammals (Silvestre *et al.*, 2004). To our knowledge, no data are available on vitrification methods for fin explants in fish. So, the aim of this work was to extend this vitrification technique to a very different group of vertebrates, the fish, using zebrafish as a model.

MATERIALS AND METHODS

Wild-type zebra fish specimens were maintained according to standard conditions (15). Fish were anaesthetised with clove oil (Grush *et al.*, 2004; Mylonas *et al.*, 2005) in system water (100µl/l) and the amputated caudal fins were bleached (2 minutes in 0.2% v/v bleach/system water) and washed 3 times in 2ml of Hanks' buffered salt solution 10% in water (H10) for 5-10 min each and finally in Hanks' buffered salt solution with antibiotics (HC). After the last HC wash, the fin explants were prepared for vitrification or directly for their in vitro culture (control groups). All aseptic procedures and sterile instruments were used in subsequent steps. It may be pointed out that any fish used during the experiment was sacrificed or died due to the anesthetic or to the amputation procedure. In addition, in zebrafish, as in other teleost

fish species, the caudal fin can completely regenerate itself after a partial amputation, in zebrafish six days approximately (Westerfield, 2000).

A vitrification procedure previously used for skin samples in various domestic mammalian species (Silvestre *et al.*, 2002) was applied, with some modifications, to small pieces of amputated caudal fin. The assayed vitrification solutions (VS) were: V1: 3.58M (20% v/v) ethylene glycol (EG) and 2.82M (20% v/v) dimethyl sulphoxide (DMSO) (11) ;V2: 3.42M (25% v/v) propylene glycol (PG) and 2.82M (20% v/v) DMSO (established in this work) and; V3: 2.73M (20% v/v) PG and 3.21M (13% v/v) methanol (Chen and Tian, 2005). All three were prepared in Hank´s buffered salt solution plus 20% FBS (HC-F). Vitrification solutions were prepared on the day of use and were stored in darkness at room temperature until use. V2 was included in the experiment because of the low toxicity of propylene glycol in zebrafish embryos (16), as in other fish cells (Zhang and Rawson, 2002).

For vitrification, 15-20 small tissue pieces (1mm of maximum length corresponding to the inner diameter of 0.25ml cryopreservation straws) from each amputated caudal fin were put into 1.0ml VS and immediately loaded into 0.25ml plastic straws (2-3 straws for each amputated fin). Straws were sealed with modelling clay, identified, and plunged vertically into liquid nitrogen. Straw loading took approximately 30s. Straws were stored in liquid nitrogen for 3 days minimum before warming.

Tissue samples were warmed following a two-step rapid-warming procedure. Briefly, every straw was held horizontally in nitrogen vapour for 10s (15cm over liquid nitrogen surface) and then warmed by immersion in a 25ºC water bath for 5s. The straw content was expelled into a watch glass**.** Recovered tissue samples were rinsed for five minutes in 1.0ml 0.125M sucrose in HC-F to remove cryoprotectants, and then, for a further 5 min, 1.5ml of HC-F were added in order to reduce the sucrose concentration progressively. Finally, the pieces were transferred to HC-F without sucrose for 5min and then to L15-F (see below). Tissue samples (5-7 tissue pieces each) were immediately placed in culture in 2ml of L15 Medium Leibovitz's (Sigma) supplemented with 0.3 g/l L-glutamine, 100units/ml penicillin, 100 µg/ml streptomycin, and 20% heat-inactivated foetal bovine serum (L15-F) at 28.5ºC (15) and counted as the initial number of pieces that were placed in culture. The number of tissue pieces fixed to the substratum and the emergence of tiny fibroblast-like cells immediately adjacent to the fin pieces were recorded at the third day (Paw and Zon, 1999). Two fresh control groups

were carried out at the same time as the experimental groups. In the first (Control I), samples were obtained and put in culture exactly in the same manner that the vitrified-warmed samples, while the second control group (Control II) was established with a different procedure, consisting of tearing and fixing the fin tissue pieces to the culture substratum, usually considered as the most favourable conditions for subsequent outgrowth. Cell survival was assessed during primary culture both by the ability of seeded pieces of caudal fin to attach to the culture substratum and by the presence of outgrowing living cells (mainly fibroblasts) acquiring a flattened appearance (Mauger *et al.*, 2006; Silvestre *et al.*, 2002; Silvestre *et al.*, 2003; Silvestre *et al.*, 2004), in our case evaluated at three days of culture.

Results were analysed by a Chi-square analysis. When a single degree of freedom was involved, the Yates' correction for continuity was carried out.

RESULTS AND DISCUSSION

The results are presented in Table 1.

Looking at the effect on the attachment rate of the three vitrification solutions tested, it can be highlighted that V3 (Propylene glycol and Methanol as cryoprotectants) leads to the worst results, allowing only a 8% of attachment rate of all the seeded tissue pieces after three days in culture. There are statistically significant differences between V1 and V2 attachment rates, favourable to V1. In addition, these two vitrification solutions allow higher attachment rates than Control I (fin tissue pieces prepared by the same procedure used for vitrification: similar size of pieces and no initial anchorage). A possible explanation could be the possible higher alteration of the tissue structure of fin explants in vitrified-warmed samples than that due exclusively to the scalpel blade cut injuries. Such additional alteration of the structure of tissue pieces provides a better direct contact of inner proliferating cells on the culture substrate and its further outgrowth. Two arguments support the proposal. Firstly, the fact that in this same experiment it was observed that outgrowing rate is directly related with the attachment rate (it is important to point out that these two parameters were assessed at the same time, after three days in culture) and, on the other hand, that Control II is the experimental group with the highest attachment rate (and outgrowth), precisely the group established with the seeding procedure (tearing and initial fixing of the fin samples) considered as the best in cell culture. It may be emphasised that outgrowing

after vitrification is detected as early as in fresh controls, suggesting a low cellular toxicity of cryoprotectants. It is still surprising that the vitrification solution which offers better attachment rates is statistically worse in outgrowing rate when compared with Control II. It is probable that the number of fixed pieces will increase further because of the short time of culture defined until assessment of this parameter. Whatever the case, it should be emphasised that the use of V1 or V2 in this vitrification procedure allows us to obtain live cells efficiently from cryopreserved tissue samples of adult zebrafish specimens.

The PG-Methanol combination has previously been proposed for cryopreservation of fish embryos due to the high permeation rate of methanol (Chen and Tian, 2005) and the low toxicity of the propylene glycol on fish cells (Zhang and Rawson, 2002). However, for somatic tissue samples, the results obtained with this vitrification procedure when methanol was used were discouraging. Despite the results using methanol, the use of DMSO in combination with EG or PG (particularly in V1) and according to the vitrification procedure tested, have shown that DMSO is adequate for epithelial tissue sample vitrification. In previous works (Silvestre *et al.*, 2002; Silvestre *et al.*, 2003; Silvestre *et al.*, 2004), this cryoprotectant in combination with EG was used to cryopreserve epithelial tissue samples from a wide number of mammalian species and has also been shown to be efficient in poultry to cryopreserve quail blastoderms (results not published). This work now extends its use to fish, taking into account, additionally, that the direct cryopreservation of tissue pieces, compared with that of cultured cells, is proved advantageous in biodiversity conservation strategies, especially when using it in the wild, due to the simplicity of procedure and of the required equipment.

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Table 1. Effect of three different vitrification solutions on the attachment and outgrowing rates of zebrafish caudal fin tissue pieces after three days of in vitro culture.

	Attachment rate	Outgrowing rate (on attached)
V1 (20DMSO+20EG)	88/140 (63%)[b]	73/88 (83%)[b]
V2 (20DMSO+25PG)	65/130 (50%)[c]	61/65 (94%)[ab]
V3 (20PG+13Methanol)	6/78 (8%)[e]	5/6 (83%)[ab]
Control (fresh) I	17/55 (31%)[d]	16/17 (94%)[ab]
Control (fresh) II	64/70 (91%)[a]	64/64 (100%)[a]

Rows with different superscripts are statistically different ($p<0.05$)

STUDY 2. Vitrification of zebrafish embryo blastomeres in microvolumes.*

Abstract

Cryopreservation of fish embryos may play an important role in biodiversity preservation and in aquaculture, but it is very difficult. In addition, the cryopreservation of fish embryo blastomeres makes conservation strategies feasible when they are used in germ-line chimaerism, including interspecific chimaerism. Fish embryo blastomere cryopreservation has been achieved by equilibrium procedures, but to our knowledge, no data on vitrification procedures are available. In the present work, zebrafish embryo blastomeres were successfully vitrified in microvolumes: A number of 0.25 ml drops, sufficient to contain all the blastomeres of an embryo at blastula stage (from 1000-cell stage to Oblong stage), were placed over a 2.5cm loop of nylon filament. In this procedure, where intracellular cryoprotectant permeation is not required, blastomeres were exposed to cryoprotectants for a maximum of 25 sec prior vitrification. The assayed cryoprotectants (ethylene glycol, propylene glycol, dimethyl sulphoxide, glycerol and methanol) are all frequently used in fish embryo and blastomere cryopreservation. Methanol was finally rejected because of the excessive concentration required for the vitrification (15M). All other cryoprotectants were prepared (individually) at 5 M in Hanks' buffered salt solution (Sigma) plus 20% FBS vitrification solutions: VS).

After direct thawing in Hanks' buffered salt solution plus 20% FBS, acceptable survival rates were obtained with ethylene glycol: 82.8%, propylene glycol: 87.7%, dimethyl sulphoxide: 93.4%, and glycerol: 73.9% ($P<0.05$). Dimethyl sulphoxide showed the highest blastomere survival rate and allowed the rescue of as much as 20% of the total blastomeres from each zebrafish blastula embryo.

Keywords: Biodiversity, vitrification, microvolume, cryoprotectant, blastomeres, zebrafish.

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INTRODUCTION

Cryopreservation of fish embryos is interesting for biodiversity preservation and in aquaculture. However, it is very difficult (Chen and Tian, 2005; Edashige *et al.*, 2006; Robles *et al.*, 2005).

In contrast to mammalian embryos, fish embryos have low membrane permeability and the presence of cell layers which act as osmotic barriers, making the permeation of cryoprotectants extremely difficult. Furthermore, other compounding features have also been identified including their large size (usually 1-7 mm diameter), the high yolk content in the egg and the high sensitivity to chilling injury (Hagedorn *et al.*, 1997a; Hagedorn *et al.*, 1997b; Janik *et al.*, 2000; Zhang and Rawson 1996). The low cryoprotectant permeation also makes cryopreservation of blastoderms very difficult, and even of blastomere clumps (Harvey, 1983). So, the cryopreservation of isolated blastomeres is at present the only available way to preserve fish genetic endowment efficiently. Blastomere cryopreservation may play an important role in biodiversity preservation when they are used via germ-line chimaerism, including interspecific chimaerism (Kusuda *et al.*, 2004; Lin *et al.*, 1992; Nakagawa *et al.*, 2002). Equilibrium procedures of cryopreservation have been used to achieve this (Calvi and Maisse, 1998; Calvi and Maisse, 1999; Strüssmann *et al.*, 1999). However, no references to blastomere cryopreservation by nonequilibrium procedures, such as vitrification, are present in the literature.

Liu *et al.* (1998) reported a 2-step zebrafish intact embryo vitrification procedure in microvolumes, where approximately 80% of later stage (50% epiboly to prim-6) embryos and 50% of early stage (1-cell and 64-cell) embryos remained morphologically intact when evaluated by light microscope inspection immediately after thawing. Although no embryos showed any developmental ability after vitrification, the results obtained in this study indicated that the vitrification procedures tested had advantages over previously reported methods (Zhang and Rawson, 1996).

In our laboratory, preliminary (non-published) assays of blastoderms and isolated blastomeres vitrification in 0.25 ml straws resulted in a total cellular lysis every time. As a consequence of such negative results and taking into account previous and successful assays in mammals (Lane *et al.*, 1999; Matsumot *et al.*, 2001; Mukaida *et al.*, 2003), vitrification in microvolumes but without cryoprotectants was also tested, with the aim of avoiding toxicity and osmolar effects of cryoprotectants. However,

vitrification of the medium was not achieved, and therefore cryoprotectants were considered to be necessary for such prolonged permeation times as are needed (Calvi and Maisse, 1999, Kusuda *et al.*, 2002), but results were also very poor. Finally, and as a novel strategy, the requirement of cryoprotectant intracellular permeation was obviated, ensuring only the extracellular medium vitrification. The promising results obtained in this final preliminary assay led to the present work to test the survival efficiency reached with the most frequent cryoprotectants used in fish embryos and blastomere cryopreservation (Harvey, 1983; Hagedorn *et al.*, 1997a; Janik *et al.*, 2000; Liu *et al.*, 1998; Robles *et al.*, 2005). The aim was to establish a definite and efficient vitrification procedure in microvolumes for zebrafish blastomeres from blastula stage embryos.

MATERIALS AND METHODS

Animal care and obtaining of blastoderm

Wild zebrafish (*Danio rerio*) dechorionated embryos from 1000-cell stage to Oblong stage (3-4 hours post-fertilisation at 28.5ºC) (Driever, 1998) were used. They were collected by siphoning from adults kept in 6:3 proportion (females/males). Granular food was supplemented with recently defrosted hen egg yolk and shrimp meat in replacement for live food (*Daphnia* or *Artemia* nauplius), which is usually recommended for egg production (Westerfield, 2000).

After washing with conventional tap water on a nylon mesh, embryos were selected under microscope and washed again with tap water. The embryos in a suitable development stage and perfectly clean were kept in system water (Westerfield, 2000). No bleaching treatment was applied, but sterility conditions of media and pipettes after dechorionation were strict.

Dechorionation was carried out by pronase treatment (1.5 mg/ml in Hanks' 10%) followed by immersion twice in Hanks´ buffered salt solution diluted 10% (v/v) in distilled water (H10; 35 mOsm). Partially or totally damaged embryos (by lysis or blastoderm-yolk disconnection) due to previous manipulation were removed and finally only intact embryos were included in the experiments.

The yolk was removed using two sterile hypodermic needles. First, the yolk was punctured, then the embryo was taken to the liquid surface in which the yolk was

removed immediately from the embryo due to the surface tension. Intact blastoderms obtained were placed in Hanks´ buffered salt solution plus 20% Fetal Bovine Serum (FBS).

Physical support and container

The cryopreservation support consisted of a filament of fishing line (0.15mm diameter; Boomerang 2000, super co-polyamide) of 2.5 cm length. This filament was glued with cyanoacrylate by its ends to one half of a 0.25 ml freezing straw, forming a loop. The container to hold physical supports was a conventional screw cap plastic container of 30 ml (25 x 90mm). The cap and container body were drilled to facilitate the entry of liquid nitrogen. Every container kept 20 physical supports without risk of losing microdrops (with blastomeres).

Vitrification solutions (VS)

Cryoprotectants initially chosen were ethylene glycol (ETOH), propylene glycol (PROH), dimethyl sulphoxide (DMSO), glycerol (GLY) and methanol (MET), because they are the most common cryoprotectants used in fish embryo and blastomere preservation (Harvey, 1983; Hagedorn *et al.*, 1997a; Janik *et al.*, 2000; Liu *et al.*, 1998; Robles *et al.*, 2005). All were prepared in Hanks´ buffered salt solution plus 20% FBS to obtain the vitrification solutions (VS), and were assayed individually.

Initial vitrification assays with each cryoprotectant were carried out in microdrops (without blastomeres) to establish the minimum concentration required for vitrification, assessed by ocular inspection of microdrop transparency (Liu *et al.*, 1998). After establishing this minimum concentration (ETOH: 4.60 M; PROH: 3.86 M; DMSO: 4.25 M; GLY: 4.80 M; MET: 10.20 M), the vitrification solutions (VS-ETOH, VS-PROH, VS-DMSO and VS-GLY) were all standardised to 5 M in order to ensure the successful vitrification of the microdrops when containing blastomeres. Methanol was not included in the final assay because of the excessive concentration required for vitrification. In fact, 15 M methanol microdrops containing blastomeres did not vitrify and this concentration of methanol also negatively affected the blastomere morphology and appearance.

Thawing medium

Microdrops held on nylon filaments as physical support were directly thawed in Hanks´ buffered salt solution plus 20% FBS. When needed, Hanks´ buffered salt solution without Ca^{++} and Mg^{++} (Sigma) was used with the aim of avoiding cellular aggregations (Calvi and Maisse, 1998; Calvi and Maisse, 1999) which would make all subsequent assessments difficult.

Microdrop volume estimation

A microcapillary pipette (10 ml; Sigma P6804) was used to estimate the microdrop volume. Four measurements were carried out. In each one, 8 microdrops were absorbed and, constantly, 2 ml was the resulting volume every time. Consequently, the volume of each microdrop was estimated at 0.25 ml.

VITRIFICATION AND THAWING PROCEDURES

Vitrification

Each blastoderm was taken with the minimum possible amount of Hanks´ buffered salt solution plus 20% FBS by means of a fire-polished Pasteur pipette and transferred to an embryological plate with VS. In this plate, the blastoderm was disaggregated into its blastomeres by using a pulled and fire-polished Pasteur pipette, which had approximately 1/5 inner diameter size of the zebrafish blastoderm. Immediately, all blastomeres were loaded into the same pulled pipette. Then, VS plus blastomeres were placed as microdrops on the nylon filament (each 2 mm apart) of the physical support. Finally, the physical support was quickly immersed in LN2. The vitrification procedure spent a maximum of 25 sec from the first contact of blastomeres with VS to immersion in LN2. This procedure was carried out under stereo microscope (90 x magnification). All physical supports were stored in LN2 for 3 days minimum before thawing.

Thawing

Direct thawing was performed in Hanks´ buffered salt solution without Ca++ and Mg++ at 25ºC. A one ml volume of this medium was established as the minimum required for thawing by this procedure. After thawing, 30min elapsed until any assessment, with the aim of counting only intact (non-lysed) blastomeres (dead or

alive). It must be emphasised that a high speed thawing is required to achieve high survival rates, so the physical support must be shaken quickly while thawing.

EVALUATION CRITERIA

Number of total intact blastomeres after thawing

These results are presented in Table 1 as "Nº of total intact blastomeres".

Every physical support was thawed in an embryological plate containing 3ml of Hanks´ buffered salt solution without Ca++ and Mg++. There, all the intact blastomeres from a physical support were counted.

The number of intact blastomeres (from each blastoderm) before vitrification was required to evaluate the lysis rate derived exclusively from the vitrification effect. Unfortunately, this lysis rate was impossible to assess because blastomeres remained in SV for a longer time than in vitrification procedure and the alteration of results in all attempts became obvious.

Blastomere survival rate after thawing.

These results are presented in Table 1 as "Blastomere survival rate". Physical supports were individually thawed in 1 ml of Hanks´ buffered salt solution without Ca^{++} and Mg^{++}. After 30 min, a sample of 20 ml with the largest number of blastomere possible was taken and mounted on a slide. Blastomere survival rate was established as the number of live blastomeres from intact blastomeres (live plus dead) recovered, by using 0.4% trypan blue (1:1 v/v). All blastomeres, dead and alive, were counted in eight random fields (100 x magnification) for each physical support. All considered fields contained at least one blastomere (dead or alive). A minimum of ten repetitions were carried out on each VS assayed.

Count of number of blastomeres from used embryos

A Thoma counting chamber was used. A pulled pipette similar to that described in the *Vitrification* procedure was used to isolate cells from one blastoderm. Fourteen repetitions were carried out. The estimated mean number of blastomeres from one zebrafish embryo was 2285.7 ± 309.3.

Statistical analysis

Data were analysed by a one-way analysis of variance (number of blastomeres) and by Chi-squared analysis (survival rates). When one degree of freedom was involved, Yates' correction for continuity was carried out.

RESULTS

Results are presented in Table 1. It may be highlighted that VS-DMSO significantly increased the number of total intact blastomeres recovered after thawing (471.8 ± 70.2), fourfold in relation to every other vitrification solution assayed (which were not statistically different among themselves). In addition, the blastomere survival rate is high with all cryoprotectants, especially with VS-DMSO (93.4%, $P<0.05$). Blastomere survival rate evaluated on number of blastomeres per embryo at blastula stage reached 20% ($P<0.05$) when VS-DMSO was used.

DISCUSSION

As indicated in Introduction, several attempts at vitrification of blastoderms and isolated blastomeres were carried out in our laboratory following (with modifications) typical vitrification procedures in 0.25 ml straws initially developed in mammals (Cervera and García-Ximénez, 2003; Leibo and Mazur, 1978; Silvestre *et al.*, 2003; Vatja *et al.*, 1996). The results obtained were discouraging. This fact brought up vitrification in microvolumes as a feasible alternative.

Several supports for cryopreserving in microvolumes have been described in mammals (Lane *et al.*, 1999; Matsumoto *et al.*, 2001), the cryoloop being one of the most widespread in use on intact embryos. In our case, due to the high number of blastomeres from each donor embryo, the cryoloop was not included in experiments because of its limited loading capacity. As an alternative, a new support was designed. This new support provided some advantages. Firstly, small-sized drops could be reduced in volume as low as needed to contribute to vitrification efficiency (by modifying the nylon filament diameter of the support and the inner bore of the disaggregation pipette used to deposit the microdrops over the support). Moreover, all required microdrops which contained all blastomeres from each blastula embryo could be loaded at the same time on a unique nylon support.

Harvey (1983) detected a scale-up effect in relation to isolated blastomeres, clumps and blastoderms, which affected the cryoprotectant permeation and thus cryopreservation success. Because of this, earlier microvolume vitrification assays carried out with the described novel cryopreservation support were initially performed reaching the maximum level of embryo disaggregation (with all cells isolated as ideal) and spent prolonged permeation times (30 min), as many authors recommend (Janik *et al.*, 2000; Robles *et al.*, 2004). Unfortunately, results obtained were unsuccessful. From this moment, and as a real innovative strategy, the requirement of cryoprotectant intracellular permeation was obviated. Two (not independent) arguments supported this innovation; firstly, in vitrification procedures the cryoprotectant concentration can be progressively reduced if cooling speed rate is increased (Mukaida *et al.*,2003); secondly, the small-sized drops (0.25 ml) and the low mass of nylon filament required made their very quick cooling at -196ºC possible, achieving successful vitrification.

This strategy required a quick blastoderm disaggregation in VS, so the complete isolation of blastomeres could not be achieved. Due to this, a relatively high number of clumps of blastomeres (usually 20 to 30 blastomeres and occasionally more than 50) appeared. Surprisingly, most of them were almost intact and with most of their cells alive after thawing. These results are in disagreement with the scale-up problem proposed by Harvey in cryopreservation of zebrafish embryo blastomeres, possibly due to the non-requirement of cryoprotectant cell permeation.

It may be pointed out that blastomere survival assessment was confirmed after observing lobopodia on blastomeres evaluated as alive by trypan blue. The lobopodia movement has already been proposed as a sign of viability in embryo blastomeres at blastula and half epiboly stages (Calvi and Maisse, 1998; Calvi and Maisse, 1999; Edashige *et al.*, 2006).

As indicated previously, methanol was not included in the final assay because VS-MET did not vitrify at 10.20M or even at 15M when blastomeres were added. Methanol is frequently used as a cryoprotectant in fish embryo cryopreservation (Chen and Tian, 2005, Hagedorn *et al.*, 1997a, Robles *et al.*, 2005). In fact, Liu *et al.* (1998) used methanol to vitrify zebrafish embryos in microvolumes. In that work, zebrafish embryos at half epiboly stage were used (a relatively later stage than embryos used in the present work). A 2-step procedure was performed (30 min at 2 M and 10 min at 10 M methanol), then the embryos (with chorion) were vitrified onto a gold electron microscope grid. Within 20 min after thawing, the collapse of blastomere plasma

membrane and rupture of the embryo yolks were observed. Perhaps our successful blastomere vitrification was achieved as a result of combining some factors, such as the short exposure time of the blastomeres to cryoprotectants (25 sec), the very small volume to be vitrified (0.25 ml), no presence of yolk and a relatively high (but not complete) disaggregation of the blastoderm.

Our results indicate that VS-DMSO achieves the lowest lysis and highest survival (on intact blastomeres) rates of all cryoprotectants tested. With the proposed vitrification procedure, combined with VS-DMSO, 20% of living blastomeres from a zebrafish embryo at blastula stage can be recovered after thawing.

Survival rates after cryopreservation by equilibrium procedures reported by different authors at MBT (or nearly) embryo stages are wide ranging. Strüssmann et al. (Strüssmann *et al.*, 1999) reported 20% to 67% survival rates from blastomeres at the 128 to 1024 cell stage in warm water fish species (whiting, medaka and pejerrey). Kusuda et al. cryopreserved chum salmon (Kusuda *et al.*, 2002) and goldfish (Kusuda *et al.*, 2004) blastomeres at blastula stage, obtaining 59.3% and 55% survival rates respectively. Harvey (1983) found as high as 85% survival rate in zebrafish embryo blastomeres at half epiboly stage. Calvi & Maisse cryopreserved trout blastomeres at 6A, 6B, and 6C stages of Ballard's classification (Calvi and Maisse, 1998) and goldfish blastomeres at blastula stage (Calvi and Maisse, 1999), achieving 95% of survival rate in trout and 94% to 96% in goldfish. These widely ranging survival rates are in consequence with different cryopreservation procedures used and with particularities of each fish species. However, high survival rates were also achieved (in a vitrification procedure) in this work (from 73.9% to 93.4%) in comparison with the reported results from these authors. Unfortunately, to our knowledge, no data on survival regarding the initial number of blastomeres from donor fish embryos are available in the works cited. In our case, 20% (400 blastomeres) of the estimated total number of blastomeres from a zebrafish blastula embryo can be recovered after thawing when VS-DMSO is used. This number seems to be enough to assay germ-line chimaerism (Lin *et al.*, 1992; Nakagawa *et al.*, 2002), which would rescue the genetic endowment of such embryos (Kusuda *et al.*, 2004). Because of this, germ-line chimaerism efficiency will be tested by using thawed blastomeres from zebrafish blastula embryos in a subsequent work.

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Table 1: Effect of four vitrification solutions on the number of intact blastomeres and blastomere survival rates after thawing

	VS-ETOH	VS-PROH	VS-DMSO	VS-GLY
Nº of total intact blastomeres	102.0±19.3 [b]	114.7±24.7 [b]	471.8±70.2 [a]	117.5±24.7 [b]
Blastomere survival rate	82.8% [b] (183/221)	87.7% [b] (272/310)	93.4% [a] (1291/1382)	73.9% [c] (150/203)
Blastomere survival/ embryo	3.7% [b]	4.4% [b]	19.3% [a]	3.8% [b]

Rows with different superscripts are statistically different ($p<0.05$)

STUDY 3. Can vitrified zebrafish blastomeres be used to obtain germ-line chimaeras?[*]

Abstract

Blastomere cryopreservation plays an important role in maintaining the genetic diversity for valuable fish species. Recently, an original procedure for blastomere vitrification in zebrafish (*Danio rerio*) was developed in our lab. In the present work, blastomeres from the *wild* strain embryos, previously vitrified-thawed by this procedure, were injected into embryos from the *gold* strain in order to assess their ability to colonise the germ-line of recipient embryos.

The blastomere survival rate at thawing (higher than 90 %) as well as the whole number of recovered blastomeres per donor embryo (around 20 %), were in the ranges previously reported for this vitrification technique. Despite this, only 2 adult chimaeric specimens were finally obtained from a total of 47 injected embryos. Signals of chimaerism were not detected at any stage of development of the chimaeric embryos (somatic chimaerism) or in adulthood (somatic and germ-line chimaerism). In relation to this, difficulties during blastomere insertion are thought to be responsible for the poor results obtained, their aspects being discussed in detail in this work. More improvements to overcome such technical difficulties are needed and, until then, blastomere vitrification may only be of interest for germplasm cryobanking.

Keywords: blastomere, vitrification, germ-line chimaerism, reproductive techniques, zebrafish.

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INTRODUCTION

Due to the difficulty of fish embryo cryopreservation (Chen and Tian, 2005; Edashige *et al.*, 2006; Martínez-Paramo *et al.*, 2009), the cryopreservation of embryo blastomeres constitutes a technical alternative in the establishment of a gene bank to maintain genetic diversity (Calvi and Maisse, 1998) for valuable fish species. Blastomere cryopreservation by equilibrium procedures has been achieved in zebrafish (Huang *et al.*, 2003; Lin *et al.*, 2009) and many other fish species (Calvi and Maisse 1998; Kusuda *et al.*, 2002; Strüssmann *et al.*, 1999), but their use in the laboratory becomes time-consuming, requiring complex and expensive equipment, and in some cases other cryopreservation techniques such as vitrification are more cost-effective. To our knowledge, only one vitrification procedure for zebrafish (*Danio rerio*) blastomeres has been developed, which proposed vitrification in microvolumes (see *Study 2*). Throughout its development, this technique led to recovery after thawing of 20 % of total blastomeres from each donor embryo, with a survival rate of 93.4 %. However, it is necessary to validate the possibilities of this vitrification procedure as an integral biodiversity conservation strategy, which supposes its combination with topical and available reproductive techniques (also for zebrafish) such as nuclear transplant (Huang *et al.*, 2003; see *Study 10*) or chimaerism (Lin *et al.*, 1992). Moreover, and in relation with chimaerism, there are strategies capable of minimising and even cancelling the cell participation of fish recipient embryos in development, such as ionising radiations (Joly *et al.*, 1999). More recently, UV radiation (see *Study 8*) is also of interest because its combination with an embryo manipulation medium of 300 mOsm in chimaerism reaches germ-line chimaerism as high as 50%. This radiation source is also cheaper and easier to use, as no special installations are required for its use. In this sense, the ability of previously vitrified-thawed blastomeres to colonise the germinal ridge lineage of penalised recipient embryos by chimaerism in zebrafish was tested in the present work.

MATERIALS AND METHODS

Animal care and obtaining of embryos

Wild (blastomere donor) and gold (recipient embryo) strains of zebrafish, *Danio rerio*, embryos were used. These two strain populations were maintained separately in 20 L aquariums at a 2:1 female:male ratio 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 (Simão *et al.*, 2007). Both wild and gold embryos were obtained by siphoning as described by Simão et al. (Simão *et al.*, 2007) or by *in vitro* fecundation (Westerfield, 2007).

Dechorionation was carried out when embryos developed near to the mid-blastula transition stage (MBT; 16), by pronase treatment of 1.5 mg/ml in H10 (Hanks' buffered salt solution diluted 10% (v/v) in distilled water; 30 mOsm) followed by washing twice in H10.

Vitrification and thawing of wild-type (donor) blastomeres

Blastomeres from wild dechorionated zebrafish embryos at MBT were vitrified and thawed as described by Cardona-Costa and García-Ximénez (see *Study 2*). The cryoprotectant was 5 M dimethyl sulphoxide (DMSO), prepared in Hanks´ buffered salt solution plus 20% FBS (H-FBS), resulting in the vitrification solution (VS). Briefly, for vitrification, each blastoderm was taken with the minimum volume possible of H-FBS and transferred to an embryological plate with VS. In this plate, the blastoderm was disaggregated into its blastomeres by using a pulled and fire-polished Pasteur pipette, which was approximately 1/5 of the inner diameter size of the zebrafish blastoderm. Immediately, all blastomeres were loaded into the same pulled pipette. Then, VS plus blastomeres were placed as microdrops (the estimated volume of each microdrop was 0.25 µl) on the nylon filament (the physical support), each 2 mm apart. Finally, the physical support was quickly immersed in LN_2. The vitrification procedure took a maximum of 25 sec from the first contact of blastomeres with VS to immersion in LN_2. This procedure was carried out under stereo microscope (90x magnification).

All loops (vitrification supports) were stored in LN_2 for 3 days minimum before thawing.

Direct thawing was performed in Hanks' buffered salt solution without Ca^{++} and Mg^{++} (H-Free) at 25ºC to avoid re-aggregation processes and substrate attachment during micromanipulation. It must be emphasised that high speed thawing is required to achieve high survival rates, so the physical support must be shaken quickly while thawing. Then, it was kept for 30 min before any other manipulation in order to ensure that only intact (non-lysed) blastomeres (dead and alive) were present (4).

UV irradiation of gold-type (recipient) embryos.

According to previous results obtained in our lab (see *Study 8*) related to improving the colonisation of transplanted cells during chimaeric embryo development, gold-type embryos were treated with UV radiation. Briefly, embryos irradiation was carried out almost to MBT stage without dechorionation. They were held in 35 mm-Petri dishes (corning) as containers with system water (no water level was finally standardised due their scant ability to absorb UV radiation; see *Study 2*). A vortex (MS1-IKA) at 200 rpm was used with the aim of homogenising the radiation area during UV exposure. A UV germicide lamp (General Electric, 30W) was used.

Irradiation was carried out at 62 cm of focus-object distance. The radiation dose applied was 0.529 mW/cm^2 and was measured by a USB 4000 (Miniature Fiber Optic Spectrometer; Ocean Optics Inc. First In Photonics, USA).

After irradiation, embryos were kept at room temperature for 30 min and then they were dechorionated (see *Study 2*).

Cell transplantation and assessment of chimaera development ability

Gold embryos partially damaged (lower developmental speed or different visual appearance in developing to that of normal zebrafish embryos; Westerfield, 2007) or totally damaged (arrested development, lysis, blastoderm-yolk disconnection; Pérez-Camps and García-Ximénez) due to previous manipulations (including UV radiation) were removed and finally only intact embryos were included in the experiment as recipients.

In each experimental chimaerism session, the required number of loops were thawed, depending on the initial number of recipient embryos to manipulate. The viability of donor thawed blastomeres was assessed by Trypan blue dye (see *Study 2*)

before each chimaerism session, to assure that the vitrification-thawing procedure was correct. After ending each session, survival assessment of donor blastomeres was again carried out taking the blastomeres remaining from the micromanipulation plate.

Additionally, another blastomere survival evaluation was performed, 2000 to 3000 of thawed blastomeres were grouped in hanging drop for 2 h in some sessions. Then, they were cultured in L-15 medium plus 20% FBS to evaluate their ability of holoblastic morula formation (Calvi and Maisse, 1998).

As an initial step for blastomere transplantation, two specific and separate culture media drops of the same osmolarity (H-Free and Hanks' saline; 300 mOsm), for blastomeres and embryos respectively, were deposited in a 9 cm Petri dish (micromanipulation plate) and covered with mineral oil. Then, *wild* thawed blastomeres were transferred to the H-Free medium drop while recipient embryos remained in the Hanks' saline drop. Micromanipulation was carried out using an inverted microscope (Nikon Eclipse TE200) equipped with Leitz micromanipulators. The outer diameter of the injection micropipette was 60-70 µm (adjusted as closely as possible to the donor cell size, but without compromising their survival ability; see *Study 7*). Due to the high survival rates of thawed blastomeres (90 %) and in order not to exceed the embryo manipulation time, no more than the recommended number of cells (around 50-100 blastomeres) were injected per recipient embryo (Lin *et al.*, 1992), under visual inspection at 200x magnification into the lower part of the blastoderm (Nakagawa and Ueno, 2003). Finally, manipulated embryos were incubated in H10 medium at 28.5 ºC. At day 5 after manipulation, well developed chimaeric larvae were transferred to a 50 L aquarium under standard conditions (Westerfield, 2007) where they were grown to adulthood.

A total of 12 chimaerism sessions were carried out.

The survival and normal development ability of chimaeras were assessed at 1 h, 24 h, 48 h and 5 d post-chimaerism. Somatic and germ-line chimaerism were assessed in adult chimaeras and by the presence of *wild*-type pigmentation in F1 larvae at 48 h of development (Lin *et al.*, 1992). Skin pigmentation of chimaeras at 48h post-chimaerism was not assessed because of the impossibility of observing partial pigmentation marks at this developmental stage in this species.

This experiment only attempted to obtain germ-line chimaeric fry from previously vitrified-thawed blastomeres. In our laboratory, germ-line chimaeras are routinely obtained with an efficacy of 50 % with fresh blastomeres (see *Study 8*), using the same

protocol as outlined here. In this case, chimaeric survival rates are: 80 % at 48 h post-chimaerism, 92 % at day 5, 31 % global survival at day 5 and 7 % to adult stage. These data constituted the benchmark to compare the results of germ-line chimaerism obtained in this work.

RESULTS

Survival assessments of thawed blastomeres

The survival rates of recovered blastomeres after thawing, both before and after each chimaerism session, were in the normal survival range previously established and reported for this vitrification technique (higher than 90%; see *Study 2*). Similar results were observed in the whole number of recovered blastomeres per donor embryo (around 20%).

It was again confirmed that blastomeres were alive throughout the chimaerism session. In addition, the observation of lobopodia was frequent in thawed blastomeres (Fig.1) as well as high blastomere re-aggregation rates, but not holoblastic morulae formation after their culture in hanging drop (Fig.1)

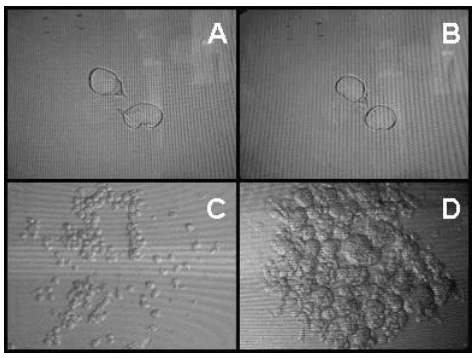

Fig. 1. Two phenomena prove the viability of previously vitrified-thawed zebrafish blastomeres. (A) and (B) show the time evolution (5 min. time-lapse) of lobopodia in two vitrified-thawed zebrafish blastomeres. (C) and (D) show the re-aggregation process time evolution when thawed blastomeres are held in hanging drop.

Chimaera survival and germ-line chimaerism

Chimaera survival and development ability results are presented in Table 1.

Results show a high number of abnormal larvae detected from 24 h to 48 h. However, the number of abnormal larvae observed at 48 h did not increase in further assessments. Another aspect to take into account was the high mortality rate at 24 h (survival rate of 69 %). Moreover, in this assessment, only 9 larvae from the 25 alive

were evaluated as normal. The final consequences that accompanied this double phenomenon were the poor survival assessed on day 5 (5 normal survivor larvae of 47 injected embryos) and the low number of adult chimaeras finally obtained (2 non-pigmented fish out of 47 injected survival embryos). Since it is possible to obtain germ-line chimaerism without the presence of associated pigmentation (Fan *et al.*, 2004; Ma *et al.*, 2001), the 2 non-pigmented adult chimaeras obtained (1 male and 1 female) were mated. After 2 months of mating period, a total of 129 F1 embryos were obtained. None of them finally presented *wild* pigmentation.

DISCUSSION

Technical difficulties that arose during this work were the major reason for the poor results obtained.

Firstly, it was difficult to regroup the thawed blastomeres in a reduced media volume for use in chimaerism, because centrifugation was not used to avoid any excessive mechanical damage, so the blastomeres were regrouped in a watch glass.

In each session, before chimaerism was performed, an increase in cell volume was observed in many thawed blastomeres and, in consequence, major bore sized micropipettes (60-70 µm outer diameter) were used in contrast with the most usual pipette size (50 µm) for zebrafish chimaerism used in our lab (see *Study 8*). Stüssmann and colleagues (1999) detected similar volume increments in fish blastomeres after thawing and suggested they were caused as a consequence of cryoinjuries, but survival tests and observations (Trypan blue dye, lobopodia, cell re-aggregation in hanging drop, substrate attachment) indicated in our case that blastomeres were alive at the micromanipulation step. Another explanation (which does not exclude the previous) may be based on the fact that DMSO induces an increment in cell size by acting on the cytoskeleton (Ashwood-Smith, 1985).

In relation with the re-aggregation process in hanging drop, it did not result in 0holoblastic morula formation, probably as a consequence of using an earlier developmental stage (MBT), and the fact that they were also cryopreserved, as Calvi and Maisse pointed out in Rainbow trout (3).

The observation of continuous and irremediable blastomere attachment to the micromanipulation plate substrate (despite the use of H-Free medium), was probably a consequence of the ionic (Ca^{++} and Mg^{++}) and proteic cell components from lysed

blastomeres at the thawing step. Under these conditions, difficulties in blastomere capture with the increased size micropipette involved, in general, a greater volume of medium load accompanying blastomeres through the micropipette.

Thus, the combination of major bore sized pipettes (60-70 µm) required for volume increased blastomeres and the greater injected volume of media due to the blastomere attachment could suppose greater internal disorders in the recipient embryo affecting development and would explain the high abnormal rates of chimaeric larval development observed at 24 h and 48 h post-chimaerism (see Table1).

As results show, both the global survival and germ-line chimaerism rates obtained in this work (11 % and 0 % respectively) were low when compared with the survival efficiencies (also at 5 days of live) and germ-line contribution achieved routinely in our lab (31 % global survival and 50 % germ-line; see *Study 8*) when the same procedure is used but with non-vitrified blastomeres as previously described.

In general, the results obtained here are poor when compared with those provided by other authors. Also, in many cases these authors reported problems related to technical effects when cryopreserved or fresh blastomeres were used. For example, in goldfish, Kusuda *et al.* (2004) proposed that the low survival rates obtained in chimaerism using cryopreserved blastomeres probably resulted from the contamination of cell suspension with dead cells and DMSO remains in the medium. In that case, the survival rates achieved to adulthood varied from 10 % to 50 %, in contrast with the lower results obtained in the present work (4 %). With an analogous result, Sawant *et al.* (2004) obtained 3 % of well developed larvae at one month of life after performing intraspecies embryonic chimaerism by injecting Rosy barb donor fresh cells into zebrafish recipient embryos. On the other hand, Takeuchi and colleagues (2001) reported a germ-line participation of 32% in rainbow trout by fresh blastomere transplantation, but to accomplish this they designed a special transplant micropipette to achieve more control during cell injection. Despite this, the percentage of hatched larvae (12 %) was similar to our results (11 %).

The appearance of problems in chimaerism experiments has been described regardless of whether isolated fresh blastomeres (Takeuchi *et al.*, 2001), cryopreserved blastomeres (Kusuda *et al.*, 2004) or ESCs (Béjar *et al.*, 2002) were used. Therefore, the final chimaera survival to adulthood is probably limited to a great extent by mechanical damage associated with manipulation during chimaerism and not so much by the possible donor cell injuries previously occurred, although they do exist. In this

sense, Kusuda *et al.* (2004) also detected a lower survival rate in chimaeric embryos transplanted with cryopreserved blastomeres (41.6 %) in comparison with their control group (57.1 %).

Cryopreserved blastomeres are able to colonise many tissue types after being transplanted into recipient embryos (Kusuda *et al.*, 2004). However, in our hands, neither somatic nor germ-line chimaerism was detected after using vitrified-thawed blastomeres as donor cells in the much reduced number of surviving adult specimens.

Whatever the case, the technical success of chimaerism is still limited to date and more efforts related to the technical aspects cited here, as well as those addressed by other authors, must be made to use this interesting reproductive technique efficiently. Until then, blastomere vitrification can only be of interest for germplasm cryobanking.

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Table1. Chimaeric embryo survival and normal developmental rates from mid-blastula transition stage to adulthood.

Initial injected survivor embryos		47		
At 1 h		36	36/47	(77%)
At 24 h	Normal	9	9/25	(36%)
	Abnormal	16		
	Total	25	25/36	(69%)
At 48 h **	Normal	5	5/20	(25%)
	Abnormal	15		
	Total	20	20/25	(80%)
At 5 d **	Normal*	5	5/20	(25%)
Global survival at 5 d **		5	5/47	(11%)
Adult chimaeras **		2	2/47	(4%)

* Growth to adulthood.

** Routine survival results (see *Study 8*) after performing chimaerism with fresh blastomeres, using the same protocol as outlined here are: 80 % at 48 h, 92 % at day 5, 31 % global survival at day 5 and 7 % of adult chimaeras obtaining.

STUDY 4. Effects on cell viability of three zebrafish testicular cell or tissue cryopreservation methods.[*]

Abstract

In this work, three cryopreservation procedures were tested in order to obtain efficiently viable testicular cells after cryopreservation. Testicular cells of *Wild* type zebrafish males were frozen using an equilibrium protocol and testicular tissue fragments were cryopreserved with equilibrium freezing and vitrification procedures. Results showed that vitrification was significantly more efficient than freezing in terms of final cell survival (cell freezing: 14.4%, tissue freezing: 77.4%-85.5%, tissue vitrification: 94%). It must be noted that, in live cells, the presence of pseudopodia was frequently observed, which indicated their spermatogonial nature.

Based on these results, the authors suggest that vitrification, with the subsequent elimination of connective tissue after warming, offers the best combination to rescue live testicular cells as a genetic conservation procedure in zebrafish.

Keywords: cryopreservation, vitrification, testicular tissue, testicular cell, zebrafish.

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INTRODUCTION

In fish, testicular cell transplantation is done on larvae because their immunologic system is still immature, so donor cells can colonize, proliferate and differentiate in recipient testes without immunologic rejection (Takeuchi *et al.* 2003, Okutsu *et al.* 2006a, 2006b and 2007). Pioneer works in fish species employing this technique had essentially focused on *salmonidae* family (Okutsu *et al.* 2006a y 2007). Furthermore, Sakai (2002 and 2006) achieved zebrafish *in vitro* co-culture of Sertoli cells with spermatogonia for *in vitro* spermatozoa production. This goal was also developed in medaka (Hong *et al.* 2004).

In this context, the availability of an efficient cryopreservation technique is an interesting tool, with the aim of rescuing live pluripotent cells from testicular tissue (i.e. spermatogonial stem cells). Several groups have used cryopreservation in different species, both spermatogonia freezing (Izadyar *et al.* 2002, Frederickx et al. 2004) and testicular tissue freezing (Milazzo *et al.* 2008, Song and Silversides 2007). Nevertheless, we did not find any reference in the literature to the possibility of using vitrification as a cryopreservation method. So, in the present work we compare the efficiencies of a vitrification technique and more conventional equilibrium procedures in testicular cell cryopreservation.

MATERIALS AND METHODS

Preparation of testicular cells and tissue

Preparation of testicular cells was based on the procedure described in other species (*salmonidae* family: Takeuchi *et al.* 2003, Okutsu *et al.* 2006a), although for zebrafish adaptation we also followed Sakai (2002, 2006).

Wild type zebrafish males were completely anaesthetised and euthanized in clove oil solution (200 µl/l). Later, testicles were removed, cut into tissue pieces and placed in Hanks' balanced salt solution without calcium and magnesium (Sigma). After initial centrifugation (5 min, 2000 rpm), the tissue pieces were incubated with trypsin-EDTA for 2 hours and blocked with 1 ml of foetal bovine serum (FBS). Then, cells were separated from connective tissue, with the help of two sterile hypodermic needles. Finally, a second centrifugation was done (8 min, 2000 rpm) to recover cells.

The procedure for testicular tissue obtaining was carried out as described above, with trypsin-EDTA treatment only being performed after thawing or warming (equilibrium freezing or vitrification). In addition, two variants were tested for each tissue cryopreservation method: with or without the removal of connective tissue.

Cell freezing

Freezing medium (13.33% dimethyl sulphoxide in Hanks' supplemented with 13.33% FBS) with testicular cells was loaded in conventional plastic straws (0.25 ml) which were frozen in a programmable rate-freezer (Planer Kryo 10 series II) at a rate of -1ºC/min since -70ºC. At this temperature, straws were plunged directly in liquid nitrogen. Thawing was done in 37º C water bath. After 6 s, straw content with cell suspension was poured into a plate with Ca++ and Mg++ free Hanks' solution.

Testicular tissue freezing

Non-trypsinized tissue pieces (1 mm maximum length corresponding to the inner diameter of 0.25 ml straws) were frozen by the same procedure as described for cell freezing (see above). In this case, freezing medium was 20% dimethyl sulphoxide (DMSO) in Hanks' with 20% FBS.

Thawing was done in 37ºC water bath for 6 s. Cryoprotectant dilution medium was Hanks' with 20% FBS (1.5 ml per straw). After 5 min in this solution, tissue fragments were washed twice in Hanks' without calcium and magnesium to remove the cryoprotectant.

Testicular tissue vitrification

The vitrification procedure assayed was previously developed in our laboratory for tissue and cells from skin and it has proven very efficient in different mammalian species (Silvestre *et al.* 2002, 2004) and more recently also in zebrafish (Cardona-Costa *et al.* 2006).

Non-trypsinized small tissue pieces were plunged in vitrification solution.

The vitrification solution contained 20% DMSO, 20% ethylene glycol (EG) and 60% Hanks' balanced salt solution plus 20% FBS with biomycine.

For vitrification, 3-5 small tissue pieces were submerged in 1.0 ml VS and immediately loaded into 0.25 ml plastic straws. Straws were sealed with modelling clay

and plunged vertically into liquid nitrogen. Straw loading took less than 30 s. Straws were stored in liquid nitrogen for 3 days minimum before warming.

For warming, every straw was held horizontally in nitrogen vapour for 10s (15 cm over liquid nitrogen surface) and then warmed by immersion in a 25ºC water bath for 5s. The straw content was expelled into a watch glass. Recovered tissue samples were rinsed for 5 minutes in 1.0 ml 0.125M sucrose in HC-F (Hanks' balanced salt solution plus 20% FBS)to remove cryoprotectants, and then, for a further 5 min, 1.5 ml of HC-F was added in order to reduce the sucrose concentration progressively. Finally, the pieces were transferred to HC-F without sucrose for 5 min and then to Hanks' without calcium and magnesium (Cardona-Costa *et al.*, 2006)

Cell survival assessment after warming

9 straws were thawed in 3 different sessions (3 straws per session) for each group (fresh cell control, cell freezing, tissue freezing and tissue vitrification). Thawing and warming was performed for each cryopreservation procedure as previously indicated. Cell survival assessment was performed as described by Cardona-Costa and García-Ximénez (2007). Briefly, a sample of 20 l with the largest number of cells possible was taken and mounted on a slide. Cell survival rate was established as the number of live cells from the whole amount of intact cells (live plus dead) recovered, by using 0.4% trypan blue (1:1 v/v). All cells, dead and alive, were counted in eight random fields (200 x magnification) and this procedure was repeated at least twice for each sample. All considered fields contained at least one cell (dead or alive).

Statistical analysis

Results were analysed by a Chi-square analysis. When a single degree of freedom was involved, the Yates' correction for continuity was carried out.

RESULTS AND DISCUSSION

Cell freezing was the first cryopreservation procedure assayed in this work. This strategy meant that cells were exposed to the freezing effects after trypsin treatment and, in our case, resulted in a high level of cell mortality (Table 1). These results obtained in zebrafish cannot be extrapolated to other species. In fact, Okutsu *et al.* (2007) achieved a spermatogonial survival rate of 45.4 % after thawing in rainbow trout. One reason

could be the removal of connective tissue by filtration. Furthermore, these authors used a similar cryopreservation procedure (-1ºC/min in a deep freezer to -80ºC), but with the use of ethylene glycol as cryoprotectant in contrast with dimethyl sulphoxide (DMSO) used in the present work.

In the case of testicular tissue cryopreservation, equilibrium freezing and vitrification procedures were tested. The tissue pieces were not trypsinized prior to cryopreservation. Thus, it avoided the negative effects both of trypsin treatment and the mechanical isolation on the cells before being cryopreserved. In this way, cell survival rates in freezing and vitrification were compared and the subsequent possibility of connective tissue removal was also taken into account (Table 2).

The connective tissue retained a significant part of the cells, making cell recovery difficult. This fact was of special importance, but unfortunately no references that compared this effect were found. Whatever the case, the removal of connective tissue did not significantly affect cell survival rates when either vitrification or freezing procedures were used. However, the subsequent elimination of connective tissue after warming made easier the cell handling. Vitrification was significantly more efficient than freezing in terms of final cell survival. Cell survival rates obtained after vitrification of testicular tissue samples were higher than results obtained by other authors with conventional freezing in fish (Okutsu *et al.* 2007) and mammal species (Mouse: Frederickx *et al.* 2004; Calves: Izadyar *et al.* 2002).

Moreover, when cell viability was evaluated, pseudopodia were frequently observed in many live cells, which indicated their spermatogonial condition (Ulvik 1983, Hill and Dobrinski 2006, Okutsu 2006a) and again confirmed their viability. However, the final test which assures the obtaining of spermatogonia and their posterior viability is germline chimaerism.

In conclusion, the availability of an efficient testicular tissue cryopreservation procedure will assure the conservation of samples belonging to different specimens and will make subsequent *in vitro* culture or testicular cell transplantation possible. On the basis of these results, the authors suggest that vitrification with the subsequent elimination of connective tissue after warming offers the best combination to rescue live testicular cells as a genetic conservation procedure in zebrafish.

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Table 1. CELL FREEZING. Cell survival before and after freezing-thawing procedure

	Pre-freezing	Post-thawing	Total
Dead cells	88	487	575
Live cells	143	82	225
Cell Survival rate	143/231 (62%)[a]	82/569 (14.4%)[b]	

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

Table 2. TESTICULAR TISSUE FREEZING AND VITRIFICATION. Cell survival after thawing or warming tissue samples.

	VITRIFICATION		FREEZING	
	Without removing connective tissue	Removing connective tissue	Without removing connective tissue	Removing connective tissue
Dead cells	18	16	30	16
Live cells	293	247	103	94
Cell survival rate	293/311 (94%)[a]	247/263 (94%)[a]	103/133 (77.4%)[b]	94/110 (85.5%)[b]

Percentages with different superscripts show that values are statistically different ($p<0.05$).

EXPERIMENT 5. Testicular cell transplantation into newly hatched larvae in zebrafish.*

Abstract

In mammals, the genetic modification and transplantation of SSCs (spermatogonial stem cells) to obtain germ line chimaeras constitutes a reliable strategy to obtain viable transgenic specimens for one or several predefined genes that can be stably inherited by their progeny. In order to test this possibility in fish, in this work a zebrafish testicular cell transplantation method was developed which permits the insertion of vitrified-warmed testicular cells from adult wild specimens near the genital ridge of newly hatched (48-72 h) *gold* embryos previously penalised by UV radiation at the MBT stage. Injected surviving larvae (176) presented low abnormality and mortality rates, however germ-line participation in surviving adults from previously vitrified-warmed testicular donor cells was not observed. Reasons for these results are discussed, but the effect of some degree of asynchrony between the germ line colonisation ability with transplanted testicular cells and receptivity of the genital ridge timings are proposed as the most plausible cause.

Keywords: vitrification, testicular cell, transplantation, zebrafish.

* This manuscript has been submitted to the journal "*CryoLetters*" with the following reference: Bono-Mestre C, Cardona-Costa J and García-Ximénez F. (2009). Testicular cell transplantation into newly hatched larvae in zebrafish. CryoLetters. (submitted).

INTRODUCTION

Nowadays, it is possible to obtain viable transgenic specimens of one or various predefined genes that can be stably inherited by their progeny (Brunetti *et al*., 2008). In order to achieve this goal, cells must be cultured and transfected *in vitro*. Then, they must participate in the germ line of viable specimens. To this end, three types of transplantation techniques have been developed: chimaerism with embryo stem cells (ESCs) (Stillwell *et al.* 2009) or induced pluripotent stem cells (iPS) (Takashi and Yamanaka 2006, 2007), somatic cell nuclear transfer (Eyestone *et al.* 1999, Indo *et al.* 2009) and the culture and further transplantation of spermatogonial stem cells (SSCs) or primordial germ cells (PGCs) (Takeuchi *et al.* 2003, 2004; Okutsu *et al.* 2007).

The SSC transplantation technique was performed for the first time in 1994 in mice (Brinster and Zimmermann 1994) and has since been extended to different species such as hamsters (Dobrinski *et al.* 1999), goats (Honaramooz *et al.* 2003), rats (Kanatsu-Shinohara *et al.* 2006), sheep (Rodríguez-Sosa *et al.* 2006) and fish (Yano *et al.* 2008).

Spermatogonia transplantation is the introduction of these cells into the testicle of a recipient animal to achieve the spermatogenesis of transplanted cells in this new host (Honaramooz *et al.* 2002) producing functional sperm (Houdebine 2003), as proven by *in vivo* and *in vitro* fertilisation assays in mammals and fish (Hill and Dobrinski, 2006; Sakai, 2002).

SSCs obtained from adults can be transplanted into adult recipients and prepuberal specimens. To date, adult testicular cell transplantation in fish has been performed on early larvae because their immunological system is still immature, so donor cells can colonise, proliferate and differentiate in recipient testes without immunological rejection (Takeuchi *et al.* 2003, Okutsu *et al.* 2006a, 2006b and 2007). Furthermore, in transplanted recipient fish spermatozoa or oocytes can be obtained depending on the recipient sex due to spermatogonial sexual plasticity (Okutsu *et al.* 2006a).

In this technique, the germ line contribution of donor cells can be increased by penalising treatments that diminish or annul the viability of recipient primordial germ cells (Honaranooz *et al.* 2005, Trefil 2006, Izadyar *et al.* 2003), or by the use of triploid sterile fish (Okutsu *et al.* 2007).

Pioneer works in fish species employing this technique had essentially focused on *salmonidae* family (Okutsu et al. 2006a and 2007) and raised important questions related to economical aspects (Takeuchi *et al.*, 2004, Okutsu *et al.* 2006b). Additionally, the use of cryopreserved spermatogonia as donor cells in fish (Avarbock *et al.* 1996) may constitute an interesting strategy for the preservation of genetic resources in teleost fish.

In a previous work, our laboratory developed and tested in zebrafish a testicular tissue vitrification technique that allowed high cell survival rates. As a continuation of that study, the present work assesses the possible integration of vitrified spermatogonial cells in the germ line of zebrafish larvae.

MATERIALS AND METHODS

DONOR CELLS

Preparation of testicular tissue and cells

Adult *wild*- type specimens were used as testicular tissue donors.

Preparation of testicular tissue was based on the procedure described in other species (*salmonidae* family: Takeuchi *et al.* 2003, Okutsu *et al.* 2006a), although for zebrafish adaptation we also followed Sakai (2002, 2006). In this way, males were completely anaesthetised and euthanised in clove oil solution (200 µl/l). Later, testicles were removed, cut into tissue pieces and placed in Hanks' balanced salt solution without calcium and magnesium (Sigma). Then, small pieces of testicular tissue were vitrified as described below.

Vitrification-warming procedure

The vitrification procedure assayed was previously developed in our laboratory for testicular tissue (Bono-Mestre *et al.* 2009). The vitrification solution (VS) contained 20% dimethyl sulphoxide (DMSO), 20% ethylene glycol (EG) and 60% Hanks' balanced salt solution plus 20% FBS (HC-F) and antibiotics.

For vitrification, 3-5 small testicular tissue pieces (non-trypsinised) were submerged in 1.0 ml VS and immediately loaded into 0.25 ml plastic straws. Straws were sealed with modelling clay and plunged vertically into liquid nitrogen. Straw loading took less than 30 s. Straws were stored in LN_2 for 3 days minimum before warming.

For warming, every straw was held horizontally in nitrogen vapour for 10s (15 cm over the liquid nitrogen surface) and then warmed by immersion in a 25ºC water bath for 5s. The straw content was expelled into a watch glass. The recovered tissue samples were rinsed for 5 minutes in Hanks' balanced salt solution plus 20 % FBS (HC-F) with 0.125M sucrose to remove cryoprotectants, and then, for a further 5 min, 1.5 ml of HC-F was added in order to reduce the sucrose concentration progressively. Finally, the testicular tissue pieces were transferred to HC-F without sucrose for 5 min and then to Hanks' without calcium and magnesium (Cardona-Costa *et al.*, 2006).

Cell survival assessment after warming

The warmed tissue pieces were incubated with trypsin-EDTA for 2 hours and the enzymatic action was blocked with 1 ml of foetal bovine serum (FBS). Then, cells were separated from connective tissue, with the aid of two sterile hypodermic needles.

Cell survival assessment was performed as described by Cardona-Costa and García-Ximénez (2007). Briefly, a sample of 20 □l with the largest number of cells possible was taken and mounted on a slide. Cell survival rate was established as the number of live cells from the whole amount of intact cells (live plus dead) recovered, by using 0.4% trypan blue (1:1 v/v). All cells, dead and alive, were counted in eight random fields (200 x magnification) and this procedure was repeated at least twice for each sample. All considered fields contained at least one cell (dead or alive).

RECIPIENT LARVAE

UV irradiation

In accordance with previous results obtained in our lab (Francisco-Simão *et al.*, 2009), *gold*-type (recipient) embryos at MBT stage were treated with UV radiation (General Electric 30W, germicide lamp) for 30 sec in order to improve the colonisation of transplanted cells during chimaeric embryo development. The focus-object distance was standardised at 62 cm and the radiation dose was 0.529 mW/cm^2 measured with a USB 4000 spectrometer (Ocean Optics, USA). Then, penalised embryos were cultured at 28.5 ºC for 2-3 days until they hatched and were used as testicular cell recipients.

TESTICULAR CELL TRANSPLANTATION

Immobilisation of recipient larvae

The larvae immobilisation procedure used was based on that proposed by Nüsslein-Volhard and Dahm (2002) for the observation of larval structures *in vivo* in zebrafish.

Our immobilisation method must consider several requirements. At the moment of transplant, the immobilisation medium must be transparent and allow the larvae respiration for almost 30 minutes (required time for the manipulation of each larvae batch); the immobilisation medium must be isosmolar (300 mOsm kg) with the donor cells to transplant and finally; this medium must allow the transplantation pipette penetration. These requirements were achieved by the use of 15% agarose in Hanks' balanced salt solution.

To reduce the larval motility, they were previously cooled at 4 ºC for 10 minutes in a fridge before the seeding of larvae on the bottom of a embryologic plate covered with agarose-based immobilisation medium and completely covered with agarose medium.

Cell transplantation procedure

Warmed *wild*-type donor cells were transplanted manually with a 50 µm diameter glass micropipette. The micropipette tip was bevelled for easier penetration into the peritoneal cavity where the genital crest is found. Due to the small size of larvae (3.5 mm), the pipette penetration must be precise to avoid damaging vital structures.

This procedure was carried out under stereo microscope (90 x magnification). The number of transplanted cells was higher than 75 per larva.

When transplantation was finished, the manipulation plate was flooded with dechlorinated and decalcified tap water (system water) to facilitate the escaping movements of larvae and larvae were released from agarose with the aid of a stretched and fire polished pipette. Then, larvae were incubated at 28.5 ºC in a plate with system water. Finally, 5 day-old larvae were grown to adulthood in a 50 l tank under standard conditions (Westerfield, 2000).

Germ line chimaerism was assessed in mature specimens. They were mated in groups to test the presence of specimens in the descendents with the skin pigmentation pattern of the *wild* donor strain (Lin *et al*, 1992).

RESULTS

Testicular cells from 12 males were vitrified and stored until transplantation. These cells were injected into 176 larvae during 12 transplant sessions (table 1).

From 122 injected normal larvae that would grow to adulthood, 12 females and 15 males finally reached the mature stage. As was expected, none of them presented somatic skin pigmentation. To assess germ line chimaerism, resultant adult males and females were grouped in 4 mating groups (2♀/3♂, 2♀/5♂, 4♀/3♂, 4♀/4♂) as they reached sexual maturity. From a total of 486 fertilised eggs, 284 survived at 48 hours. However, the skin melanocyte presence of the larvae corresponded to that of the *gold* strain zebrafish (from recipient own cells), that is, they had fewer melanophore-producing cells than normally present in *wild*-type zebrafish donor cells (Saito *et al.* 2008). These larvae were grown until adulthood to reassess their pigmentation pattern. Finally, 85 adults survived and all had *gold* pigmentation.

DISCUSSION

In this work, a methodology for zebrafish testicular cell transplantation was developed. This procedure was efficient from a technical point of view, which supposes larvae inclusion in agarose and manual injection with the use of reduced diameter micropipettes (50 μm). In this respect, the low abnormality and mortality rates in 12 transplant sessions (176 injected larvae) should be noted.

Newly hatched larvae used in this work as recipients have also been employed previously in salmonids (Okutsu *et al.*, 2007; Takeuchi *et al.*, 2004), but Saito (2008) suggested that it is quite likely that the method established in salmonid fish cannot be applied to all other fish species, especially those that have small eggs and larvae compared with salmonids, as is the case of zebrafish. In fact, to our knowledge adult testicular cell transplantation in newly hatched zebrafish embryos has never been performed.

In the present work, no germ line chimaerism was observed from *wild* donor testicular cells. This fact could be due to the heterogeneity of transplanted testicular cells, although Okutsu *et al.* (2006a) obtained almost 25 % colonisation efficiency in rainbow trout transplantation with unsorted testicular cells containing somatic cells, spermatogonia and other testicular germ cells.

Another reason for the negative testicular colonisation might be the cell death caused in the vitrification-warming procedure. Nevertheless, it must be noted that, as observed by Bono-Mestre et al. (2009), the presence of pseudopodia was frequently observed in living cells, both before vitrification and after warming, which indicated their spermatogonial nature (Ulvik 1983, Hill and Dobrinski 2006, Okutsu 2006a), and again confirmed their viability and the possibility of gonadal tropism (Okutsu 2006a).

Gonad development in zebrafish begins during embryogenesis (von Hofsten J and Olsson PE 2005) and it may be that the development stage used was too late because the testicular organogenesis had already finished and because of this, the cell cannot be inserted into testicles. Perhaps the transplant stage (2-3 day larvae) was not the best and earlier embryos might be better recipients.

Another plausible reason that may explain the negative results obtained here could be that the transplanted spermatogonia did not have enough time to colonise the recipient testicular tissue and remained ectopically as a consequence of the rapid larvae development in zebrafish. This is because the colonisation procedure is not immediate, whereas zebrafish gonad development takes place quickly. It could explain why in slowly developing species, such as trout, the gonadal colonisation of transplanted cells is efficient. Perhaps a slower development by temperature reduction (Francisco-Simão *et al.* 2007) could facilitate the required synchrony in zebrafish.

In conclusion, the methodology employed here presents useful technical aspects, but more studies are necessary in order to achieve germ line participation from previously vitrified-warmed testicular cells in zebrafish.

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Table 1. Survival ability of injected (UV radiated) larvae with (vitrified-warmed) testicular cells 1 h after manipulation.

Session number	Injected larvae	Recovered larvae	Dead larvae	Abnormal larvae	Normal larvae
1	5	5 (100 %)	1	0	4 (80%)
2	12	12 (100 %)	1	0	11 (92%)
3	17	17 (100 %)	0	0	17 (100%)
4	8	8 (100 %)	1	1	6 (75%)
5	16	16 (100 %)	2	9	5 (31%)
6	14	14 (100 %)	9	0	5 (36%)
7	13	13 (100 %)	0	0	13 (100%)
8	12	12 (100 %)	4	4	4 (33%)
9	11	11 (100 %)	2	5	4 (36%)
10	23	23 (100 %)	2	4	17 (74%)
11	26	26 (100 %)	5	2	19 (73%)
12	19	19 (100 %)	1	1	17 (89%)
Total	176	176 (100%)	28 (16%)	26 (15%)	122 (69%)

III.2. Chimaerism in zebrafish.

Throughout last years, different chimaerism techniques have been developed for all cellular types used in our lab (blastomeres, testicular cells, blastema), with the exception of epithelial fibroblasts (where the use of NT is required, and also developed in our lab). With respect to chimaerism using blastema cells (Pérez-Camps M. Francisco-Simão M, Cardona-Costa J and García-Ximénez F. Short communication. Evaluation of presumptive caudal fin blastema cells as candidate donors in intraspecies zebrafish (*Danio rerio*) chimaeras. Spanish Journal of Agricultural Research 6(4): 610-614 (2008)), the doctorandus did not participate in a special relevance. For this reason, its inclusion in this thesis was obviated. Otherwise, the doctorandus did actively participate in another three works in order to develop an easy, save and efficient germ-line chimaerism technique for blastomere transplantation.

In the previous part, it was described the chimaerism technique for blastomere and testicular cell transplantation after their cryopreservation. So, in this section there are presented the set of works which lead to an efficient chimaerism technique for germ-line contribution at MBT stage of embryo development. In this sense, a first step was the establishment of an ultraviolet radiation (UV) dose for recipient embryo penalization, aimed to cancelling their primordial germ cell participation in development and in their further gamete production.

Despite the UV penalization protocol was laborious, some interesting observations were recorded (ie: the different zebrafish strains tolerance to UV radiation) and discussed in the manuscript "**Ultraviolet radiation dose to be applied in recipient zebrafish embryos for germ-line chimaerism is strain dependent**". Results obtained were: the establishment of a radiation dose (0.529 mW/cm^2 of 60s and 30s for *wild* or *gold* MBT embryos respectively) to be applied on MBT recipient embryos.

Other interesting and unexpected aspects regarding chimaerism were also detected, particularly those related to osmolar effects on donor cells and recipient embryo survivals when chimaerism was performed in higher or lower osmolar conditions (300 vs 30 mOsm/kg). As known, ideal osmolar conditions for zebrafish intact embryos (30 mOsm/kg) and isolated blastomeres form MBT embryos (300 mOsm/kg) are different. Aimed to looking for the conciliation of both osmolar requirements, there were assayed a series of experiments covered in the manuscript

"**Micromanipulation medium osmolarity compromises zebrafish embryo and cell survival in chimaerism**". Relevant results were obtained, for example the better survival rates when chimaerism is performed in 300 mOsm/kg, the fact that too stretched micropipettes excessively damage donor cells, or no osmolar negative effects on donor cells during transplantation.

The establishment of a reliable germ-line chimaerism technique was finally accomplished in the third work entitled "**Ultraviolet radiation and osmolarity media affect germ-line chimaerism success in zebrafish experiments**", in which all previous main findings were tested together. As result, it was observed that recipient embryo UV radiation and their further manipulation in 300 mOsm/kg medium, achieved as high as 50 % germ-line participation (and 13% somatic chimaerism). Although, an interesting question was not solved: why sexual imbalance to males did appear? Perhaps, the answer is more related to the system and factors of phenotypic sex determination in zebrafish than those related to the chimaerism technique.

- In these three works, the doctorandus contributed in all experimental works (UV radiation, embryo micromanipulation, somatic and germ-line chimaerism assessment) as well as in the manuscript preparation.

STUDY 6. Ultraviolet radiation dose to be applied in recipient zebrafish embryos for germ-line chimaerism is strain dependent.*

Abstract

Germ-line chimaerism is a powerful technique that has proven to be useful to produce viable gametes when transplanted blastomeres colonise the germinal ridges in recipient embryos and obtaining offspring from such transplanted cells. In fish, ionising radiations were commonly used for embryo penalization to cancelling the cell participation of recipient embryos in development and in gamete production. The ultraviolet radiation when compared with other radiation types is cheaper, easier and no special installations are required for its use. So, the aim of this work was to establish the optimal ultraviolet radiation dose to be applied in zebrafish embryos at mid blastula transition stage of development, in order to use them as penalised recipient embryos in futures chimaerism assays. An ultraviolet germicide lamp was used as radiation source (0.529mW/Cm^2). Four exposure levels and three exposure times of ultraviolet radiation were tested. The survival rates obtained with the ND (non dechorionated embryos without lid) group suggested that it could be the optimal exposure level to achieve the objective proposed. With the obtained results, we concluded that this ultraviolet radiation dose for 60s and 30s are optimal parameters to penalise recipient wild and gold strain zebrafish embryos respectively in chimaerism assays but without involving their survival and apparently normal development.

Keywords: UV radiation, chimaerism, strain, embryo penalization, zebrafish**.**

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INTRODUCTION

Germ-line chimaerism is a technique that has proven to be useful to produce viable gametes when transplanted cells colonise the germinal ridges in recipient embryos and, in a final step, to obtain offspring from such transplanted cells.

This technique has been applied in mammals (Rossant *et al.*, 1982), poultry (Naito *et al.*, 1994) and fish (Nilsson and Cloud, 1992; Wakamatsu et al, 1993; Luo and Li, 2003; Sawant et al, 2004), but the success rates are still quite limited, and many efforts have been made to improve them. Strategies usually employed to accomplish this aim have focused on minimising and even cancelling the cell participation of recipient embryos in development and, particularly, in gamete production (Naito *et al.*, 1994) to increase the contribution of transplanted cells in embryo development, especially in establishing the germ line (Carsience et al, 1993). This end can be achieved by chemical treatments such as culture atmosphere, media toxicity by the addition of substances such as ethanol or busulfan (Brown *et al.*, 1979; Swartz, 1980), by physical treatment (Aige-Gil and Simkiss, 1991; Strahle and Jesuthasan, 1993; Ikegami et al, 1997; Li *et al.*, 2001) or by ploidy manipulation (Luo and Li, 2003).

In fish and poultry, ionising radiations were commonly used for embryo penalization (Joly *et al.*, 1999; Li *et al.*, 2002). Gamma, X and laser radiations were also used to inactivate the oocyte and spermatozoa genome to obtain androgenetic or gynogenetic development in fish embryos (Mims and Mc Kinnell, 1971; Graham *et al.*, 1996; Bhise and Khan, 2002). However, the security requirements of facilities, expensive equipment and difficult handling make the use of this type of radiation awkward and dangerous. In contrast, the use of other radiation types, such as ultra violet (UV), could be of interest because it is cheaper, easier and no special installations are required for its use.

As occurred with other ionising radiation types, spermatozoa and oocyte genomic inactivation in fish, but without involving the loss of oocyte activation and subsequent embryo development ability, was also achieved by UV radiation (Ijiri and Egami, 1980)

Despite this, no references related to improved germ-line chimaerism efficiencies in fish by embryo penalization with UV radiation were found. So, the aim of this work was to establish the optimal UV radiation dose to be applied in zebrafish

embryos at mid blastula transition stage (MBT; Westerfield, 2000), in order to use them as penalised recipient embryos in chimaerism assays.

MATERIALS AND METHODS

Animal care and embryo obtaining: Adult zebrafish (*Danio rerio*) from two different strains (*wild* and *gold*-types) were maintained separately in 20 L aquariums and kept in 6:3 proportion (females/males) per aquarium. Both populations were fed with granular food supplemented with recently defrosted hen egg yolk and shrimp meat (Francisco Simão *et al.*, 2007). Embryos obtained by siphoning were washed with conventional tap water on a nylon mesh and selected under a stereo microscope and washed again with tap water. Only well developed and perfectly clean embryos (intact embryos without detritus adhered to the chorion) were kept in dechlorinated and decalcificated tap water (system water) (Westerfield, 2000). No bleaching treatment was applied, but sterilised media and materials were used and aseptic conditions were established. All chemical products and culture media were from Sigma-Aldrich (Madrid, Spain)

Dechorionation:

Dechorionation was carried out at MBT stage of embryo development by pronase treatment (1.5 mg mL^{-1} in Hanks´ buffered salt solution, CH, diluted 10% in volume in distilled water, H10) followed by immersion twice in H10. Embryos that were mechanically damaged (blastoderm-yolk disconnections, yolk injuries, etc.) were discarded and finally only intact embryos were included in the experiments.

Ultraviolet radiation treatment:

Embryos irradiation was carried out nearly to MBT stage. A UV germicide lamp (General Electric, 30W) was used. Irradiation was carried out at 62 cm of focus-object distance. The radiation dose applied was 0.529 mW/cm^2 and was measured by a USB 4000 (Miniature Fiber Optic Spectrometer; Ocean Optics Inc. First In Photonics, USA).

As containers, 35 mm-Petri dishes (corning) with system water for non dechorionated embryos or H10 medium for dechorionated embryos were used. The level of media column was standardised at 6 mm due to the ability of water to absorb

UV radiation. A vortex (MS1-IKA) at 200 rpm was used with the aim of homogenising the radiation area during UV exposure. After irradiation, embryos were kept at room temperature for 30 min and then the remaining groups with chorion were dechorionated and finally damaged embryos from all groups were discarded. So, the initial number of embryos was defined as the whole number of non-lysed embryos 30 min after UV treatment and dechorionation.

Chimaerism technique:

Donor blastomeres (non radiated cells) from *wild* specimens were obtained by blastoderm disaggregation in CH medium without Ca^{++} and Mg^{++}. Briefly, the yolk was removed using two sterile hypodermic needles, and then the embryo was taken to the liquid surface in which the yolk was removed immediately from the embryo due to the surface tension (see *Study 2*).

The chimaerism was performed using a Nikon inverted microscope equipped with two Leitz micromanipulators. During the manipulation process, the embryos were held with a 260 µm outer diameter holding pipette and the cells were picked and injected into the embryos by means of a 50 µm inner diameter microinjection pipette. Two separated drops were deposited in a Petri-dish (90 mm) and covered by mineral oil. One of them contained the isolated blastomeres composed by CH medium without Ca^{++} and Mg^{++}. The other drop was the handling drop, which means the place where the chimaerism was performed and it was composed by HC medium (Pérez-Camps and García-Ximénez, 2008). The number of injected cells per recipient embryo ranged from 50 to 100 cells and they were deposited into the animal pole as was described by Lin *et al.* (1992). Finally, embryos were cultured in 35 mm- cell culture dishes (corning, Sigma) at 28.5ºC for 6 days in H10 (35 mOsm/Kg) to carry out the monitoring and assessments during the experiments.

Embryo viability rates were assessed at 24, 48 and 72 h post-chimaerism. The initial number of manipulated embryos was established as the whole number of micromanipulated embryos after performing the chimaerism.

Experimental design.

In order to define the most favourable level of UV radiation, four different and sequential experiments were carried out. Experiments 1, 2 and 3 were performed using

wild-type embryos because it is the standard type for this species. Finally, the fourth experiment was realised with *gold*-type embryos (see Fig. 1.).

Reciprocal crossing between the two lines used in this study has proven that *wild* type is dominant over *gold* type in terms of body pigmentation (Nagakawa *et al.*, 2002).

Experiment 1.

The aim of the first experiment was to define the UV radiation treatment which involves the highest survival rate at 100% epiboly stage, but the lowest rate at 24 hours, which meant an irreversible damage of endogenous blastomeres but an increase in the capacity of the injected blastomeres (non radiated) to colonise recipient (radiated) embryo. Chorionated and dechorionated embryos were handled as previously described. To this end, four exposure levels of UV radiation were tested: non dechorionated embryos without Petri dish lid (ND) and with lid (ND-L), dechorionated embryos without lid (D) and with lid (D-L). Three exposure times were tested in all exposure levels of UV radiation: 2, 4 and 6 min. Embryo survival rate was evaluated at 100% epiboly stage and at 24 h post-fertilisation.

Experiment 2.

The UV radiation level which caused the highest survival rate at 100% epiboly stage but the lowest at 24 h from experiment 1 was selected for experiment 2. This exposure level was the ND (see results). The aim of the second experiment was to value the survival rates and normal embryo development (at 24 h, 48 h and 72 h) of the ND embryos when they were used as recipients in chimaerism experiences. In this case, the embryos were only radiated for 6 or 2 min (E1 and E2 respectively) and chimaerism assays with *wild* blastomeres were carried out. Two control groups of radiated but non micromanipulated embryos were established: Control 1 (6 min of UV radiation exposure) and Control 2 (2 min of UV radiation exposure).

Experiment 3.

Taking into account the results from experiment 2 (see results), the irradiation time in the ND group was reduced to only 60 s (E3). The chimaerism and its evaluation were carried out as described in experiment 2, but in this case three control groups were established: radiated for 60 s but non micromanipulated (Control 3), non radiated but

micromanipulated (Control 4) and non radiated and non manipulated (Control 5).

Experiment 4.

Wild cells were injected into *gold*-type embryos used as recipients in chimaerism. To prevent a possible difference in sensitivity between *gold* and *wild* embryos, *gold* embryos close to MBT stage were exposed to the radiation for 60 s and also for 30 s (E4 and E5 respectively). As a control, non radiated gold embryos were also used as recipients in chimaerism (Control 6).

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.

RESULTS

Experiment 1.

According to the obtained results, the combination of times and the different exposure levels (chorion and Petri lid) from the ND-L and the D-L groups were discarded due to the high rates of larval survival and the D groups due to the excessive damage at 100% epiboly stage (Table 1). The survival rates obtained with the ND groups suggested that it could be the optimal combination to achieve the objective proposed because the survivals at 100% of epiboly were high but the larval rates (at 24 h post-fertilisation) were low (Table 1). It is important to point out that morphologically abnormal larvae appeared in ND and D groups at 24 h post-fertilisation (body deformity, curly tail, heart dilation, bifid heart, absence of eyes).

Due to no differences were observed in the ND group, in the second experiment only the ND group was considered for testing by chimaerism but testing the two extremes of time (6 and 2 min) of UV radiation exposure.

Experiments 2 and 3.

Regarding the second experiment, no embryo from the ND group developed as normal (Table 2), either when the time of exposure was 6 min or when it was reduced to 2 min. The survival rate was almost null, even when they were not micromanipulated (Table 2). This result means that the transplanted (and non radiated) blastomeres did not reduce the damage caused in the recipient embryos by the UV radiation. For this reason, the UV radiation exposure time was reduced to 60 s in the third experiment (Table 3). In this case, the survival rates were higher than before (Table 2) in both radiated groups (E3 and Control 3), but they were significantly lower than the two non radiated groups (Control 4 and Control 5).

Experiment 4.

The differences observed in the survival rate between the groups exposed at UV radiation for 60 or 30 s reached significant levels at 24 h post-fertilisation. However, the group radiated for 30 s did not differ significantly from the control group (Control 6) in which the embryos were not exposed to the UV radiation, although the survival rate of radiated gold embryos was progressively higher as of 24 h post-fertilisation (Table 4).

Surprisingly, a strong difference was detected in the survival rate between the wild and gold strain embryos subjected to 60 s of UV radiation (Tables 3 and 4), because no normal gold embryo survived 60 s of UV radiation, in contrast to the 40% of radiated wild embryos that developed as normal and reached the larval stage (72 h post-fertilisation) (Tables 3 and 4).

DISCUSSION

Harmful effects produced by UV radiation on embryonic development of teleost fish and other species have been studied for several decades (Ijiri and Egami, 1976; Williams and Smith, 1984). In this way, such effects were employed to establish the most suitable penalization level in zebrafish embryos, with the aim of increasing the transplanted germ cell colonisation in future chimaerism works.

In the first experiment, in accordance with the measuring equipment, it was possible to prove that the Petri lid absorbed 100% of UV radiation. This fact was in concordance with groups radiated with lid (ND-L and D-L), which showed high

survival rates (Table1). In contrast to these results, the groups without lid (ND and D), showed evidence of damage due to the UV radiation treatment, such as paralysation of embryo development and even embryo lysis. Results obtained were not affected by the system water or the H-10 medium that covered the embryos, due to the low level of radiation absorbed by the water column (4,8 mm over the embryos; embryo outer diameter: 1,2 mm). In ND and D groups, the low survival rates showed abnormalities in embryo development at 24h of developmental stage. These phenomena were probably manifested as a consequence of UV radiation, as occurred in other fish species (Loach, *Oryzias latipes*) (Bass and Sistrun, 1997; Fujimoto *et al.*, 2007). The measuring equipment could not assess the possible protecting effect of the embryo chorion, but an immediate protective effect by the chorion was detected in the 100% epiboly stage assessment in ND group. This difference was significantly higher than the dechorionated group (D), although this difference disappeared at 24h post-fertilisation. Although the ND group was finally selected as candidate for Experiment 2 assays, no embryo developed as normal when 2 or 6 min of UV radiation were tested. Akimoto *et al.* (2003) and other authors (Lesser *et al.*, 2001; Bates, 2004) detected intracellular and embryological changes in other species (Urchin *Strongylocentrotus droebachiensis,* Ambystoma maculatum, Molgula pacifica) due to the oxidative damages in different organic compounds (DNA, lipids, proteins, etc.) as a consequence of UV radiation. In fact, Xu *et al.* (2006) observed that UV radiation induced apoptosis in *in vitro* cultured ESCs. These damages could be part of the reasons of our bad results, because no improvement was achieved, even when non radiated cells were injected into these treated embryos.

The low and non-significant differences observed between E3 and Control 3 and also between Control 4 and Control 5 (both non radiated), could be attributed to mechanical handling damage in chimaerism assays. Surprisingly, a great difference related to the sensitivity to UV radiation between wild and gold strain embryos was detected when data from Table 3 and 4 were compared. They showed a clear disadvantage in gold strain embryo development. These results were in agreement with Hyodo-Taguchi (1983), who detected significant differences in several embryo strains of *Oryzias latipes* when they were exposed to UV radiation. Unfortunately, few studies have been reported in fish.

In this way, the significant differences in UV radiation sensitivity between these two zebrafish strains used as recipients are of relevance, because it would condition the

efficiency of germ line chimaerism. This sensitivity difference takes on more importance when chimaerism technique is performed to recover endangered species from a limited source (Nagakawa *et al.*, 2002; Nagakawa and Ueno, 2003).

It is known that, in addition to the above mentioned, different sensitivity levels to radiation among cells in a same embryo were present, Jacquet (2004) observed in mouse that precursor cells of PGCs would be more sensitivity to gamma radiation than others, being a fact of importance in the context of germ line chimaerism.

Taking into account the results obtained from this work, we conclude that an UV radiation dose of 0.529mW/Cm^2 for 30s are optimal parameters to penalise recipient gold strain zebrafish embryos in chimaerism assays but without involving their survival and their normal development.

Nevertheless, the issue of whether the parameters established in this work may finally improve the germ line contribution of transplanted cells remains to be confirmed in a subsequent work.

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Table: 1. Wild zebrafish embryos survival after different times of UV radiation exposure. ND-L: non dechorionated embryos with Petri lid; ND: non dechorionated embryos without lid; D-L: dechorionated embryos with tip; D: dechorionated embryos without lid.

	Time of exposure to UV radiation (min)	Number of initial whole embryos	Number of embryos at 100% of epiboly	Number of embryos at 24h post-fertilisation
ND-L	2	38	36(95%)[a]	30(83%) [a]
	4	37	36(97%)[a]	30(83%)[a]
	6	44	40(91%)[a]	31(78%)[a]
ND	2	38	38(100%)[a]	7(18%)[b]
	4	36	35(97%)[a]	7(20%)[b]
	6	45	45(100%)[a]	2(4%)[b]
D-L	2	23	20(87%)[a]	17(85%)[a]
	4	12	10(83%)[ac]	9(90%)[a]
	6	11	10(91%)[ad]	9(90%)[a]
D	2	20	11(55%)[bcd]	1(9%)[b]
	4	27	14(52%)[bcd]	4(29%)[b]
	6	26	9(35%)[b]	0(0%)[b]

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

Table 2: Wild zebrafish embryos survival of ND group (ND: non dechorionated wild embryos without lid) after 6 or 2 min of UV radiation exposure. Non radiated blastomeres were injected in both experimental groups (E1 and E2). Neither of the control groups (Controls 1 and 2) was subjected to blastomere injection.

		6 min of UV radiation exposure		2 min of UV radiation exposure	
		E1	Control 1	E2	Control 2
Initial whole embryos		39	20	43	43
Whole embryos at 24h post-chimaerism	Normal	0/39 (0%)	0/20 (0%)	0/43 (0%)	0/43 (0%)
	Abnormal	2	-	7	11
Whole embryos at 48h post-chimaerism	Normal	-	-	-	-
	Abnormal	-	-	6	9
Whole embryos at 72h post-chimaerism	Normal	-	-	-	-
	Abnormal	-	-	-	2

Columns are not statistically different ($P \geq 0.05$)

Table 3. Wild zebrafish embryos survival of ND group (ND: non dechorionated wild embryos without lid) after 60s of UV radiation exposure. Non radiated blastomeres were injected in the experimental group (E3) and in the control group 4. Control groups 3 and 5 were not subjected to cell injection.

		60s of UV radiation exposure		Non radiated embryos	
		E3	Control 3	Control 4	Control 5
Initial whole embryos		57	43	48	21
Embryos at 24h post-chimaerism	normal	29/57 (51%) [a]	29/43 (67%) [a]	42/48 (88%) [b]	20/21 (95%)[b]
	abnormal	15	9	7	1
Embryos at 48h post-chimaerism	normal	25/29 (86%)	28/29 (97%)	42/24 (100%)	20/20(100%)
	abnormal	14	6	1	1
Embryos at 72h post-chimaerism	normal	23/25 (92%)	25/28 (89%)	41/42 (98%)	19/20 (95%)
	abnormal	14	9	0	1
Global survival		23/57 (40%)[a]	25/43 (58%) [a]	41/48 (85%) [b]	19/21 (90%)[b]

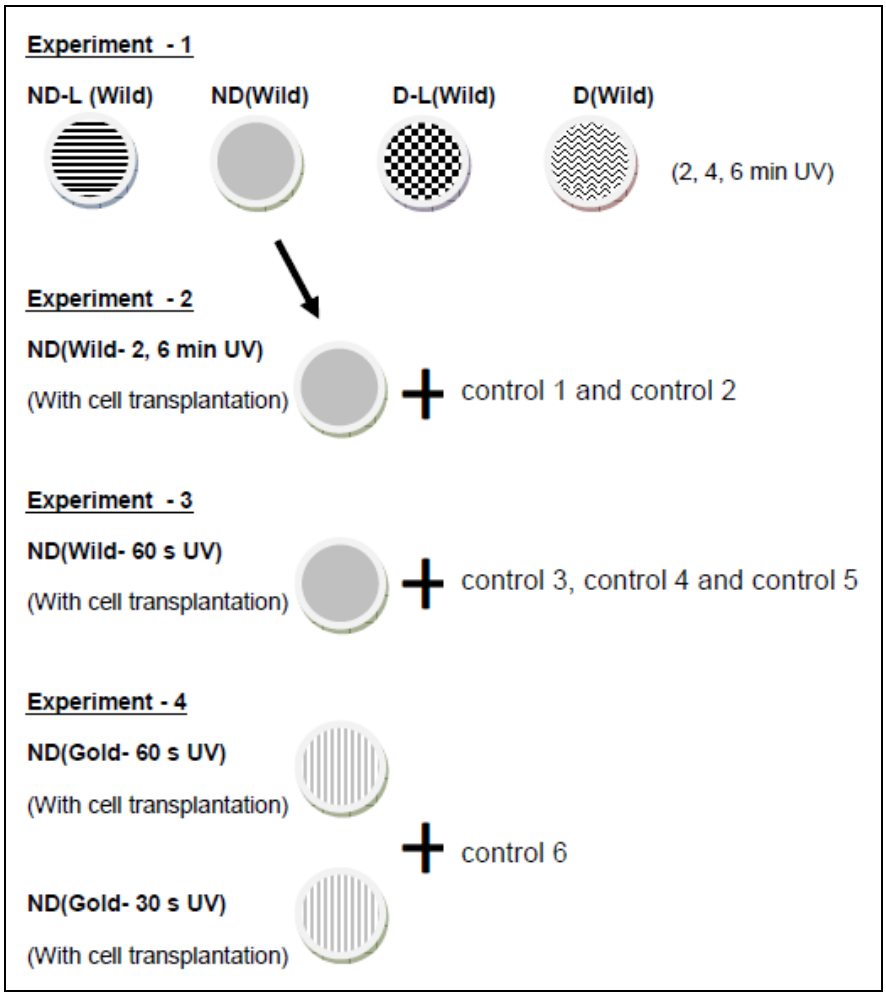
Columns with different superscripts are statistically different ($P<0.05$).

Table 4. Gold zebrafish embryos survival after 60s (E4) or 30s (E5) of UV radiation exposure. The control group (control 6) was not subjected to UV radiation. Wild blastomeres were injected in all three groups.

		Radiated embryos		Non radiated embryos
		E4 (60 s)	E5 (30 s)	Control 6
Initial whole embryos		37	83	31
Embryos at 24h post-chimaerism	normal	0/32(0%)[a]	57/66(86%)[b]	19/26(73%)[b]
	abnormal	4	2	0
Embryos at 48h post-chimaerism	normal	-	55/57(96%)	19/19(100%)
	abnormal	2	3	0
Embryos at 72h post-chimaerism	normal	-	55/55(100%)	19/19(100%)
	abnormal	0	3	0
Global survival		0/37(0%)[a]	55/83(66%)[b]	19/31(61%)[b]

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

Fig. 1. Diagram representing the experimental design.



Legend: ND-L, nondechorionated embryos covered with Petri dish lid; ND, non-dechorionated embryos without Petri dish lid; D-L, dechorionated embryos covered with Petri dish lid; D, dechorionated embryos with out Petri dish lid. Experiment 1: The four groups were divided into three exposure times (2, 4 and 6 min) to UV radiation. Survival rates are shown in Table 1. Experiment 2: Treatment selected (ND) at 24 h. ND group was divided into two exposure times (2 and 6 min) to UV radiation, prior to wild donor cells transplantation. Survival rates are shown in Table 2. Experiment 3: Exposure time of ND group was reduced to 60 s prior to wild donor cells transplantation. Survival rates are shown in Table 3. Experiment 4: Gold embryos were used in ND group and were divided in two exposure times (60 s gold) and (30 s gold) prior to wild donor cells transplantation in both cases. Survival rates are shown in Table 4.

STUDY 7. Micromanipulation Medium Osmolarity Compromises Zebrafish (*Danio rerio*) Embryo and Cell Survival in Chimaerism Experiments.[*]

Abstract

In zebrafish chimaerism experiments, the cell injection can involve intra-embryonic cell lyses by osmolar effects. Moreover, the donor cells can be injured during manipulation due to osmolar changes into the transplant pipette. So, the present study aimed to assess the effects of the manipulation media osmolarity on embryonic survival and donor cell viability.

In Experiment-I, 0.1µl to 0.15µl approximately of an isosmolar solution (300 mOsm) were injected into recipient embryos, which were kept at 300 (E1) or 30 mOsm (E2). Survival at day 1 was significantly higher in E2 group than in E1 (E1: 68 % vs E2: 81%, $p<0.05$) but at 5 days embryo survival of E1 group was slightly higher. In Experiment-II, donor cells from zebrafish embryos were exposed (or not) to a possible osmolarity change (inner pipette medium: 300 mOsm vs external medium: 30 or 300 mOsm) using two different micropipette outer diameters, 40-50 and 60-70 µm. Cell mechanical damages were detected in the 40-50 µm pipette ($p<0.05$), but not by the handling medium osmolarity. Results recommend the use of a 300 mOsm manipulation medium and bore sized pipettes adjusted as closely as possible to the donor cell size.

Keywords: chimaerism, osmolarity, blastomere, embryo, zebrafish.

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INTRODUCTION

In zebrafish, fertilization and subsequent embryo development take place in a hyposmolar environment with respect to the embryo internal osmolarity. The inner osmolarity of a zebrafish embryo is around 300 mOsm, similar to that required for isolated somatic cells or blastomeres in culture, while the suitable osmolarity for an intact embryo is 30 mOsm (Westerfield, 2000). The osmolarity difference between that of the media required by embryos and for isolated embryonic cell culture should be taken into account in chimaerism experiments in zebrafish, where it is necessary to reconcile the osmolar requirements of both cells and embryos. In this way, for chimaerism, common practice involves the employment of two media drops during manipulation, one for donor cells and the other which contains the recipient embryos and where the cell injection is done (Hong et al., 1998; Ma et al., 2001). However, the use of a single medium is also carried out when chimaerism is performed by aspirating cells directly from a donor embryo and introducing them into the recipient embryo. Whatever the case, the cell injection is usually done in a low osmolarity environment (30mOsm) according to the requirements of the intact embryos (Lin et al., 1992; Nakagawa and Ueno, 2003). However, in this case, no attention is paid to the external osmotic barrier breakdown in recipient embryos by the transplant pipette, and its interaction with the low osmolarity of the manipulation medium, which could temporally modify osmolar characteristics of embryos along the pipette entry channel, causing intra-embryonic cell lyses. Moreover, injuries could appear in intermediate steps in which donor cells are loaded into the transplant pipette and injected into the embryo, due the osmolar changes produced by ion exchanges between the inside (300mOsm) and outside (30mOsm) of the pipette opening.

In a previous work, we tested that the survival and further development were not affected by the culture of embryos at mid blastula transition state (MBT) in an isosmolar medium (300 mOsm) for 1 hour (Pérez-Camps and García-Ximénez, 2008) and, no references related to these manipulation particularities in chimaerism were found in the reviewed literature. So, the aim of this study was to assess donor cell viability and chimaeric embryo survival when different manipulation media osmolarities are used in chimaerism assays.

MATERIALS AND METHODS

Care of zebrafish specimens, *Danio rerio*, and embryo collection were carried out as described by Francisco-Simão et al. (2007). Embryos near the mid blastula transition stage (MBT) were dechorionated by pronase (1.5 mg mL-1 in H10), being H10 Hanks' buffered salt solution (HBSS) diluted 10% in distilled water, v/v. Then, dechorionated embryos were washed twice in H10. Damaged embryos were discarded and only intact embryos were used in the experiments.

All chemicals and culture media were from Sigma-Aldrich (Madrid, Spain).

Experimental design and procedures:

Experiment I. Effect of handling medium osmolarity on injected embryo viability

In order to assess embryo viability after chimaerism, two experimental groups were established depending on the medium osmolarity in which recipient embryos were manipulated (E1: HBSS, 300 mOsm; E2: H10, 30 mOsm). Osmolarity was measured using a cryoscopic osmometer (Osmomat 030; Gonotec, Berlin, Germany).

Micromanipulation was carried out using an inverted microscope (Nikon ECLIPSE TE200) equipped with Leitz micromanipulators. Embryos were placed in the different manipulation mediums described and covered by mineral oil. Embryos were held with a 260 μm outer diameter holding pipette. The outer diameter of the injection pipette was set between 50-60 μm. Embryo manipulation consisted of the injection of cell medium (around 0.1 to 0.5 μl of HBSS, 300 mOsm), but not cells, into the marginal zone of the recipient embryo blastoderm (15 embryos per batch).

After manipulation, embryos from the two groups were incubated at 28.5ºC in H10. Two control groups were established, composed of non-injected embryos from each batch of E1 and E2 experimental groups. They remained in the same manipulation medium (C1: 300 mOsm; C2: 30 mOsm) for as long as the injection process took, and then were incubated at 28.5ºC in H10.

At least 125 embryos were injected in both experimental groups, in different sessions.

Embryo survival rate was assessed after 1h post-injection and at larval stage (5 days). Only embryos with no malformations were considered well developed embryos.

Results were analysed using the Chi-square test. When a single degree of freedom was involved, Yates' correction for continuity was performed.

Experiment II. Evaluation of a possible osmolar change effect on the donor cells in chimaerism experiments.

Donor blastomeres were obtained from 5-10 embryos (per batch) at the MBT stage as described by Cardona-Costa and García-Ximénez (see *Study 2*). They were kept in HBSS without Ca^{2+} and Mg^{2+} (cell medium, 300 mOsm).

With the aim of simulating the chimaerism process, blastomeres were exposed (A) or not (B) to an osmolar change, using in each group two different micropipette outer diameters, 40-50 μm (I) and 60-70 μm (II), so four experimental groups were established (A-I, A-II, B-I and B-II).

In the A group, around 50-100 blastomeres were aspirated with a micropipette (I and II) from the cell medium and were held near the micropipette opening. Then, the micropipette was immersed for 10 seconds in H10 medium (30 mOsm) and blastomeres were finally transferred to HBSS (300 mOsm). The B group was not exposed to a possible osmolarity change, so the blastomeres were aspirated with a micropipette (I and II) from the cell medium (300 mOsm) and directly transferred to HBSS medium (300 mOsm).

After each batch, a sample of 20μl from HBSS medium containing the largest number of cells possible was immediately taken and mounted onto a slide. Cell survival rate was established as the number of live cells from intact cells (live plus dead) recovered, using 0.4% trypan blue dye (1:1 v/v). Unfortunately, it was impossible, in our experimental conditions, to identify and quantify the immediate cell lyses because they disappeared rapidly as ghosts. Only intact cells could be assessed at the end of the process. In this way, dead and alive intact cells were counted in eight random fields (100 x magnification) as Cardona-Costa and García-Ximénez (see *Study 2*) described.

For control, a sample of cells that remained the whole time in the initial medium, cell medium, were directly recovered with a Pasteur pipette at the end of each batch.
Results were analysed by the Chi-square analysis. When a single degree of freedom was involved, Yates' correction for continuity was performed.

RESULTS AND DISCUSSION

I. Handling medium osmolarity in chimaerism experiments affects embryonic survival

Results are presented in Table 1.

In the first assessment (1 h post-injection), embryo survival was significantly lower in E1 group, manipulated in the higher osmolarity medium (E1: 68 % vs E2: 81%; $p<0.05$). This low percentage could be attributed to a momentary harmful effect due to a sharp osmolar change (E1 group was transferred from H10 to HBSS medium for manipulation and to H10 again for incubation). Moreover, the survival differences at 1 h between the control groups (C1 and C2) supported this possibility because significant differences in survival rates were also detected among them in favour of H10 medium (C1: 72% vs C2: 91%; $p<0.05$). In addition, mechanical damage during manipulation would enhance this effect. In fact, after injecting 0.1µl to 0.15 µl approximately of HBSS (300 mOsm) into the basal area of the blastoderm, the immediate survival (1 hour) was reduced both in the E1 and E2 experimental groups, when they were compared with the respective control groups not handled (E1: 68% vs C1: 72%; E2: 81% vs C2: 91%), although these differences did not reach levels of significance.

At 5 days post-injection, differences between the E1 and E2 experimental groups did not reach significance levels. Despite this, the survival rate of the first group was slightly higher (E1: 78% vs E2: 69%). This fact implies the possibility that, when embryos are micromanipulated at low osmolarity (E2, 30 mOsm), additional damage could be sustained as consequence of the external osmotic barrier breakdown during the injection process and affect them until their re-sealing. This would permit the osmolar interchange between the internal (300 mOsm) and external (30 mOsm) media, causing intra-embryonic cell lyses. In this case, and as results suggested, these effects are not immediate but presented in later stages of development.

In terms of percentage, the survival rate in the E1 group increased at 5 days post injection (HBSS-1h: 68% vs HBSS-5days: 78%), in contrast with embryos from E2 group, where the survival rate even decreased (H10-1h: 81% vs. H10-5days: 69%). These results suggest that the final survival rate increases when chimaerism is performed in HBSS medium (E1, 300 mOsm) if compared with H10 medium (E2, 30 mOsm). Moreover, the fact of keeping the embryos in HBSS only during

micromanipulation and transferring them to H10 medium for incubation (with the consequent sharp osmolarity change), did not prevent embryos continuing their development without any delay (Pérez-Camps and García-Ximénez, 2008). So, these results are of interest to take into account in future chimaerism works.

II. Handling medium osmolarity in chimaerism experiments does not affect viability of transplanted cells

In fish, to achieve germ-line chimaerism success, the final number of living cells to be inserted into the recipient embryo is an important aspect (Fan *et al.*, 2004; Hong *et al.*, 1998; Ma *et al*., 2001). Therefore, to assess the osmolar effect on cells in chimaerism, two different transplant pipette outer diameters were used in this Experiment II. Results are presented in Table 2.

After assessing the number of live versus intact cells that survived the passage from the cell medium (HBSS without Ca^{2+} and Mg^{2+}) to the two different handling mediums (HBSS or H10), results obtained did not show differences in terms of the use of different handling osmolarities (A: H10, 30 mOsm; B: HBSS, 300 mOsm). In fact, differences related with the possible osmolar damage between the use of two different pipette bore sizes, presumably greater as diameter increased, were not detected. However, cell survival differences between A-I and B-I groups (both with 40-50 μm outer diameter), showed levels of significance ($p<0.05$) when compared with respective control groups. It seems that these cell mortalities, occurring during transfer by pipette, is a consequence of the mechanical damage incurred during aspiration and when cells are expelled through the pipette opening. Thus, they increased when the pipette bore size decreased, favouring in our case the A-II and B-II groups (60-70 μm outer diameter, without significant differences compared with to their control groups). So, higher diameters avoid mechanical damages on donor cells during their manipulation but, at the same time, they can produce higher mechanical embryo disorganization and perhaps osmolar effects into recipient embryos.

In conclusion, the use of a 300 mOsm manipulation medium and bore sized pipettes adjusted as closely as possible to the donor cell size may be recommended as the best combination for chimaerism assays.

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Table 1: Embryo survival rates after being injected with 0.1µl to 0.15 µl approximately of Hanks' saline (300 mOsm) in two different handling media (E1: 300 mOsm; E2: 30 mOsm). Control groups (C1: 300 mOsm; C2: 30 mOsm) were parallel carried out to their respective experimental groups.

Treatment	E1	E2	C1	C2
Initial embryos	142	126	69	79
At 1h	96 (68%)^{c}	102 (81%)^{ab}	50 (72%)^{bc}	72 (91%)^{a}
At 5d	75 (78%) ^{ab}	70 (69%)^{b}	41 (82%)^{ab}	60 (83%)^{a}

Between columns, data with different superscripts are statistically different ($p<0.05$)

Table 2: Cell survival in the cell Control groups (cells maintained in the initial drop of Hanks' saline without Ca^{2+} and Mg^{2+}) and Experimental groups, cells picked up from the initial drop and manipulated on two handling media (A: 30 mOsm; B: 300 mOsm) and with different bore diameters of transplant micropipettes (I: 40-50 µm; II: 60-70 µm).

	Living cells/Intact cells	
(Handling medium/bore size)	Experimental groups	Control groups
A-I (30 /40-50)	93 % (294/315)^{a}	98 % (1512/1542)^{b}
A-II (30 /60-70)	98 % (1162/1186)	97 % (1886/1943)
B-I (300/40-50)	92 % (322/349)^{a}	96 % (1700/1764)^{b}
B-II (300/60-70)	97 % (651/673)	97 % (1488/1530)

Between columns, data with different superscripts are statistically different ($p<0.05$)

STUDY 8. Ultraviolet radiation and handling medium osmolarity affect chimaerism success in zebrafish.*

Abstract

The effects of a predefined ultraviolet radiation dose (0.529 mW/cm^2 for 30s) together with two different micromanipulation media osmolarity (30 mOsm/kg vs 300 mOsm/kg) were tested on embryo survival at different developmental stages and on the somatic (skin) and germ-line chimaerism rates.

Somatic (13 %, 6/47 adults) and germ-line chimaerism (50 % pigmented F1 larvae) were detected only in the UV treated recipient embryos micromanipulated in a 300 mOsm/kg medium. From the results obtained, we concluded that the conditions cited above were the most suitable to improve somatic and germ-line chimaerism rates in zebrafish.

Keywords: germ-line chimaerism, embryo, osmolarity, zebrafish

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INTRODUCTION

The chimaerism technique has proven useful to obtain offspring in which a part of the gametes comes from embryonic cells or embryo stem cells (Ma *et al*., 2001; Fan *et al.*, 2004).

The colonisation of transplanted cells in the presumptive chimaera, both at somatic and germ-line levels, depends on the preponderance of transplanted cells over the recipient cells. To facilitate colonisation, different treatments can be applied with the aim of penalising the recipient embryo or some specific structures, as occurs with PGCs precursors (Carscience *et al.*, 1993). To this end, chemical products (Swartz, 1980) or ionising radiations (Joly et al., 1999; Li et al., 2002) have commonly been used. The use of ultraviolet (UV) radiation is of interest because it is cheaper, easier and less dangerous than other kinds of radiation, and no special installations are required for its use. In our lab, a UV radiation dose to penalise recipient embryos was defined specifically for *wild* and *gold* zebrafish strains (see *Study 6*). On the other hand, in another work, it was observed that the micromanipulation medium osmolarity (30 vs 300 mOsm/kg) could affect recipient embryo survival, possibly due to the rupture of the osmolarity barrier when the microinjection pipette punctured the outer embryonic layer (see *Study 7*).

In this context, the aim of the present work was to test the effect of the previously defined radiation dose (0.529 mW/cm^2 for 30s) together with the micromanipulation medium osmolarity (300 or 30 mOsm/kg) on the germ-line chimaerism efficiency in zebrafish.

MATERIAL AND METHODS

Embryos at the early blastula stage from two different strains (*wild*: donors; *gold*: recipients) were used. All chemical products and culture media were from Sigma-Aldrich (Madrid, Spain)

UV irradiation of gold (recipient) embryos

According to previous results obtained in our lab (see *Study 6*), gold-type embryos were treated with UV radiation to improve the colonisation of transplanted cells during chimaeric embryo development. Briefly, embryo irradiation was carried

out almost to mid blastula transition (MBT) stage without dechorionation. They were held in 35 mm-Petri dishes (corning) as containers with system water. A vortex (MS1-IKA) at 200 rpm was used with the aim of homogenising the radiation area during UV exposure. A UV germicide lamp (General Electric, 30W) was used. Irradiation was carried out at 62 cm of focus-object distance. The radiation dose applied was 0.529 mW/cm^2 and was measured by a USB 4000 (Miniature Fiber Optic Spectrometer; Ocean Optics Inc. First In Photonics, USA). After irradiation, embryos were kept at room temperature for 30 min and then dechorionated.

Chimaerism technique

Donor MBT blastomeres (non radiated cells) from *wild* specimens were obtained by blastoderm disaggregation, in modified Hanks' buffered salt solution (HBSS) medium free of Ca^{++} and Mg^{++} (see *Study 2*).

The chimaerism was performed using a Nikon inverted microscope (Nikon Europe B.V, Badhoevedorp, Netherlands) equipped with two Leitz micromanipulators (Leica, Wetzlar, Germany). Two separated media drops were placed in a Petri-dish (90 mm) and covered by mineral oil. One of them composed of HBSS (300 mOsm/kg) medium free of Ca^{++} and Mg^{++} contained the isolated blastomeres and the other one was the handling medium in which the chimaerism was performed, composed of HBSS (300 mOsm/kg) or HBSS-10% (30 mOsm/kg) medium (Pérez-Camps and García-Ximénez, 2008) depending on the experimental group carried out. During the manipulation process, the cells were picked with a microinjection pipette of 50 µm inner diameter and injected into the embryos held with a 260 µm outer diameter holding pipette. The number of injected cells per recipient embryo ranged from 50 to 100 cells and they were deposited into the animal pole as described by Lin *et al.* (1992); specifically, into the lower part of the blastoderm (Nakagawa and Ueno, 2003). Manipulated embryos were placed in 35 mm cell culture dishes at 28.5ºC for 5 days in HBSS-10% (30 mOsm/kg).

Surviving embryos at 30-60 minutes were considered as the initial number. The further survival rates were assessed at 24h, 48h, 72h and 5 days post-chimaerism. Then, surviving embryos were raised to adulthood where skin pigmentation from adult chimaera and their F1 progeny was registered.

Experimental design

Four experimental groups were established by combining embryo recipient UV radiation (30s UV vs non radiated) and the micromanipulation medium osmolarity (30 vs 300 mOsm/kg). Differences among groups in survival rates of different stages were tested. Somatic and germ-line chimaerism were evaluated in adults.

Overall germ-line chimaerism rate estimation

In this work, the parameter used to compare the osmolarity media and UV effect on germ-line chimaerism rates assumed that all the adult specimens obtained (male and female) in each experimental group provided a single "hermaphrodite and simultaneous" gonad. In this way, depending on the treatment applied, the relative frequency of gametes produced (whether sperm or eggs) from donor cells (*wild*) or from recipient (*gold*) could be estimated by melanocyte presence in the larval skin, because the marker from *wild* specimens (pigmentation) is dominant over *gold* specimens, so only offspring from *gold-gold* gametes pairing will be non pigmented. To this end, embryos from the four experimental groups were collected for 8 weeks and their skin pigmentation (*wild or* gold) was evaluated at 48 h developmental stage (Lin et al., 1992).

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.

RESULT AND DISCUSSION

Results from experiment I are shown in Table I. In the irradiated groups, significant differences were observed in the survival rate at 24h between the two handling media osmolarity (300 mOsm/kg: 50% and 30 mOsm/kg: 36%; $p<0.05$). However, this difference gradually disappeared from the 48h to 5 days stage, and even in the global survival (cumulative survival). Moreover, in the non radiated groups, micromanipulation media osmolarity did not affect embryo survival rates at any developmental stage. These results could indicate that the osmotic shock produced when chimaerism is performed in 30mOsm/kg micromanipulation medium osmolarity does not apparently affect long term survival in a relevant manner (see *Study 7*). As

was expected, global survival (cumulative survival) rates in the irradiated groups were significantly lower than in non-irradiated (see *Study 2*).

It may be noted that the number of males was higher than females in all experimental groups. No interpretation of this observation could be made because the system and factors of phenotypic sex determination are unknown in zebrafish (Saito *et al.*, 2007).

Only 6 (4 males and 2 females) from the 47 total adults showed *wild* skin pigmentation and all of them belonged to the 300mOsm-30s UV experimental group (Table 2). Moreover, it should be emphasised that high rates of *wild* offspring (50%) were also only observed in the 300mOsm-30sUV group (Table 3). This fact confirms that the presence of pigmentation acts as an excellent sign of germ-line chimaerism in zebrafish (Lin et al., 1992). In medaka fish, the gamma irradiation of recipient embryos also favoured the appearance of large pigmentation signals from donor cells and, in parallel, a significant increase in germ-line chimaerism (Joly et al., 1999).

According to the results obtained, the penalization of recipient embryo with a radiation dose of 0.529 mW/cm^2 for 30s together with the manipulation in 300mOsm/kg handling medium osmolarity was the combination that obtained the best somatic and germ-line chimaerism rates.

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Table 1: Survival rates of transplanted embryos. Embryos were irradiated or not (controls) and manipulated in different osmolarity media (30 and 300 mOsm/kg).

	30s of UV		Non radiated	
	300 mOsm/kg	30 mOsm/kg	300 mOsm/kg	30 mOsm/kg
Initial number of embryos	188	232	54	108
Normal embryos at 24h	93/188 (50%)[a]	82/232 (36%)[b]	37/54 (69%)[c]	59/108 (55%)[ac]
(abnormal)	(30)	(38)	(6)	(16)
Normal embryos at 48h	74/93 (80%)[a]	69/82 (84%)[ab]	35/37 (95%)[ab]	55/59 (93%)[b]
(abnormal)	(21)	(31)	(4)	(9)
Normal embryos at 72h	64/74 (87%)	62/69 (90%)	28/35 (80%)	53/55 (96%)
(abnormal)	(22)	(24)	(3)	(5)
Normal embryos at 5 days	59/64 (92%)	54/62 (87%)	26/28 (93%)	48/53 (91%)
(abnormal)	(12)	(23)	(5)	(3)
Global survival at 5 days	(31%)[a] 59/188	(23%)[a] 54/232	(48%)[b] 26/54	(44%)[b] 48/108

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

Table 2: Sex distribution and pigmented marks in adult presumptive chimaeras.

	Nº of final adults fishes			Nº of adults with pigmented marks		
Experimental groups	Total	Male	Female	Total	Male	Female
30 mOsm - Non UV	13	13	0	0	0	0
30 mOsm - 30s UV	11	7	4	0	0	0
300 mOsm - Non UV	9	7	2	0	0	0
300 mOsm - 30s UV	14	8	6	6	4	2
Total	47	74% 35/47	26% 12/47	13% 6/47	67% 4/6	33% 2/6

Table 3: *Wild* skin pigmentation rates in F1 larvae assessed at 48 h.

Experimental groups	Total embryos	Gold pigmented embryos	Wild pigmented embryos
30 mOsm – Non UV(*)	105	105	0(0%)[a]
30 mOsm – 30s UV	25	24	1(4%)[a]
300 mOsm – Non UV	36	36	0(0%)[a]
300 mOsm – 30s UV	494	254	240(50%)[b]

Data in rows with different superscripts are statistically different (P< 0.05)

(*) As all were males, gold females were introduced to make the germ-line chimaerism assessment feasible.

III.3. Nuclear transplant in zebrafish.

This is certainly the most complex part.

The nuclear transplant (NT) technique in zebrafish is very difficult and need of solving several technical barriers throughout its development. Moreover, other complementary techniques are also required. They can vary in difficulty level from the mere obtaining of non-activated sperm and eggs and further *in vitro* fertilization (IVF), or more sophisticated and original techniques as the application of complementary egg activation stimuli by electric pulses, in which the improvement of the NT technique is pursued.

This part is the current working area of the LARB. In this context, the NT technique has also opened a new research front of much expected importance: the possibility of egg "fertilization" with a donor nucleus coming from a primary cell culture of parthenogenetic haploid larvae. To this respect, and because of the major complexity of this part 3, their following description is presented in four differenced sub-sections: firstly, the study of gametes aging and their importance in NT; secondly, the definition of NT techniques. The third sub-part is referred to egg electroactivation stimuli and their possible coupling to NT techniques. Finally, in the fourth sub-part, there are presented all preliminary results of NT using cell cultures of parthenogenetic haploid larvae as nuclei donors.

First:

Throughout initial assays of IVF in our lab, there were observed that temporal limitations in the use of zebrafish gametes after their extraction were very different than those usually recommended in the zebrafish laboratory guides. In order to cover this controversy, there was defined a medium to preserve eggs from activation (Hanks' buffered salt solution plus 1.5g BSA and 0.1g ClNa; 320 mOsm, pH 7.4, storage temperature of 8 ºC) which also served for sperm storage. Results suggested us that the information given in zebrafish guides must be re-evaluated in each laboratory as well as the possible strain difference in the fertilization response. Under our storage conditions, technical results obtained were the efficient storage of sperm at 8 ºC for up to 24 h and the observation of a rapid egg aging (even less than 1 h). The methodology employed and results obtained are described in the manuscript "**Effect of gametes aging on their activation and fertilizability in zebrafish (*Danio rerio*)**". However, it has recently

observed that Chinook salmon ovarian fluid allows the efficient egg storage for up to 5 h at room temperature [Siripattarapravat K, Busta A, Steibel JP, Cibelli J. Characterization and in vitro control of MPF activity in zebrafish eggs (2009). Zebrafish 6(1):97-105]. Unfortunately, this medium is only available to few researchers because it is not commercialized.

- The doctorandus did participate in the development of egg and sperm obtaining, sperm motility assessment and IVF. Moreover, he actively participated in all experimental phases, as well as in the manuscript preparation

Second:

Recent advances in medaka fish (*oryzias latipes*) NT in contrast with the disappointing results given in current zebrafish NT, aimed us to set a double strategy for the zebrafish NT studies to carry out in our lab. On the one hand, the enucleation requirement was obviated because the di- or tetraploid (or mixoploid) condition of reconstructed embryos in medaka fish was favourable to their further development, and also fertility, that is not the case of triploidy. Also, some authors pointed out that female pronucleus could degenerate some cleavages after egg activation. On the other hand, the assessment of different NT procedures where the donor nucleus was inserted before, during or after egg activation was of great interest. In our case, it was firstly followed the strategy of injecting the donor nucleus into the central region of non-activated eggs (due their interest in reprogramming studies). We assumed that injected nucleus would carry together with intracytoplasmic flows (produced throughout egg fertilization or activation) and oriented to animal pole (the desired target of deposition place). It is important to remark that obtained results highlighted that premise, regardless of whether pre- or post-fertilized, or solely activated eggs by water stimulus, obtained embryo developments. The other two NT procedures were also useful under a technical point of view because they did not require the micropile detection, which it is the unique referent in non-activated zebrafish eggs, for further metaphase II plate (female pronucleus) destruction.

The definition of these different NT techniques is described in detail in the manuscript "**Definition of three somatic cell nuclear transplant methods in zebrafish (*Danio rerio*): before, during and after egg activation by sperm fertilization**". Further, another work was performed and presented in "**Transplant of**

adult fibroblast nuclei into the central region of metaphase II eggs resulted in mid blastula transition (MBT) embryos". Among all NT combinations assayed or developed before, only nuclear transplanted eggs reached adult stage when fertilization with (non UV radiated) sperm was used. In this work, results showed that it was passed the lower developmental limit (of similar result to a parthenogenetic activation by water), reaching as high as 20% rates to MBT stage using water as the only activating stimulus. Also, it was observed that a limited egg ageing (2h) penalization did not improve the activation process in zebrafish NT.

If the recent work of Siripattarapravat and colleagues related to nuclear transfer in zebrafish [Siripattarapravat K, Pinmee B, Venta PJ, Chang CC, Cibelli J. Somatic cell nuclear transfer in zebrafish (2009). Nat Methods 6(10):733-735], they only obtained adult clones with employing highly sophisticated techniques (as destruction of metaphase II eggs by laser-ablation technique) and using embryonic cells as nuclear donors, so results obtained in our lab encourages us to deepen this findings.

- The participation level of doctorandus regardless the works related to the NT techniques development, has not only relation to specific aspects (sperm obtaining and *in vitro* fertilization) but also in preparing the manuscripts under equal conditions with the rest of team members.

Third:

As occur in mammals, the limited developmental rates of nuclear transplanted eggs in zebrafish are (in part) consequence of an activation deficit stimulus of eggs. The group of Cibelli [Siripattarapravat K, Pinmee B, Venta PJ, Chang CC, Cibelli J. (2009). Somatic cell nuclear transfer in zebrafish. Nat Methods 6(10):733-735] obtained a complete embryo development to adulthood in zebrafish with the only activating stimulus of system water. In our case and others, the unique activating stimulus whereby it is observed a complete development is that promoted by sperm (either genetically inactivated or not). However, sperm stimulus is solely applicable to non activated eggs and only one time. The electric stimulus overcomes this limitation, therefore acquiring special interest. In our case, in the manuscript "**Electroactivation of zebrafish (*Danio rerio*) eggs**" it is shown the strategy followed for the definition of an egg electroactivation stimulus: on the one hand, the use of ionic pulsing medium, system water, due it was the optimal medium of activated eggs and embryos. On the

other hand, we tried to extend the electric stimuli throughout the zygotic stage until the first cleavage.

The obtained results showed that the established electric stimulus sequence (1 electrical DC pulse of 5.4V and 20µs duration at 0, 10, 20 min post- egg activation in water) was efficient in terms of egg activation and to begin parthenogenetic development, reaching some cleavages apparently normal in first instance, although no normal development was finally achieved. This electric treatment was proposed as the most suitable for non-micromanipulated eggs. However, when it was applied to nuclear transplanted eggs, produced high lysis rates (near to 100 %) and thus forcing us to the future re-definition of electric pulses to specifically apply them together with NT techniques. It is no doubt that the intensity reduction of the applied stimulus is needed.

The different procedures tested and their obtained results are discussed in detail in the manuscript "**Comparison of different activation stimuli efficiency in zebrafish nuclear transplant**". Moreover, that work reported other useful observations, for example the obtaining of intermediate embryonic developments with radiated sperm between those reached by non-radiated sperm, and those observed by the parthenogenetic activation exclusively induced by water stimulus.

- The procedure of an egg electroactivation technique came from the development of a doctorandus idea. Throughout its development, the doctorandus did participate in a major extent. Regarding the participation level of doctorandus in the subsequent work, related to different activation stimuli efficiency in NT, has not only relation to specific aspects (sperm obtaining and *in vitro* fertilization) but also in preparing the manuscripts with the rest of team members in a lesser extent.

Fourth:

As said before, a newly research front has been recently opened in our lab: the use of primary cell cultures from haploid parthenogenetic larvae as donor nuclei in NT.

Preliminary results are presented in the manuscript entitled **"Reconstruction of heteroparental gynogenetic diploid condition by nuclear transplant in zebrafish: preliminary results"**. In this work, it is carefully described how haploid somatic cells from primary cultures derived from gynogenetic larvae at 24h stage were obtained. Then, their nuclear transplantation into non-enucleated metaphase II oocytes and early

zygotes, aimed the study of the reconstruction ability, in zebrafish, of the heteroparental gynogenetic diploid condition.

As important preliminary results, to highlight that 27% of reconstructed embryos reached the 100% epiboly stage when early zygotes were used as recipients. From them, 2 embryos developed to 24h and 1 survived to 5 days, although it showed morphologic abnormalities. These preliminary results obtained allow suggesting the zebrafish as a model system for imprinting studies.

- This original and interesting idea was initially proposed by the doctorandus, although its following development also required the participation of the whole research team. In this sense, the development of genetically inactivation sperm technique to obtain gynogenetic haploid embryos, their further identification by morphologic indicators at 24 h, the obtaining of primary cell cultures from these haploid embryos and the NT technique, were result of the whole collaboration of LARB members.

STUDY 9. Effect of gametes aging on their activation and fertilizability in febrafish (*Danio rerio*).[*]

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.

Keywords: IVF, aging, gametes, zebrafish.

[*] This paper has been published in the journal "*Zebrafish*" with the following reference: Cardona-Costa J, Pérez-Camps M, García-Ximénez F and Espinós FJ. (2009). Effect of Gametes Aging on their Activation and Fertilizability in Zebrafish (*Danio rerio*). Zebrafish 6(1): 93-95.

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, 2007) 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 (Ransom and Zon, 1999; Sakai, 1997; Nüsslein-Volhard and Dahm, 2002; Westerfield, 2007) particularly in NT protocols.

MATERIALS AND METHODS

Animal care and obtaining of gametes

Zebrafish (Danio *rerio*) specimens from the gold strain were maintained in 20 L aquariums under standard conditions (Westerfield, 2007) 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 (Grush *et al.*, 2004; Mylonas *et al.*, 2005) in dechlorinated and decalcified tap water (system water; Westerfield, 2007). 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 (2007) 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, 2007), 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.

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.

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.

RESULTS AND DISCUSSION

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

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 (2007) 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.

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 30ºC 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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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 ($p<0.05$)

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 (49.1%) [a]	44/189 (23.3%) [b]	5/272 (1.8%) [c]	0/49 (0%)[c]	0/18 (0%)[c]
Larvae 24h	89/267 (33.3%) [a]	18/189 (9.5%) [b]	2/272 (0.7%) [c]	0/49 (0%)[bc]	0/18 (0%)[bc]
Larvae 5 d	55/267 (20.6%) [a]	11/189 (5.8%) [b]	2/272 (0.7%)[c]	0/49 (0%)[bc]	0/18 (0%)[bc]

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

STUDY 10. Definition of three somatic adult cell nuclear transplant methods in zebrafish (*Danio rerio*): before, during and after egg activation by sperm fertilization. [*]

Abstract

Zebrafish somatic nuclear transplant has only been attempted using pre-activated eggs. In this work three methods to carry out the nuclear transplant using adult cells before, during and after the egg activation/fertilization were developed in zebrafish with the aim to be used in reprogramming studies. The donor nucleus from somatic adult cells was inserted: (method A) in the central region of the egg and subsequently fertilized; (method B) in the incipient animal pole at the same time that the egg was fertilized and; (method C) in the completely defined animal pole after the fertilization. Larval and adult specimens were obtained using the three methods. Technical aspects related to temperature conditions, media required, egg activation/fertilization, postovulatory time of the transplant, egg aging, place of the donor nucleus injection in each methodology are presented. In conclusion, the technical approach developed in this work can be used in reprogramming studies

Keywords: Nuclear Transplant, Microinjection, Egg activation, Fertilization, Zebrafish.

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INTRODUCTION

After 1952, when Briggs and King obtained normal hatched tadpoles by blastomere nuclear transfer, nuclear transplant (NT) technology began to be developed for reprogramming studies (Kikyo *et al.*, 2000, Wade and Kikyo, 2002; Li, 2002, Giraldo *et al.*, 2008). Mammalian cloning by nuclear transfer has been successfully achieved in several species (Wilmut *et al*., 1997, Wakayama *et al.*, 1998, Byrne *et al.*, 2007, French *et al.*, 2008) with varied somatic cell types as donors (Campbell *et al.*, 1996; Wilmut *et al.*, 1997, Wakayama *et al.*, 1998, Shiga *et al.*, 1999).

Although fish cloning is less developed, several recent works in medaka have been reported, in which both blastomeres (Bubenshchikova *et al.*, 2005) and somatic larval and adult cells (Bubenshchikova *et al.*, 2005, Bubenshchikova *et al*., 2007, Kaftanovskaya *et al.*, 2007) were used as donors, and non enucleated and activated eggs were used as recipients in all cases. In this species, embryonic nuclear transplants using functionally enucleated and non activated eggs have been achieved only with blastomeres as nuclei donors (Wakamatsu *et al*., 2001).

On the contrary, in zebrafish, the first successful embryonic (10-15 somites) somatic cloning by nuclear transplant with mechanically enucleated and previously activated eggs was described by Huang in 2003. Since this work and to date, to our knowledge, no additional improvement in nuclear transplant techniques in zebrafish has been published by these or any other authors.

To date, due to technical (Nüsslein-Volhard and Dahm, 2002) or biological (Westerfield, 2000) limitations, fish somatic nuclear transplant in these two laboratory species with somatic embryonic (10-15 somites; Huang et al., 2003) or adult cells (Bubenshchikova *et al.*, 2007) has only been attempted using pre-activated eggs as recipients. These limitations have hindered the study of the reprogramming effects of cytoplasmic factors characteristic of the metaphase II status in the oocyte, the effects of the synchrony degree between activation and nuclear transplant, egg aging, or the previous donor cell reprogramming treatments. This is not the case in mammals, in which these reprogramming factors have been studied because somatic adult nuclear transplant has been more easily carried out before, at the same time and after oocyte activation (Cambell *et al.*, 1996; Wilmut *et al*., 1997).

Taking into account that zebrafish is a powerful genetic and developmental model system whose genome has already been sequenced, the aim of this work was to

develop three methods to enable nuclear transplant to be carried out using adult cells prior, simultaneously or posterior to the egg activation/fertilization in zebrafish to be used in reprogramming studies.

MATERIAL AND METHODS

Care and maintenance of zebrafish colony.

Two zebrafish (*Danio rerio*) colonies (*wild* and *gold* strains) were established in our laboratory from specimens purchased in a specialized establishment and kept in closed reproduction for five years. Adult zebrafish were kept in 20 L tanks in a 2:1 ratio (females/males) and fed on granular food supplemented with recently defrosted hen egg yolk and shrimp meat (Simao *et al*., 2007). The light cycle was regulated at 14h light/10h dark.

Non activated eggs and sperm collection.

Eggs were collected after evaluating the sexual behavior of both gold strain males and females at dawn. Only females that manifested this behavior were anesthetized in an oil clove solution (100 µL in 1L of dechlorinated and decalcified water: system water) for a few minutes and the eggs were obtained by gentle extrusion of the ovary. It is important to prevent eggs coming into contact with fresh water, because they activate immediately. Only good eggs (yellow and translucent color) were kept in Hanks' buffered salt solution supplemented with 1.5% (v/v) of BSA and 0.1 g of NaCl/100 cc of Hanks' medium (egg medium; ph: 7.4 ;osmolarity: 310-320 mOsm) at 8ºC of temperature until their use (1h and 30 minutes as maximum time).

The *gold* zebrafish males that showed reproductive behavior were also anesthetized as described before. The abdominal region was gently pressed while the sperm was being recovered from the genital pore in individually glass microcapillaries (1×90 mm Narishige Scientific Instrument Lab.). A pool from 2-3 different males (0.5-2 µL/male) was diluted in 200 µL of egg medium, which can also keep the sperm in a non activated status and then, the dilution was stored at 8ºC until use.

In vitro fertilization.

In zebrafish, the eggs quickly lose their postovulatory ability to be fertilized (90 min). Moreover, the time between the complete egg activation and in vivo fertilization

is extremely short (seconds) in zebrafish (Nüsslein-Volhard and Dahm, 2002). Non activated eggs and sperm were mixed in egg medium and stored at 8ºC until fertilization, whatever the nuclear transplant method (see experimental design). To activate both gametes, 1 mL of system water at room temperature was added to the egg-sperm mixture. After 2-3 min, time required for the fertilization in zebrafish, the 35-mm Petri-dish was full filled with water system for achieving well developing embryos. Further culture was done at 28 ºC.

Primary culture and somatic cell collection.

Somatic cells used as nuclei donor came from *wild* zebrafish caudal fin primary cultures. The tissue was obtained by caudal fin amputation of adult specimens after being anesthetized in oil clove solution. The tissue was cleaned with a 0.2% bleach solution for 2 min, then washed twice in 10% Hanks´ buffered salt solution (H10) and then each tissue fragments were plated individually in a 35 mm Petri-dish (corning). Next, the tissue was incubated in Leibovitz medium supplemented with 20% of FBS and 0.036 g/L of glutamine (L15-FBS) at 28.5ºC (Westerfield, 2000).

Before use, donor cells were incubated in Hanks´ buffered salt solution without Ca^{2+} and Mg^{2+} at room temperature for 30 minutes before performing the nuclear transplant. No additional detachment treatment was realized. Once the cells had come off the substrate, L15-FBS was added and the cell dish was preserved at 5ºC throughout the daily experimental session.

Somatic cell nuclear transplant equipment.

The nuclear transplant was performed using a Nikon inverted microscope equipped with two Leitz micromanipulators. During the manipulation process, the non-dechorionated eggs were held with a 260 µm outer diameter holding pipette and the cells were picked, lysed and injected into the eggs by means of a 10-12 µm inner diameter microinjection pipette. The microinjection pipette was fire polished, beveled and sharpened.

To perform the nuclear transplant, two separated drops were deposited in a Petri-dish (90 mm) and covered by mineral oil. One of them contained the donor somatic cells and was composed by L-15-FBS medium (300 mOsm). The other drop was the handling drop, which means the place where the nuclear transplant was performed, so

the medium was the correspondent depending on the nuclear transplant method tested in each case (see below).

The donor cell was picked up and lysed by aspiration with the injection micropipette before injection. The exact place where the cellular content is to be deposited was dependent on the nuclear transplant method tested in each case (see below).

Nuclear transplant was performed at different temperatures depending on the nuclear transplant method (see below).

Nuclear transplant methods.

In order to carry out the NT whatever the status of the egg activation, three NT methods were developed in which the somatic nuclear transplant was performed prior, simultaneous or posterior to the egg activation by the spermatozoa. Since the aim of this work was to establish these methods technically and they were independently performed, no comparison of their technical efficiencies was made.

Method A: nuclear transplant prior to the egg activation/fertilization.

The somatic cell nucleus was inserted in the central region of the egg. To prevent egg activation, the transplant was performed in a handling drop composed of egg medium and the micromanipulation area was cooled down to 8 ºC. This temperature around the handling zone was reached by air cooled with N_2l. Then, transplanted eggs were individually in vitro fertilized and cultured at 28.5ºC in system water (Westerfield, 2000).

Method B: nuclear transplant simultaneously to the egg activation/fertilization.

In this case, previously mixed non activated eggs and sperm were kept at 8ºC and individually deposited in the handling drop containing system water so that gametes were activated and fertilized. The micromanipulation area was not cooled (room temperature). The donor nucleus was injected in the incipient animal pole, just where zygote nucleus was being constituted (Wolenski and Hart, 1987). The reconstructed embryos were incubated under the same described conditions.

Method C: nuclear transplant posterior to the egg activation/fertilization

In order to carry out the NT after fertilization, eggs and sperm were previously mixed and activated, then fertilized at room temperature as described in the second technique but, in this case, the NT was realized a few minutes after fertilization, just the time required for visualizing the completely defined animal pole. After injecting the

donor nucleus in the animal pole, reconstructed embryos were incubated at the same above described conditions.

Experimental design.

The three techniques tested (A, B and C) were not carried out simultaneously, but were developed and assessed independently. In the three cases, in order to evaluate how post-ovulatory aging affects reconstructed embryo survival, two batches consisting of 3-5 eggs each were transplanted sequentially and compared in each session (A1, A2; B1, B2; C1, C2). Overall, the length of each experimental session did not exceed up 90 min in any case, the maximum time for efficient egg fertilization (Nüsslein-Volhard and Dahm, 2002). In this way, A1, B1 and C1 were manipulated during the first 45 min and A2, B2 and C2 during the last 45 min. A non-manipulated control group was fertilized at the end of each experimental session, at 90 min (CA, CB and CC) to test the egg ability to be fertilized at this time.

Given that the aim of the present work was mainly technical, only the embryo and larval survival rates of reconstructed embryos from the three techniques (A, B and C) were evaluated at different developmental stages: at mid blastula transition (MBT) stage (2h after NT), at 50% epiboly stage (7h after NT), at 24h post-NT, at 48h post-NT and at larval stage (5 days after NT) (Westerfield, 2000). Moreover, at 24h, 48h and at 5 days post-NT, normal and abnormal development was registered. In the non-manipulated control group, only the fertilization rate was evaluated by the survival rate at MBT stage.

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

RESULTS AND DISCUSSION

Three methodologies for zebrafish somatic nuclear transplant prior, simultaneously and posterior to the egg activation/fertilization in their technical aspects have been established in our laboratory. All three were developed and evaluated using non irradiated eggs activated/fertilized by non irradiated spermatozoa. In this way, the effects on survival and further embryo and larval development can be attributed exclusively to the transplant methodology employed, because the background noise due

to the exigency of the spermatozoa for egg activation in zebrafish (Lee *et al.*, 1999) and the developmental limitations caused by a haploid condition (Nüsslein-Volhard and Dahm, 2002) would not exist in this case. On the subject, it has to taken into account that in these fish species, the tri-or tetraploid condition, which could occasionally be derived from the addition of a somatic nucleus to the resident zygote pronuclei, does not affect the embryonic, and even larval, development in a relevant way (Diter et al., 1988; Peruzzi and Chatain, 2003).

As a first general comment regarding the efficiency of the in vivo artificial collection of ovarian oocytes, the sexual behavior synchrony showed by the separate fish colonies must be pointed out, in such a way that a large number of eggs could be collected in some sessions whereas no eggs might be obtained in others. Another relevant consideration concerns the fact that MBT nuclear transplant embryos were obtained in the great majority of the daily experimental sessions, whatever the transplant method used.

A technical advantage common to the three methodologies developed in our laboratory was the avoidance of previous de-chorionation. In fish nuclear transplant, when the oocyte is activated but not enucleated, the donor nucleus is usually inserted in the perinuclear region of the oocyte, the closest as possible to the female nucleus, which is located in the cytoplasm subjacent to the plasma membrane under the micropyle (Amance and Iyengar, 1990). In various teleost species, such as medaka, catfish or tilapia, the animal pole position can easily be detected in pre-activated oocytes because the micropyle can be visualized at this stage, although, unfortunately, this is not the case in zebrafish (Poleo *et al.*, 2001).

One possible way to obviate such biological difficulty in zebrafish was attempted in method A, in which the somatic cell nucleus was inserted in the central region of the egg. This point of transplant was chosen because, in terms of probability, the central region will be closer to the female nucleus, whatever its real localization. So, the donor nucleus will be more likely to migrate to the microvillae cluster in animal pole, the place where the fertilization occurs (Wolenski and Hart, 1987), through the cytoplasmic flows together with the pronuclei at the time of the activation.

The earliest manipulated group (A1) reached significant higher survival rates compared with the most aged group (A2) both at epiboly and 24h stage (Table 1).

However, in more advanced stages (48h and larval stage), the observed differences did not reach significant levels, probably due to the low number of embryos

that developed to these stages. The egg control group (CA) fertilized at the end of each experimental session showed MBT rates equal or higher than the two experimental timing groups, indicating the maintenance of the egg fertilization ability until the end of the transplant session. Anyway, the larval survival rate was 7% (A1:6 larvae from 82 manipulated) in the first group and 5% (6 larvae from 132 manipulated) taking into account the overall two groups.

It must be underlined that to cool down to 8ºC the temperature of the micromanipulation area was decisive to maintain the egg in a non activated state during transplant.

This initial strategy permits the impregnation of the donor nucleus in the reprogramming factors present in the egg at metaphase stage, and in further experiments the effect of different times of donor nucleus impregnation before the activation will be tested.

In method B, the nuclear transplant and fertilization were performed at the same time, which meant transplanting the donor nucleus while the egg was activating. Egg activation and fertilization are both marked by elevation of the chorion and a dramatic reorganization of the yolk cytoplasm. In this way, the animal pole is segregated through the place where the female nucleus will be located (Wolenski and Hart, 1987). So, this enabled detection of the incipient animal pole to deposit the somatic nucleus in the female perinuclear region.

The survival rate differences between the first (B1) and second group (B2) did not reach significant levels in any assessment. However, it must be pointed out that these differences decreased over time and the survival rates were finally similar at 48 h stage (B1: 19% vs B2: 20%). In this case, the fertilization rate of the final control group (CB) was lower than the Total B, even than the B2, which involved an obvious effect of the egg aging and a very slightly negative effect of the assayed nuclear transplant technique.

When the NT was performed posterior to the egg fertilization, method C, the variability in the time required for showing the perivitelline space as an activation signal should be pointed out. This represented a critical point due to the technical difficulty in fixing the egg with the holding pipette because the egg rotated inside the chorion while this space was increasing. With the activation, the micropyle can be more easier detected but, a few seconds after the fertilization, the chorion hardened and the micropyle sealed (Poleo *et al.*, 2001). This made it very difficult to insert the donor

nucleus through this point even if the microinjection pipette was fire polished, beveled and sharpened, as in our case. A possibility to obviate this difficulty could be to de-chorionate the egg after fertilization but, this technique is time-consuming and the first cleavage is very early (minutes) in zebrafish. Moreover, the reduction of the temperature to arrest development before the MBT stage involves embryo lethality (Francisco-Simao *et al.*, 2007).

Regarding the survival differences between both handling groups (C1 and C2), it should be emphasized that, as in the technique B, the differences observed did not reach significant levels in any case. However, these differences were relevant, ranged from a differential of 10% at MBT stage to up 15 points at the larval stage. The explanation for not reaching significance level could be the low number of surviving embryos that developed to this stage owing to the aforementioned technical difficulty. The survival rate of the control group (CC), compared with the C1 and C2 groups, showed again a considerable egg aging effect, which means that the time for performing the NT in zebrafish must be shorter in order to avoid (or minimize) such a pronounced negative effect.

Adult specimens showed *gold* phenotype. This fact does not discard a possible mosaicism or that the reconstructed embryos with the donor nucleus incorporated more efficiently did not reach adult stages. However, it has to be into account that the nuclear fate of the transplanted nuclei was not analyzed because, as mentioned previously, the main aim of this work was to establish three nuclear transplant protocols in zebrafish by a technical approach. By this way, after the establishment of these three techniques presented, the nuclear fate will be studied, as well as its integration degree and form in the specimens.

So, the reasonable technical efficiencies achieved in the present work make the use of these three methods interesting for future reprogramming studies by nuclear transplant in this species.

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Table1. Method A: survival rates of nuclear transplant prior to the egg activation/fertilization.

		A1	**A2**	**Total A**	**CA**
Nº of transplanted eggs		82	50	132	301
Nº of embryos at MBT stage		23 (28%) [a]	6 (12%) [a]	29 (22%)	90 (30%)
Nº of embryos at epiboly stage		18 (22%) [a]	3(6%) [b]	21 (16%)	-
Nº of embryos at 24h post-transplant	normal	12 (15%) [a]	1 (2%) [b]	13 (10%)	-
	abnormal	2	1	3	-
	total	14	2	16	-
Nº of embryos at 48h post-transplant	normal	8 (10%) [a]	1 (2%) [a]	9 (7%)	-
	abnormal	3	1	4	-
	total	11	2	13	-
Nº of survival larvae		6 (7%) [a]	0 (0%) [a]	6 (5%)	-

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

Table 2. Method B: survival rates of nuclear transplant simultaneously to the egg activation/fertilization.

		B1	**B2**	**Total B**	**CB**
Nº of transplanted eggs		59	54	113	53
Nº of embryos at MBT stage		32 (54%)[a]	19 (35%)[a]	51 (45%)	15
Nº of embryos at epiboly stage		22 (37%)[a]	15 (28%)[a]	37 (33%)	(28%) -
Nº of embryos at 24h post-transplant	normal	14 (24%)[a]	11 (20%)[a]	25 (22%)	-
	abnormal	6	14	10	-
	total	20	15	35	-
Nº of embryos at 48h post-transplant	normal	12 (20%)[a]	11 (22%)[a]	23 (20%)	-
	abnormal	5	1	6	-
	total	17	12	29	-
Nº of survival larvae		11 (19%)[a]	11 (20%)[a]	22 (19%)	-

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

Table3. Method C: survival rates of nuclear transplant posterior to the egg activation/fertilization.

		C1	**C2**	**Total C**	**CC**
Nº of transplanted eggs		49	22	71	374
Nº of embryos at MBT stage		26 (53%) [a]	9 (41%) [a]	35 (49%)	147(39
Nº of embryos at epiboly stage		21 (43%) [a]	7 (32%) [a]	28 (39%)	%)
					-
Nº of embryos at 24h post-transplant	normal	16 (33%) [a]	4 (18%) [a]	20 (28%)	-
	abnormal	5	1	6	-
	total	21	5	26	-
Nº of embryos at 48h post-transplant	normal	14 (29%) [a]	3 (14%) [a]	17 (24%)	-
	abnormal	3	1	4	-
	total	17	4	21	-
Nº of survival larvae		14 (29%) [a]	3 (14%) [a]	17 (24%)	-

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

STUDY 11. Transplant of adult fibroblast nuclei into the central region of metaphase II eggs resulted in mid blastula transition (MBT) embryos.[*]

Abstract

Recently, a novel technical method to perform somatic nuclear transplant in zebrafish using non activated eggs as recipients without the need to detect the micropyle was developed in our lab. However, the use of spermatozoa as activating agent prevented to know if the inserted nucleus conditioned embryonic and early larval developmental ability. The aim of the present work was to test the developmental ability of the embryos reconstructed by transplanting adult fibroblast nuclei into the central region of the metaphase II egg but subsequently activated by only water. In addition, since oocyte ageing facilitates the activation in mammalian oocytes and also leads to metaphasic plate disorganization, this work also pursued to test whether limited aged eggs used as recipients improve the activation and involve some functional enucleation in zebrafish. The adult somatic nucleus located in the central region of the non activated egg resulted in the 12% of mid blastula transition embryos *vs* the 20% when the transplant is in the animal pole ($p \geq 0.05$). This fact suggests that the central region of the non activated metaphase II eggs can be a suitable place for nucleus deposition in nuclear transplant in zebrafish. These results reinforce the possibility to use non activated metaphase II eggs in subsequent reprogramming studies by adult somatic nuclear transplant in zebrafish. Unfortunately, in contrast to mammals, a limited egg ageing (2h) penalized did not improve the activation process in zebrafish nuclear transplant, moreover the hypothesis about a possible functional enucleation was also ruled out.

Keywords: nuclear transplant, activation, oocyte, reprogramming, zebrafish.

[*] This manuscript has been submitted to the journal "*Zebrafish*" with the following reference: Pérez-Camps M, Cardona-Costa J, and García-Ximénez F. (2009). Transplant of adult fibroblast nuclei into the central region of metaphase II eggs resulted in mid blastula transition (MBT) embryos. Zebrafish. (submitted).

INTRODUCTION

In the few nuclear transplant (NT) studies reported to date in zebrafish (Li et al., 2000; Hu et al., 2002; Lee et al., 2002; Luo et al., 2009), the location of the donor nucleus injection place, the animal pole, requires previous oocyte activation because of the micropyle cannot be easily detected in this species (Poleo et al., 2001).

Recently, a novel method developed in our lab made it technically possible to perform the somatic nuclear transplant in zebrafish using mature non activated eggs without the need to dechorionate or localize the micropyle (see *Study 10*). In this study, non enucleated oocytes were activated by sperm fertilization after the nuclear transplant. Such strategy was based on the fact that diploidized eggs have been shown as suitable recipients for nuclear transplant in medaka due to the more stable ploidy status, among other aspects (Bubenshchikova *et al.*, 2007). But, the activation by sperm fertilization prevented to know if the donor nucleus conditioned embryonic and early larval developmental ability.

In fish somatic cloning, as in mammals, it is assumed that the nuclear transplant have to do using enucleated oocytes. However, in zebrafish, the resident pronucleus in non enucleated and non fertilized egg receptors seems to degenerate after the nuclear transplant during the first cleavages (Li et al., 2000; Li et al., 2003). This particularity in zebrafish, not showed in other species as medaka, loach or amphibious (Hu et al., 2002), could avoid the need to use whatever enucleation method (mechanical, ionizing and ultraviolet radiations, etc.) including laser.

Since oocyte ageing facilitates the activation in mammalian oocytes, but also leads to metaphasic plate disorganization in this species (Kim et a., 1996), it can be hypothesized that a limited ageing in zebrafish eggs could improve the activation response and, perhaps, induce some type of “aging” functional enucleation.

Our lab is focused on developing in zebrafish an efficient somatic cell nuclear transplant technique to be used for reprogramming studies using non activated metaphase II eggs as recipients. The aim of the present work was to test the developmental ability of the embryos reconstructed by transplanting the adult fibroblast into the central region of the metaphase II egg subsequently activated by only water. In addition, this work pursued to test whether limited aged eggs used as recipients improve the activation and involve some functional enucleation in zebrafish.

MATERIAL AND METHODS

Non activated eggs and sperm collection

Eggs and sperm were collected after evaluating the sexual behaviour of both *gold* strain males and females at dawn. Eggs were obtained by gentle extrusion of the ovary and sperm was recovered from the genital pore in individual glass microcapillaries (see *Study 10*). Then, non activated gametes were kept in egg medium (Hanks' buffered salt solution supplemented with 1.5% (v/v) of BSA and 0.1 g of NaCl/100 cc of Hanks' medium; ph: 7.4 ; osmolarity: 310-320 mOsm) at 8ºC (see *Study 9*).

In vitro fertilization

Non activated eggs and sperm were mixed in a 35-mm Petri-dish and both gametes activated by the addition of a 1 mL of system water. After 2-3 min, the dish was filled with system water to achieve well developing embryos. Further culture was done at 28.5 ºC (see *Study 9*).

Donor cells culture.

Adult somatic cells used as nuclei donors came from *wild* zebrafish caudal fin primary cultures. Tissue was obtained and treated as Pérez-Camps *et al.* (see *Study 10*) described and cultured in Leibovitz medium supplemented with 20% of FBS and 0.036 g/L of glutamine (L15-FBS) at 28.5ºC (see *Study 9*).

Somatic cell nuclear transplant.

Somatic cell nuclear transplant technique, methods and equipment employed was described by Pérez-Camps *et al.* (see *Study 10*). Briefly:

Method A-Nuclear transplant prior to egg activation/fertilization.

To prevent egg activation, eggs were kept in egg medium at 8ºC until the nuclear transplant, which was performed in a handling drop of egg medium in a petri dish under mineral oil and the micromanipulation area was cooled to 8 ºC. The somatic cell nucleus was inserted in the central region of the metaphase II egg. Then, transplanted

eggs were individually either activated by only water or fertilized by sperm and cultured at 28.5ºC in water (see *Study 10*).

Method B-Nuclear transplant simultaneous with egg activation/fertilization

When performing the NT during the activation only by water, eggs were also kept in egg medium at 8ºC until NT. They were individually deposited in the handling drop containing the water so that oocytes were activated at the moment of transplant. When performing the NT simultaneously with the fertilization, non activated eggs and sperm were previously mixed and kept at 8ºC and, as before, individually deposited and transplanted in the handling drop. Donor nuclei were injected in the incipient animal pole in both cases, just where either the female pronucleus or the zygote (male and female) pronuclei were being constituted (Wolenski and Hart, 1987).

Experimental design

With the aim of testing the developmental ability of embryos reconstructed by transplanting the somatic nucleus into the central region of the non activated egg, the NT were carried out before (method A) and simultaneously (method B) to the egg activation both by sperm and only water as activating stimulus. All nuclear transplants were realized in different sessions immediately after the ovarian egg extraction (0h). In the 0h NT groups, although the literature indicates that eggs can be fertilized until 90 min after their ovarian extraction (Westerfield, 2007; Nüsslein-Volhard and Dahm, 2002), our results in a previous work indicated that, in our hands, development ability drops when NT is performed after the first 45 min following extraction (see *Study 10*). So, we established that the 0h experimental sessions did not exceed 45 min in any case. The nuclear transplants with 2h aged eggs were performed in the same daily session with eggs from the same laying. In this way, to study the egg aging effect on the activation response, results from the 2h NT groups activated only by water were compared with the 0h NT groups activated by water too.

An initial fertilizability control group was done immediately when eggs were extracted to test the egg quality, whatever the time and method of transplant. Nuclear transplant sessions were considered only when the initial fertility rates were higher than 70%. Activations with water were carried out in parallel to check a possible parthenogenetic development beyond some abortive divisions. Moreover, in all

experimental sessions, some oocytes were punctured without nuclear transplant to evaluate the activation efficiency of the micropippete puncture (control groups of activation).

Reconstructed embryos were considered as the developing embryo only when the number of embryonic cells was more than 4 (Lee *et al.*, 1999). In all experiments, survival rates from early embryonic cleavages (>4cell) to larvae (5 days) were evaluated.

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.

RESULTS

Central nuclear injection in non activated eggs resulted in embryonic development.

Embryo survival rate differences between the two nuclear transplant methods (transplant before or simultaneously to egg activation) activated by sperm fertilization or by water as the only stimulus were compared (Table 1). Results showed that there were no significant differences in survival rates between the two nuclear transplant methods when the oocytes were activated by sperm. Differences in the embryo survival rates between the two nuclear transplant methods when oocytes were activated by only the water stimulus were not found either.

On the other hand, as was expected, embryonic survival rates differences between reconstructed embryos activated by sperm fertilization and by only water were significant, wherever the nucleus deposition took place (Table 1). Moreover, reconstructed embryos activated only by water reached, at maximum, the MBT stage.

It has to be noted that eggs activated but non transplanted did not show embryonic development. In fact, only very few eggs remained, at most, at the two cell stage. Moreover, no development signs were observed in punctured non transplanted metaphase II eggs.

Partial egg ageing did not improve the activation.

Because the NT in aged eggs were carried out in the same daily session with eggs from the same female than in the groups previously described at 0h, data corresponding to the freshly eggs were the same presented in the table 1. Differences in

the survival rates at early cleavages (> 4 cells) were relevant, although they did not reach significance levels, when young and aged eggs were compared, whatever the NT method, in favour of young eggs (12% vs 3% in NT before activation; 23% vs 7% in NT during activation; $p>0.05$). Differences were also observed at MBT stage but, in this case, significant levels were reached (12% vs 0% in NT before activation; 20% vs 0% in NT during activation). It must be emphasized that none of reconstructed embryos reached the MBT stage when aged eggs (2h) were used. These results suggest that, in contrast to mammals, egg ageing does not involve an improvement in the parthenogenetic development progression or in nuclear transplant efficiency in zebrafish.

The results obtained in this experiment also allowed us to test the possible functional enucleation by ageing. The null survival rates obtained in the 2h transplants ruled out considering a functional enucleation in 2h-aged eggs.

DISCUSSION

In a previous work, we demonstrated that the nuclear transplant in zebrafish before activation of metaphase II eggs was technically possible (see *Study 10*) but, in such work, the use of spermatozoa as activating agent made it difficult the study, the possible integration of the donor nucleus in the reconstructed embryo. In zebrafish eggs, the mere contact with water causes chorion expansion and cytoplasm segregation, but they either remain at the one cell stage or, in some cases, eggs may develop to some abortive cleavages. In this way, a fertilized embryo is considered as developing when it passes from 2 to 4 well organized cells (Lee *et al.*, 1999). In the present work, we obtained MBT reconstructed embryos by the transplant of adult somatic nuclei in the centre of the non activated eggs, subsequently activated with water as the only activating stimulus. Moreover, there was no disadvantage at all for the central injection of the nucleus with respect to the nucleus injection in the incipient animal pole.

of the egg with the micropipette during the transplant. In medaka, unfertilized eggs are activated by pricking with a fine glass needle, but most eggs cannot develop further and remain at the one cell stage (Yamamoto, 1944). No data on this phenomenon were found in the reviewed literature in zebrafish. In fact, the only way in which gynogenetic zebrafish embryos have been obtained was by using genetically inactivated sperm by radiation as activating agent (Westerfield, 2007). Moreover, the

results here obtained from the puncture of non activated eggs plus water activation (control group of activation) indicate that the only mechanical action of injecting the nucleus does not stimulate by themselves the activation process. So, it can be proposed that, in our case, when no sperm was used as activating stimulus, the nucleus transplanted exerted an activating effect on the nuclear transplant embryo, participating in the development, at least, until MBT stage.

As we hypothesized in method A (central nucleus transplant before the activation), the results obtained suggest that the nucleus transplanted in the centre of the metaphase II egg migrates to the animal pole, may be, through the cytoplasmic flows at the time of activation (Wolenski and Hart, 1987). This result opens the possibility of exposing the donor nucleus to an metaphase II ooplasmic environment for longer and controlled periods of time before activation, a requirement that has proven effective for reprogramming improvement in mammals (Wilmut *et al.*, 1997). Unfortunatelly, the egg culture medium used in the present work is only able to preserve the non activated eggs in good conditions for 1 hour (see *Study 9*). A recent study has detected that the Chinook salmon ovarian fluid (CSOF) can preserve non activated eggs for longer periods of time (Siripattarapravat *et al.*, 2009a), but they commercial availability is, at this time, practically null.

Although karyotyping analysis have not realized due to the difficulty in this specie (Hu *et al.*, 2002), the fact that in zebrafish, when nuclear transplant is performed into non enucleated eggs involves the degeneration of the female pronucleus (Li *et al.* 2000; Li *et al.* 2003), would lead to generate diploid reconstructed embryos, and not triploid, as occurs in other species (Hu et al., 2002). Such phenomenon of ploidy correction is not innusual. In this sense, in medaka, there also seem to be mechanisms that operate in the rearrangement of the chromosome status after the NT (Kaftanovskaya *et al.*, 2007; Bubenshchikova *et al.*, 2007). Mechanisms of this type working in different ways have also been described in mammals (human: Tesarik and Mendoza, 2003; pig: Somfai *et al.*, 2008).

To date, in zebrafish, nuclei from embryonic cells (Hu *et al.*, 2002), from 5-somite embryos (Lee *et al.*, 2002) and from kidney cells of adult male (Luo *et al.*, 2009) have been transplanted into enucleated pre-activated eggs. In our knowledge, in the present work, fibroblasts from primary cultures are used for the first time as donor nuclei in zebrafish nuclear transplant without activation by sperm fertilization. The percentages of reconstructed embryos achieved were similar to those obtained by Lou *et*

al. (2009), being even higher when water activation was previous to the NT (20%). However, as Lou *et al.* (2009) the survival rates dropped drastically after reaching the blastula stage.

P.S: During the elaboration of the present manuscript, a new zebrafish nuclear transplant study was published by Cibelli and colleagues (Siripattarapravat et al., 2009b), in which adult specimens were obtained from larval cells but not from adult cells.

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Table 1. Survival rates of somatic cell nuclear transplants into non enucleated freshly eggs activated by sperm or by water.

	Method A NT before the activation		Method B NT during the activation	
	Activated by sperm fertilization	Activated by only water	Activated by sperm fertilization	Activated by only water
Eggs transplanted	20	34	20	30
MBT embryos	9 (45%) [a]	4 (12%) [b]	8 (40%) [a]	6 (20%) [ab]
1day embryos	7 (35%) [a]	0 (0%) [b]	4 (20%) [a]	0 (0%) [b]
5 days larvae	4 (20%) [a]	0 (0%) [b]	3 (15%) [ab]	0 (0%) [b]

Between columns, data with different superscripts are statistically different ($p<0.05$).

Table 2. Survival rates of somatic cell nuclear transplants into non enucleated fresh and 2h-aged eggs activated by water. Data of freshly egg are the same that in table 1.

	Freshly eggs		2h-aged eggs	
	Method A NT before the activation	Method B NT during the activation	Method A NT before the activation	Method B NT during the activation
Eggs transplanted	34	30	34	30
Embryos with more than 4 well orientated cells	4 (12%) [ab]	7 (23%) [a]	1 (3%) [b]	2 (7%) [ab]
MBT embryos	4 (12%) [abc]	6 (20%) [a]	0 (0%) [c]	0 (0%) [bc]

Between columns, data with different superscripts are statistically different ($p<0.05$).

STUDY 12. Electroactivation of zebrafish (*Danio rerio*) eggs.*

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 (inactivated or not) is not used as activating agent, zebrafish cloning (and other NT techniques) is very inefficient.

In this work, three experiments were performed: In the first, 6 treatments were compared (Voltage x Pulses: 2.76x1; 2.76x2; 2.76x3 and 5.40x1; 5.40x2; 5.40x3). The group 5.4x3 showed the best results (32% activated). In the second experiment, 1 or 3 consecutive electric pulses of 20 µs each were applied repeatedly at 0 min, 10 min and after 20 min post-activation. Two voltage levels (2.76 V and 5.4 V) were assayed. The number of pulses negatively affected the rates of damaged and lysed eggs. Moreover, only the B2 ([2.76 x 3] x 3; activated eggs: 43%) group showed significant differences with Control B non manipulated group (18% activated eggs). In the third experiment, negative effects of egg ageing were observed.

The best treatment established here was able to activate 66% of eggs, but without accomplishing normal parthenogenetic embryo development.

Keywords: activation, egg, partenogenetic, zebrafish.

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INTRODUCTION

In zebrafish, egg activation takes place after they are discharged from the ovarian stroma and come into contact with the spawning medium (Sakai, 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. These steps are followed by normal cleavages when a spermatozoon, either genetically inactivated or not, has fertilized the egg (Lee, *et al.,* 1999; Nüsslein-Volhard and Dahm, 2002). If sperm is not present in the spawning medium, an incomplete parthenogenetic activation takes place, undergoing some abortive cleavages in the best of cases, but without reaching further development (see *Study 9*), or even the configuration of four well organized cells (Lee, *et al.*, 1999).

In mammals, oocyte activation is routinely induced or reinforced by electrical pulses (and/or chemicals) after performing somatic cloning by nuclear transplant, SCNT (Okahara-Narita, *et al.*, 2007; Onishi, *et al.*, 2000), intracytoplasmic sperm injection, ICSI (Mansour, *et al.*, 2009; Zhang, *et al.*, 1997) or in the obtaining of parthenogenetic haploid/diploid embryos (Elsheikh, *et al.*, 1995; Escribá and García-Ximénez, 2000). Despite this, little attention has been paid to what benefit could be provided by an artificial activation procedure by electric pulses in zebrafish. More recently, NT techniques before, during and after zebrafish egg activation by sperm fertilization have been developed in our lab (see *Study 10*). However, when no sperm is used as activating agent, zebrafish cloning is very inefficient (Huang, *et al.*, 2003), as also occurs with other techniques such as ICSI (Poleo, *et al.,* 2001). So, the development of these and other reproductive techniques in this species (i.e. round spermatid injection, ROSI; round spermatid nuclear injection, ROSNI), depends mainly of the availability of an efficient procedure for artificial egg activation (Bubenshchikova, *et al.,* 2007; Huang, *et al.,* 2003; see *Study 10*). Moreover, electroactivation as an alternative egg activation method to the use of irradiated sperm in zebrafish would also permit the experimental evaluation and study of egg activation abilities throughout their egg ageing process, even exceeding the limits of egg fertilizability.

In fact, to our knowledge this methodology has only been attempted in fish for egg activation in NT experiments on medaka fish (Bubenshchikova, *et al.,* 2007; Wakamatsu, 2008). In this context, the aim of the present work was to establish electrical parameters for the activation of zebrafish intact (non micromanipulated) eggs as well as their activation response throughout their ageing process, but without involving the sperm-mediated stimulus.

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).

Obtaining of gametes and fertilization

The procedure carried out was described in detail by Cardona-Costa, *et al.*, (see *Study 9*). Briefly, gametes were extracted at the same artificial "dawn" time from those aquariums where fish displayed reproductive behaviour. Both males and females were simultaneously anaesthetized with clove oil solution of 100 µl/l (Grush *et al.,* 2004; Mylonas, *et al.,* 2005) in dechlorinated and decalcified tap water (system water; Westerfield, 2007). All aseptic procedures and sterile instruments were used in subsequent steps. Sperm and eggs were in vivo extracted and maintained inactivated until use at 8 ºC in a modified Hanks 'solution designed as CH (100 ml of Hanks´ supplemented with 1.5 g BSA and 0.1 g ClNa; 320 mOsm, pH 7.4). The motility of selected sperm was 80-100% and eggs were of good quality (translucent, granular and yellowish appearance; Westerfield, 2007).

Control groups of fertilized eggs were formed at the beginning of experiments. To this end, those sessions where the fertilization control was lower than 70 % were discarded. The fertilization procedure was done in a 35 mm Petri dish (Corning) after carefully mixing 15-20 eggs and sperm solution. Then, 3 to 5 system water drops were added to fertilize eggs (sperm concentration in fertilization was around 2.8 x 10^8 spz/ml,

measured with a Thoma counting chamber). Finally, the plate was filled with system water after 5 min.

Control groups activated only by water stimulus were transferred by the same procedure to a Petri dish (Corning) with system water where they were activated only by the water stimulus.

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

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 (per batch) 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 electric pulse (direct current, DC) was applied. This pulse was systematically checked through the oscilloscope. Finally, treated eggs were transferred to Petri dishes (Corning) with system water and incubated at 28.5 ºC.

Control groups (see experimental design) came from the rest of the same batch of eggs.

Assessments related to damage/lysis, activation and developmental signs took place immediately after each pulse and at 1 h and 2 h post-activation (after the first contact with system water).

Eggs were considered “activated eggs” when they showed at least one mitotic cleavage apart from the normal activation signs (already described in the introduction; 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 pulse of 8.0 V/cm 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 (x1), 2.76 (x2), 2.76 (x3), 2.76 (x4) and 2.76 (x5)).

Experiment A

In line with results obtained in the preliminary assays, the following electrical parameters were finally tested: 1, 2 or 3 consecutive pulses of 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 vs E_2 = 16 V/cm; 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 x Pulses: 2.76x1; 2.76x2; 2.76x3 and 5.40x1; 5.40x2; 5.40x3). 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 and colleagues (2009) established an elapsed time of 30 min (at 28 ºC 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 electric pulses of 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 x Pulses] x 3 sequences: B1= [2.76x1] x3; B2= [2.76x3] x3 and B3= [5.4x1] x3; B4= [5.4x3] x3).

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 8ºC in CH medium (non activating medium, see *Study 9*) for different times.

In accordance with results obtained from Experiment A and B (see results), two combinations from among all the experimental groups tested were selected for their best and similar egg activation rates by electric pulses (B2= [2.76x3] x3 and B3= [5.4x1] x3).

The experimental groups were carried out as a result of joining the different egg ageing times tested (0 h, 1h and 2h post- ovarian extraction) and the two best electric treatments (B2 and B3 as cited). In addition, three control groups of eggs activated in system water at 0 h, 1h 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 or C), a minimum of three replicates were done in alternative sessions. Results were analysed using the Chi-square test. When a single degree of freedom was involved, Yates' correction for continuity was performed.

RESULTS

From 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 in tables).

From Experiment A

Results are presented in Table 1. As shown, the differences in the percentage of activated eggs only raised levels of significance between the 5.4x3 group when compared with Control A (only activated in system water) and the 2.76x1 group.

From Experiment B

In this experiment, the electrical treatment was extended to a sequence of three pulse 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 (2.76x1 vs 5.4x3) established in experiment A (Table 2). First, it was observed that the number of pulses applied negatively affected the percentage of 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 another sense, and 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).

From 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.

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.

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 and colleagues (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, we also used an ionic medium (system water in our case) as the starting point of our electroactivation assays in zebrafish eggs.

Recent studies by Siripattarapravat, *et al.*, (2009) reported that no significant differences were found in the initial dropping of MPF activity in zebrafish eggs activated by either fertilization or water. 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 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: (2.76x3)x3 and B3 (5.4x1)x3 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 (see *Study 9*), 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 (Collas and Robl, 1990; Kaufman, 1981; Stice and Robl, 1988; 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 inactivated sperm (Nüsslein-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 (see *Study 10*) 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.

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

Voltage x Pulses	Control A	2.76x1	2.76x2	2.76x3	5.4x1	5.4x2	5.4x3
Initial	65	81	80	63	92	96	60
Activated	14%[a] (9)	10%[a] (8)	17%[ab] (14)	19%[ab] (12)	21%[ab] (19)	19%[ab] (18)	32%[b] (19)

Between columns, data with different superscripts are statistically different ($p<0.05$).

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 pulses) at two voltage levels (2.76 V and 5.4 V). In Control B, eggs were only activated by system water.

(Voltage x Pulses) x 3 sequences	Control B	B1 (2.76x1)x3	B2 (2.76x3)x3	B3 (5.4x1)x3	B4 (5.4x3)x3
Initial	62	44	58	44	30
Damaged/lysed	0%[a] (0)	0%[a] (0)	17%[b] (10)	2%[a] (1)	100%[c] (30)
Activated	18%[b] (11)	18%[b] (8)	43%[c] (25)	36%[bc] (16)	0%[a] (0)

Between columns, data with different superscripts are statistically different ($p<0.05$).

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: 2.76V x 3Pulses; B3: 5.4V x 1Pulse). In Controls, eggs were stored in CH medium at 8 ºC and activated in system water.

Egg ageing time	0 h			1 h			2 h		
Exp. Groups and Control groups	Control C– 0h	B2	B3	Control C – 1 h	B2	B3	Control C – 2 h	B2	B3
Initial	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 superscripts are statistically different ($p<0.05$).

STUDY 13. Comparison of different activating stimuli efficiency in zebrafish nuclear transplant.[*]

Abstract

The oocyte activation is one of the most important step for the nuclear transplant (NT) technique outcome. In previous works, we used sperm fertilization and water as activating agent and established an activation technique by electric pulses using non manipulated eggs. To day, the only way by which haploid larvae can be obtained is using radiated sperm as activating agent. Firstly, in the present work, we adapted the UV radiation dose to be applied to *gold* sperm for using it as activating agent in NT experiments. Secondly, we compared the embryo survival rates and the developmental degree derived from the transplant of adult fibroblast nuclei before or during egg activation associated to four activating stimuli (fresh sperm, radiated sperm, electroactivation and water). Unfortunately the electroactivation technique established for non manipulated eggs lysed all eggs when it was associated to the NT. Results from the other three stimuli together with the two NT methods showed higher embryo survival rates when the activation was by sperm (24h larvae: fresh sperm 31% and 17%; radiated sperm 20% and 11%; water 0% in both). Regarding the developmental degree, embryos activated by fresh sperm reached the free stage (5 days), those activated by radiated sperm the 24h stage and embryos activated by water only reached the MBT stage wherever the NT method.

Keywords: Activation, Haploid development, Nuclear transplant, Sperm, Zebrafish.

[*] Manuscript in preparation.

INTRODUCTION

The oocyte activation is a critical step for the outcome of the nuclear transfer (NT) technique, both in mammals and in fishes.

In fishes, the stimulus of the spermatozoa is required to obtain a complete activation, although the initial contact with fresh water initiates the process. In zebrafish, Pérez-Camps et al. (see *Study 10*) developed a technical method for performing the NT using metaphase II eggs. In such study, fresh sperm was used as activating agent both after and simultaneous to the nuclear transplant. The sperm as activating agent in NT into non enucleated eggs supposed an initial tetraploid condition that favours a better chromosomic status for like-clones obtaining, as occurs by diploidising eggs in medaka (Bubenshchikova et al., 2007). However, the sperm used as activation agent prevented to know if the inserted nucleus in the conditioned embryonic and early larval developmental ability.

However, the water as the only activating agent resulted less efficient than the sperm stimulus, obtaining only embryos at mid blastula transition stage (see *Study 11*).

If radiated sperm, the only way to activate partenogenetically zebrafish eggs to date (Westerfield, 2007), is used as activation stimulus of non enucleated metaphase II eggs after the NT or during the transplant, the participation of the spermatozoa nucleus would not exist so, a hypothetic triploid condition could be generated when non enucleated eggs were transplanted. This condition supports an embryo development in a lot of fish species. However, in zebrafish, as the female pronucleous seems to degenerate in the first cleavages after the transplant (Li et al., 2000; Li et al., 2003), it may be possible to obtain a high activating stimulus without the participation neither the receptor nor spermatozoa nuclei.

As a possible alternative to the use of inactivated sperm, an electroactivation method recently developed in our laboratory which improved the activation response of non manipulated zebrafish eggs (see *Study 12*) can be proposed to be associated to the NT. This fact would also allow a reinforcement of other primary stimuli, as the water, in the NT experiments whatever the egg status.

So, the aim of the present work is to compare the efficiencies of activation and embryo development reached when the four activation stimulus (fresh water, radiated sperm, non radiated sperm and electric pulses) are associated to two methods of nuclear transplantion, before or simultaneously to activation.

MATERIAL AND METHODS

Care and maintenance of zebrafish colony

Adult zebrafish were kept in 20 L tanks in a 2:1 ratio (females/males) and fed on granular food supplemented with recently defrosted hen egg yolk and shrimp meat (Simao *et al*., 2007). The light cycle was regulated at 14h light/10h dark.

Non activated eggs and sperm collection.

Eggs and sperm were collected after evaluating the sexual behavior of both gold strain males and females at dawn. Eggs were obtained by gentle extrusion of the ovary and sperm was recovered from the genital pore in individually glass microcapillaries (Westerfield, 2007). Then, non activated gametes were kept in egg medium at 8ºC (see *Study 9*).

Nuclei donor cells.

Somatic cells used as nuclei donor came from *wild* zebrafish caudal fin primary cultures. The tissue was obtained and treated as Pérez-Camps et al. (see *Study 10*) described and cultured in Leibovitz medium supplemented with 20% of FBS and 0.036 g/L of glutamine (L15-FBS) at 28.5ºC (Westerfield, 2007).

Somatic cell nuclear transplant

The two somatic cell nuclear transplant techniques, as well as the equipment employed were described by Pérez-Camps et al. (see *Study 10*). Method A: nuclear transplant prior to the egg activation: The somatic cell nucleus was inserted in the central region of the egg. To prevent egg activation, the transplant was performed in a handling drop composed of egg medium and the micromanipulation area was cooled down to 8 ºC. Method B: nuclear transplant simultaneously to the egg activation: the donor nucleus was injected in the incipient animal pole, just where zygote nucleus was being constituted. The handling drop was composed by fresh water so that the eggs became the activation and the micromanipulation area was not cooled (room temperature).

Activation by sperm fertilization.

The activation was carried out as Pérez-Camps et al. (see *Study 10*) described. When the method A was used, non activated transplanted were individually in vitro fertilized and cultured at 28.5ºC in fresh water. In the case of using the method B, non activated eggs and sperm were previously mixed and kept at 8ºC, next they were individually deposited in the handling drop containing system water so that gametes were activated and fertilized.

Activation by fresh water.

The nuclear transplant before or during egg ctivation by only fresh water was described by Pérez-Camps et al. (see *Study 10*). Non activated eggs were activated by fresh water after the NT when the method A was used. Regarding the method B, eggs were already activated in the transplant handling medium (see above).

Activation by UV radiated sperm.

We were looking for the optimal UV radiation dose of *gold* fresh sperm to be used as activation stimulus in NT, since a effect of UV radiation was proven to be strain dependent in zebrafish. Once determined this time, the two NT methods (A and B) were carried out in a similar way than the activation by non radiated sperm (see above).

A sample of 100 µm of the sperm diluted in Hanks' balanced solution (see *Study 9*) was radiated using a UV 85 germicide lamp (General 86 Electric, 30W). Irradiation was carried out at 62 cm of focus-object distance. The radiation dose applied was 0.529 mW/cm2 87 and was measured by a USB 4000 (Miniature 88 Fiber Optic Spectrometer; Ocean Optics Inc. First In Photonics, USA). A vortex (MS1-92 IKA, Wilmington, CA, USA) at 200 rpm was used with the aim of homogenising the radiation area during UV 93 exposure.

Preliminary assays determined the maximum time of sperm radiation by which non activation signals (motility) were observed. This time was established in 18 minutes. So, 4 groups were irradiated (2, 5, 10 and 15 min) from 15 min, as a maximum time, to the 2 min, the time used by other authors (Westerfield, 2007) for the partenogenetic haploids production with *wild* sperm.

The survival rates were evaluated at MBT stage, 24 h and 5 days were, as well as the abnorlamity and degeneration rates at 24h. Abnormality was evaluated by the haploid syndrome. Haploid embryos have a characteristic syndrome. The body is

shorter and thicker than a diploid; the brain is less clearly sculptured; the ears are variable in number; and the heart beats in a swollen pericardial cavity. Haploid cells are smaller than diploid cells and there are problems with organogenesis (Westerfield, 2007).

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 the model 453 from ECM. With the aid of a Pasteur pipette, previously stretched and fire polished, eggs were directly transferred from the NT plate to the the pulsing chamber, containing system water as electroactivation medium. Immediately, the electric pulse predefined by Cardona-Costa et al. (see *Study 12*) in non manipilated eggs was applied. This pulse, which activated the 66% of the non manipulated eggs, consisted in 20 µs of 5.4 V electric pulses in a sequence of 20 min (0 min post-NT, at 10 min and finally at 20 min). The pulse was systematically verified through the oscilloscope.

Embryo culture.

Transplanted and activated eggs were incubated in system water at 28.5 ºC (see *Study 10*).

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.

RESULTS AND DISCUSSION

Electroactivation of NT eggs

The electric pulse applied on transplanted eggs was established on non manipulated oocytes in a previous work (see *Study 12*). Unfortunately, this electroactivation treatment resulted in the lysis of all eggs previously transplanted. Most of the eggs were lysed even before the second pulse was applied. Such results led us, in a following assay, to test the minimum electric pulse assayed in our previous work. This

lower electrical stimuli pulse consisted in a 20 µs pulse of 2.7 V applied 10 min post-activation in fresh water. This second assay resulted in the lysis of 15 from 17 transplanted eggs and the 2 surviving eggs showed only abortive cleavages. These results indicate that the electric parameters previously defined to activate non manipulated eggs were not useful for the electroactivation of transplanted eggs. So, the experimental electroactivation group initially proposed to be compared with the other was ruled out. Moreover, the results obtained in the assays of electroactivation forces us to develop, in future work, an activation treatment specific for zebrafish NT experiments. However, the definition of an electric pulse associated to the NT in zebrafish exceeded the objective of the present work.

UV radiation of gold sperm for egg activation in NT

Although, it is widely established the parthenogenotes obtaining by sperm or egg radiation in *wild* zebrafish (Nüsslein-Volhard and Dahm, 2002; Westerfield, 2007), the use of UV radiated *gold* sperm to activate *gold* eggs after or simultaneously to the NT has not reported to date. After finding sensibility differences to the UV radiation between *wild* and *gold* embryos (see *Study 6*) the aim of this experiment was to establish the optimal UV radiation for *gold* sperm to achieve the higher fecundation ability but the lower embryo development and the high abnormality and degeneration rates. Fresh *gold* sperm was radiated for 2, 5, 10 and 15 minutes.

Significant differences were observed among the four groups in the embryo survival at MBT stage. The number of embryos that reached this stage was decreasing when the radiation times was increasing (2 min: 67%; 5 min: 45%; 10 min: 18%; 15 min: 4%; $p<0.05$). Differences between survival rates were significant neither at 24h nor at 5 days post-fertilization. This result, together with the high degeneration and abnormal rates at 24h in the group of embryos fertilized with sperm radiated for 2 min, led us to establish in 2 min the optimal radiation time of *gold* sperm to be used as activation stimulus in NT.

Comparison between activation stimuli

In the present work, three activation stimuli have tested in reconstructed eggs obtained by two NT methods in zebrafish, NT before or during egg activation (Table 2). Related to the two NT methods, no significant differences were observed when the three activation stimulus (fresh sperm, radiated sperm or fresh water) were compared at MBT

stage, epiboly stage, 24h and 5 days. Regarding the NT performed before the activation (method A), it has to point out that the methods of activation based in sperm, radiated or not, were in general more efficient than the activation by only fresh water. When the NT was carried out during the egg activation (method B), no differences in the survival rates were observed between the three activation stimulus neither at MBT stage nor at 24h. However, these results must be taken cautiously into account due to the low number of embryos that reached at these stages.

There are different degrees of oocytes response depending on the activating stimulus. Viable larva were only obtained when the activation were performed using fresh sperm as stimulus with rates of 15% for the method A (NT before activation) and 13% for the method B (NT during activation), values that were not significantly different (Table 2). By using radiated sperm, the reconstructed embryos by the two methods of NT reached the 24h stage but they could not continue their further development.

When non inactivated sperm fertilizes the recipient oocytes and participates in the embryo and larval development, zygotic nuclei derived may help the somatic nucleus integration through a nuclear helper role (Howlett et al., 1987; Eakin and Hadjantonakis, 2006) and they may form diploid chimaeras (Bubenshchikova et al., 2007). On the other hand, when the sperm nucleus is inactivated, there is not a nuclear helper role, but there are sperm derived factors which are responsible for triggering the calcium transient in the activation of a lot of species from sea urchin and ascidian oocytes (Dale *et al.*, 1985; Dale, 1988) to human (Tesarik *et al.*, 1994, Dale *et al.*, 1996). However, zebrafish egg activation does not involve the need of any sperm-derived factors (Swann, 1993) since when eggs come in contact with water they activate (Hart and Yu, 1980; Hart and Fluck, 1995; Sakai et al., 1997), even in absence of sperm, they undergo some abortive cleavages (Lee et al., 1996). In the present work, parthenogenetic development was not observed, neither using non motile radiated sperm (with derived-factors) as activating agent (results not shown) nor applying an electroactivation protocol to non activated eggs (see *Study 12*). In fact, to date, the only way to activate zebrafish eggs to accomplish full parthenogenetic development is by radiated but motile sperm. So, it can be proposed that in NT experiments, the sperm-oocyte interaction, rather than the sperm factors, induces the activating response. The limited embryo and larval development using water or radiated spermatozoa could be

ascribed to the inability to maintain embryo development by the scarcely reprogramming of transplanted somatic nucleus.

The major problem when radiated sperm is used is the indetermination regarding the sperm nucleus inactivation or not. It has noted that haploid larvae can be easily detected by the presence of haploid syndrome characteristics (Westerfield, 2007), but it is obvious that, when the NT is associated, the reconstructed embryo lacks these characteristics.

To date, only one reference of egg activation by electric pulses associated to the NT has found in medaka (Bubenshchikova et al., 2007). Recently, Cibelli and collegues (Siripattarapravat et al. 2009) in a NT method developed in zebrafish refer to an activation protocol, but the fact is that they activate reconstructed eggs by only "egg water" (60µg ml^{-1} sea salt), equivalent to the usual system water employed.

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Table 1. Embryo survival rates after *in vitro* fecundation of *gold* eggs by *gold* sperm radiated for 2, 5 10 or 15 min. (Radiation dose: 0.529 mW/cm2)

Developmental stages	2 min	5 min	10 min	15 min
MBT/initial	92/138 67%[a]	73/163 45%[b]	18/102 18%[c]	3/74 4%[d]
24h/MBT	27/92 29%	32/73 44%	8/18 44%	2/3 67%
5days/MBT	27/92 29%	28/73 38%	6/18 33%	2/3 67%
Abnormal and degenerated/24h	22/27 81%	22/32 69%	4/8 50%	2/2 100%

Between columns, data with different superscripts are statistically different ($p<0.05$).

Table 2. Survival rates of reconstructed embryos derived from nuclear transplants, before (method A) or during (method B) egg activation by fresh sperm, radiated sperm or water as activating agents.

	Transplant before egg activation (Method A)			Transplant during activation (Method B)		
	Activation by fresh sperm	Activation by radiated sperm	Activation by water	Activation by fresh sperm	Activation by radiated sperm	Activation by water
Initial number	26	20	25	23	19	25
MBT/initial	11/26 42%[a]	8/20 40%[abc]	3 12%[bc]	8/23 35%[abc]	9/19 47%[a]	5/25 20%[abc]
Epiboly/initial	8/26 31%[a]	6/20 30%[a]	0 0%[bc]	4/23 17%[abc]	7/19 37%[a]	0/20 0%[b]
24h/initial	8/26 31%[c]	4/20 20%[abc]	0 0%[ab]	4/23 17%[abc]	2/19 11%[abc]	0/20 0%[b]
5days/initial	4/26 15%	0 0%	0 0%	3 13%	0 0%	0/20 0%

Between columns, data with different superscripts are statistically different ($p<0.05$).

STUDY 14. Reconstruction of heteroparental gynogenetic diploid condition by nuclear transplant in zebrafish: preliminary results.[*]

Abstract

The gametic imprinting has commonly studied in mammal. However, the limited parthenogenetic development reached by these species make difficult the use of partenogenetic embryonic cells as donor nuclei in nuclear transplant experiments. The parthenogenetic developmental ability in zebrafish allows obtaining haploid embryonic cells as well as haploid somatic cells. So, the aim of this work was the reconstruction, in zebrafish, of the heteroparental gynogenetic diploid condition by nuclear transplant of haploid somatic nuclei into metaphase II oocytes and early zygotes. Haploid somatic cells from primary cultures derived from gynogentic larvae at 24h stage were obtained. The 27% of the reconstructed embryos reached the 100% epiboly stage when early zygotes were used as recipient. From them, 2 embryos developed to 24h and 1 survived to 5 days, although it showed morphologic abnormalities. The preliminary results obtained from this work allow suggesting the zebrafish as a model system for imprinting studies.

Keywords: parthenogenetic, gametic imprinting, hemicloning, zebrafish.

[*] Manuscript in preparation.

INTRODUCTION

Since the 1940's, an incipient interest for studying the parthenogenetic development in diverse species, especially in mammals, was observed (Surani and Barton, 1983; Fundele et al., 1989). Associated to this type of development, a specific pattern of genetic expression in the germ line, called "gametic imprinting" was observed, which conditioned the development depending on the paternal or maternal embryonic cells origin (Norris et al., 1990; Surani, 1991). Gametic imprinting is a form of epigenetic gene regulation that results in expression from a single allele in a parent-of-origin dependent manner. In addition, imprinted genes undergo to wide de-methylation that involves the erasure of the epigenetic marks in the early embryo, and the novo acquisition of imprints in both male and female gametes.

The imprinting phenomenon has been commonly studied in mouse (Howlett et al., 1989; Surani et al., 1990; Ferguson-Smith and Surani, 2001). However, some limitations exists in it use due to the low haploid embryo developmental ability (only few cells), both in androgenote and gynogenote embryos, that does not reach preimplantation stage. This fact made difficult, in this specie, the heteroparental diploid reconstruction from parthenogenetic nuclei transplanted in into partially enucleated zygotes.

In our lab, rabbit parthenogenetic embryo development until more advanced stages (32-64 cells) were achieved, so these haploid cells were suitable nuclei donors to reconstruct the heteroparental diploid condition by electrofusion with a hemienucleated zygote, resulting even in alive and normal offspring (Escribá and García-Ximénez, 2001).

In zebrafish, haploid parthenogenetic larvae (even 5 day stage) can be easy obtained (Nusslein-Volard and Dahm, 2002). So, zebrafish points to be a good model for evolution studies of gametic imprinting.

There are routine techniques for androgenetic and gynogenetic haploid zygotes (thus haploid larvae) based on inactivating eggs and sperm respectively.

Nuclear zebrafish transplant (NT) techniques have been recently developed in our lab, using somatic cells (adult fibroblasts) as nuclei donors. The NT technique opens the possibility to reconstruct the heteroparental diploid condition using nuclei from cells derived from zebrafish haploid somatic cells. In this context, the development of this

technique will therefore allow studying: (i) all combinations between resident and donor nucleus (gynogenote recipients-gynogenote donor; gynogenote recipients-androgenote donor; androgenote recipients-gynogenote donor; androgenote recipients-androgenote donor); (ii) the different epigenetic status of the donors nuclei imprinting in the different developmental stages, from embryonic stage to 5 days of haploid development.

There seems to be indications about the gametic imprinting phenomenon throughout the zebrafish development which concerns to relevant genes expression as specifically occurs in mammals among other species (McGowan and Martin, 1997; Hahn et al., 2005; Tsalavouta et al., 2009).

The availability of this methodology will allow deepening in the imprinting mechanisms in this species. To this end, this work pursued the reconstruction of the heteroparental gynogenetic diploid condition by nuclear transplant in zebrafish, as a first methodological step, using mature metaphase II oocytes as recipients and nuclei from primary cell cultures derived from haploid gynogenetic larvae as donors.

MATERIAL AND METHODS

Recipient eggs.

Eggs were collected after evaluating the sexual behavior of both gold strain males and females at dawn. Eggs were obtained by gentle extrusion of the ovary (Westerfield, 2007). Then, non activated eggs were kept in egg medium at 8ºC (see *Study 9*).

Haploid somatic cells.

Gold male sperm was recovered from the genital pore in individually glass microcapillaries (Westerfield, 2007). Then, non activated gametes were kept in egg medium at 8ºC (see *Study 9*).

A sample of 100 µm of the *gold* sperm diluted in Hanks' balanced solution (see *Study 9*) was radiated using a UV 85 germicide lamp (General 86 Electric, 30W). Irradiation was carried out at 62 cm of focus-object distance. The radiation dose applied was 0.529 mW/cm2 87 and was measured by a USB 4000 (Miniature 88 Fiber Optic Spectrometer; Ocean Optics Inc. First In Photonics, USA). A vortex (MS1-92 IKA, Wilmington, CA, USA) at 200 rpm was used with the aim of homogenising the radiation area during UV 93 exposure. The time of sperm radiation was 1 min.

In vitro fertilization was carried out as Westerfield (2007) describes. Non activated eggs and sperm were mixed in a 35-mm Petri-dish and both gametes activated by the addition of a 1 mL of system water. After 2-3 min, the dish was filled with system water to achieve well developing embryos. Further culture was done at 28.5 ºC.

Larvas at 24 h that showed the "haploid syndrome" (Westerfield, 2007) were selected for *in vitro* culture.

Somatic cells used as nuclei donor came primary cultures of *gold* zebrafish gynogenetic larvae at 24h stage. The tissue was cultured in Leibovitz medium supplemented with 20% of FBS and 0.036 g/L of glutamine (L15-FBS) at 28.5ºC (Westerfield, 2007).

Somatic cell nuclear transplant.

The two somatic cell nuclear transplant techniques, as well as the equipment employed were described by Pérez-Camps et al. (see *Study 11*). Method A: nuclear transplant prior to the egg activation: The somatic cell nucleus was inserted in the central region of the egg. To prevent egg activation, the transplant was performed in a handling drop composed of egg medium and the micromanipulation area was cooled down to 8 ºC. Non activated eggs were activated by fresh water after the NT Method B: nuclear transplant simultaneously to the egg activation: the donor nucleus was injected in the incipient animal pole, just where zygote nucleus was being constituted. The handling drop was composed by fresh water so that the eggs became the activation and the micromanipulation area was not cooled (room temperature). Eggs were activated in the transplant handling medium by the water drop.

Two experimental groups were established, transplant of haploid somatic cells into the central region of the metaphase II eggs (Method A) and transplant of haploid somatic cells into the animal pole during the egg activation (Method B).

An initial fertilizability control group was done immediately when eggs were extracted to test the egg quality, whatever the time and method of transplant. Nuclear transplant sessions were considered only when the initial fertility rates were higher than 70%. Survival rates were evaluated at MBT stage, 24h and 5 days.

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.

RESULTS AND DISCUSSION

Regarding the MBT stage developmental rates, significant differences ($p<0.1$) were observed in favour the NT performed simultaneously to egg activation (Table 1). This differences were more evident ($p<0.05$) at 100% epiboly stage. The 27% of embryos transplanted simultaneously the activation reached the 100% epiboly stage, however, embryos transplanted before activation stopped their development at MBT stage (Table 1). Two of the surviving embryos at 100 % epiboly continued developing to 24 h and only one survived until 5 days. In this case, the larva showed morphologic anomalies, although it presented heartbeat.

To this respect, it is important to point that if gametic imprinting certainly exists, the embryonic condition derived from coupling two haploid female nuclei would not be the most favourable to propiciate further embryonic development. The objective of this work was the methodologic establishment of heteroparental diploid condition by nuclear transplant in zebrafish. So it was chosen gynogenetic receptor-gynogenetic donor condition, due to its intermediate situation related to the difficulty of parthenogenetic larvae obtaining between those embryos derived from gynogenetic receptor-androgenetic donor and androgenetic receptor-androgenetic donor couplings.

It has be noted that, in previous work (see *Study 11*), in which diploid adult somatic cells were used as nuclei donors, reconstructed embryos only reached MBT stage. Several reasons can be proposed for the results obtained in the present work: (i) The first more plausible explanation is the use of larval and not adult cells. In fact, in NT experiments in fish, as in mammals, the developmental rates are higher with early developmental stages donors (Lee et al., 2002; Kaftanovskaya et al., 2007; Luo et al., 2009; Siripattarapravat et al., 2009b; (ii) Other possible explanation comes from the differences in the reconstructed embryo ploidy. In first instance, the diploid nucleus transplantation into a non enucleated oocyte should result in a triploid condition, although some authors indicate that resident pronucleus degenerate after scarce cleavages (Li et al., 2000; Li et al., 2003). If same phenomenon occurs with the transplantation of an haploid nucleus, a worst embryo development should be expected. However, this is not observed after comparing the results from the present work with those obtained in the transplant of diploid adult somatic nuclei (see *Study 11*). In the case of transplanting haploid nucleus, we suggest that the pronucleous resident does not degenerate.

The developmental stage differences between the two NT methodologies assayed here (previous or simultaneously to egg activation) using haploid nuclei as donors, are not according with previous results obtained when diploid nuclei donors were used (see *Study 11*). At this moment, any plausible explanation can be given.

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Table 1. Survival rates of reconstructed embryos derived from the transplant of haploid somatic nuclei into metaphase II eggs or early zygotes in zebrafish.

	NT before egg activation	NT during egg activation
MBT*	14 %	36 %
	5/36	8/22
100 % EPIBOLY	0 %[a]	27 %[b]
	0/36	6/22

Between columns, data with different superscripts are statistically different ($p<0.05$).

* These data statistically differ at 10 % ($p<0.1$)

III.4. Zygotic Electrofusion in P. oyster.

From the beginning of doctorandus collaboration in the LARB, there were proposed some embryological and micromanipulation techniques to develop in zebrafish and P. oyster. They were expected to not solely serve for biomedical research areas (ie: biomedicine, developmental biology, cell reprogramming), but also for aquaculture. Unfortunately, events succeed further avoided their possible testing for aquacultural implications. Only the case of tetraploidy induction by electrofusion technology was finally carried out. The reasons given were many, being the financial support denial by the CICYT (from Ministerio de Ciencia e Innovación del Gobierno de España) the most severe.

So, this part 4 can be defined as aborted with respect to the initial aims expected.

Despite this short-haul proposal, there have been successfully achieved some interesting results.

Firstly, it is presented the manuscript "**Seasonal evolution of gonadal maturation, gametes quality and fertilizability of Pacific oyster (*Crassostrea gigas*) in the west coast of Mediterranean Sea"**, where the reproductive seasonality of *Crassostrea gigas* was studied throughout October'07 to November'08 in the west coast of Mediterranean Sea (Burriana, Spain). In this work we mainly pursued the gonad maturation degree and sex evaluation for the forecast availability of viable gametes (both sperm and eggs) along the year to perform technologic assays (as electrofusion). Results obtained showed that the percentage of males increased from January-February (1%) to July-August (62%) and females presented the same tendency, reaching the maximum values also in July-August (32%). Also, both males and females showed significantly maximum gonad maturation degree in July-August. The obtaining of both gametes could only be achieved from March to October, only observing normal larvae from July to October. These results indicated that oysters from different origins and maintained in natural conditions were an alternative to perform either laboratory studies or assays, but only during few months: from July to October.

The conclusions obtained in this work allowed us to define the period in which the second work from this experimental part (III.4) was then carried out. This work is entitled "**Definition of fusion medium and electric parameters for efficient zygote electrofusion in the Pacific oyster (Crassostrea gigas)**". In this work, we pursued the establishment of fusion medium and electric parameters in the electrofusion of Pacific

oyster zygotes (prior to the completion of the first mitotic division) as a novel approach to obtain tetraploid oyster by electrofusion. In this way, the fusion medium (ionic vs non ionic) together with combinations of electric parameters: electric field intensity (400 or 600 V/cm) and number of electric pulses applied (1 to 3 square DC pulses for 50 µs each at 26ºC) were defined and tested.

Original results were obtained in relation with the possibility of tetraploidy obtaining, despite further assays for their ploidy assessment must be done; and also, the unexpected conditions in which aggressive procedures as it is cell electrofusion can be performed in molluscs. For example: the better embryo response to electric stimuli when an ionic fusion medium (seawater) is used in contrast with non ionic fusion media (commonly used), transcending expected biological patterns in its use and thus allowing the use of highly ionic media as the seawater (1130 mOsm/Kg); the obtaining of a high percentage of fusion rates (up to 79 %), as well as the either embryo survivals achieved or their normal morphology to D-larval stage (around 44 %).

- The experimental development of this fourth part has been exclusively carried out by the doctorandus. In relation to the manuscripts preparation, the collaboration of the other two authors has been essential.

STUDY 15. Seasonal evolution of gonadal maturation, gametes quality and fertilizability of Pacific oyster (*Crassostrea gigas*) in the west coast of Mediterranean Sea.[*]

Abstract

Reproductive seasonality of *Crassostrea gigas* was studied throughout October'07 to November'08 in the west coast of Mediterranean Sea (Burriana, Spain). The gonad maturation degree and sex were assessed. When both gametes were present fertilization assays were performed.

The percentage of males increased from January-February (1%) to July-August (62%). Females presented the same tendency, reaching the maximum values also in July-August (32%). Both males and females showed significantly maximum gonad maturation degree in July-August. The obtaining of both gametes could only be achieved from March to October, only observing normal larvae from July to October.

Breeding periods established in this work coincided with those reported by other authors in different coastal regions of the Mediteranean and Atlantic seas. These results indicated that oysters from different origins and maintained in natural conditions are an alternative to perform either laboratory studies or assays, but only during few months: from July to October.

Keywords: Crassostrea gigas, seasonality, sex ratio, fertilizability, gonadal maturation.

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INTRODUCTION

Development of reproductive technologies in Pacific oyster (*Crassostrea gigas*), require the gametes availability, but the reproductive seasonality of this species (Utting and Millican, 1997) limits their obtaining. Such problem can be avoided by subjecting oysters to an artificial conditioning in a controlled area. This option brings the opportunity to obtain gametes regularly along (almost) the year (Chávez-Villalba *et al.*, 2002), but the oyster conditioning requires both highly water volumes and renovation rates (Helm and Bourne, 2004; Utting and Millican, 1997), being the seawater availability/proximity and its quality another important limitation. Also, the artificial feeding of oysters along their conditioning period becomes one of the major costs (Helm and Bourne, 2004).

An option to assure both gametic and embryonic material regularly throughout the year is the gametes (both sperm and oocytes) and embryo cryobanking, as it has been proposed many times (Adams *et al.* 2004, Smith *et al.* 2001, Tervit *et al.* 2005).

Whatever the case, under the perspective of facilitating the study and development of laboratory techniques that require Pacific oyster gametes, the study of the natural period of the complete gonad development is essential.

According with this, the study of availability and quality of gametes obtained from animals throughout the year in the west Mediterranean Sea climatic conditions constituted the objective of present work, because to our knwoledge, only a work studied the gonadal maturation evolution *Crassostrea gigas* in the Mediterranean Sea, Tunisia (Dridi *et al.*, 2007).

MATERIALS AND METHODS

Oyster source

The experiment was carried out from October'07 to November'08.

Two-three year old Pacific oysters (*Crassostrea gigas*) were provided weekly from a commercial long-line culture (ACUIMA S.L), located in Burriana, Comunidad Valenciana, Spain with an annual temperature range at 10m deep of 11.2 ºC in December to 26 ºC reached between July-August. After the oyster collection, they were transported to our lab, located at 60 Km from the company, where they were carefully cleaned and stored "dry" in a fridge at 4-5 ºC until their gonadal evaluation.

Gonad maturation assessments

The *apparent gonad maturation degree* was assessed by visual inspection after removing the flatter shell valve. Each oyster gonad was ascribed to one of the five maturation degrees established and described as follows: “NO”: empty gonad; “EA”: early gonad activity, there are white ramifications in the gonad; “BF”: gonad begins to fill, the digestive glandule is perfectly seen; “PD”: gonad poorly developed, the digestive glandule is partially seen; “D”: gonad developed, it covers perfectly the digestive gland; “TD”: gonad totally developed, it stands voluminously. These gametic developmental stadiums are a modification of those previously described by Marteil in 1976.

Gonads from all oysters except those classified as “NO” were biopsied with a sterile hypodermic needle and gametes were removed by exerting gentle suction with a sterile Pasteur pipette inserted beneath the overlying gonad epithelium (Helm and Bourne, 2004). After this, the sex recognition was assessed by the presence of spermatozoa or oocytes under a 100x magnification microscope (Davenela *et al.* 2006).

Fertilization assays

Fertilization assays were only realized when both sperm and oocytes were obtained in the same session and were replicated thrice. They were carried out independently of their *apparent gonad maturation degree.*

Males were only selected when sperm had a minimum of 70 % motility with a high degree of lineal motility. Motility was estimated visually at 200x magnification and was expressed as the percent of spermatozoa actively moving in a forward direction. Sperm vibrating in place were not considered to be motile (Dong *et al.*, 2005a). On the other hand, in the selected females, the viable oocytes (defined as fertilizable oocytes) were around 30-60 µm diameter (Lango-Reynoso *et al.* 2000) and rounded out completely some minutes after their first contact in seawater, thus observing the germinal vesicle breakdown at 100x magnification (Dong *et al.*, 2005a, Eudeline *et al.,* 2000a; Eudeline *et al.,* 2000b). In both males and females, the cleanliness of samples obtained was also a quality criterion.

Recovered sperm was diluted with microfiltered seawater (0.22 µm Millex GP Filter Unit; Millipor Express) in order to make a “cloudy suspension” (Helm and Bourne, 2004). The procedure carried out to clean oocytes consisted of introducing them into a 20 ml sterile vial with microfiltered seawater (0.22 µm Millex GP Filter

Unit; Millipore Express) and replacing the supernatant with microfiltered seawater once the oocytes dropped to the recipient bottom (around 10 min). After 2-3 washes by this procedure, oocytes looked clean and well rounded out.

Fertilization took place in 3.5 cm Petri dishes (corning, Sigma) with microfiltered seawater at room temperature. In each plate, around 50 oocytes were deposited and sperm was added, achieving a final concentration of 1-10 spz/oocyte (Cadoret, 1992; Chao *et al.* 1997). After the 1st and 2nd PBs expulsion, the plates were incubated at 28 ^{0}C (Chao *et al.* 1997; Lin *et al.* 1999) for 24 h. Then, it was noted the presence or not of D-larvae (either normal or abnormal) in plates.

Experimental design

Obtained data were grouped and presented bimonthly to avoid an excessive data dispersion, therefore 6 temporal periods were established: January-February, March-April, May-June, July-August, September-October, November-December.

The effect of these different annual periods was analysed separately in both males and females on the following aspects: The number of both identified males and females from the total; the number of both males and females showing maximum *apparent gonad maturation degree* (D+TD) from the total number of both recognised males and females; the number of selected males with more than 70% motile sperm with high lineal motility and the number of selected females with fertilizable oocytes.

Finally, and in relation to the gametes availability to perform fertilization assays, there were recorded these sessions where only sperm, only oocytes or both gametes were obtained, as well as their temporal unavailability throughout the year. Also, when both gametes were present there were carried out fertilization assays, and was registered the number of those assays where the either normal (D-shaped larvae after 24h culture) or abnormal larvae were obtained. Also, the effect of *apparent gonad maturation degree* on males and females rates with viable gametes was also estudied.

Results were analysed with the Chi-square test. When a single degree of freedom was involved, Yates' correction for continuity was performed.

RESULTS AND DISCUSSION

In relation with the effect “annual period”, results obtained are shown in Table 1A. In the percentage of sampled specimens identified as males throughout the different annual periods, it was shown an increasing progression from the lowest values registered in January-February (1%) until the maximum values, corresponding to the period of July-August, reaching a 62% of total specimens recognised as males (Table 1A).

In relation with the percentage of sampled specimens identified as females, it was observed the same tendency as in males, reaching the maximum values of recognised females also in July-August (32%); although their registered values were substantially lower than in males for all annual periods assessed. These imbalanced values in favour to males could be explained as consequence that many bivalves will mature in their first year of life as males; further, an increasing percentage may switch sex and become females (protandric hermaphroditism, Helm and bourne, 2004).

The percentage of males with the maximum *apparent gonad maturation degree* (D+TD) from the total of identified males was significantly higher in July-August (Table 1B). The same statement is given in the case of females with maximum (D+TD) *apparent gonad maturation degree*.

The rate of selected males (with a minimum of 70% motile sperm) did not significantly vary throughout all annual periods (Table 1C). Although in the case of females, there existed significant differences in the rate of selected females (with fertilizable oocytes) in favour to the whole period from May-October.

In the reviewed bibliography, only one work was found in relation with the gametogenic cycle of *C. gigas* in the Mediterranean area, concretely in Tunisia (Dridi *et al.*, 2007). In this work, the phase of gonad inactivity coincided with such here reported (November-February, Table 1A) as well as the concordance in months related to the major or minor frequency in the detection of a percentage of sexually active specimens. Seems to be that not strictly identical climatic conditions do not affect in a determinant way the reproductive phases in this species. In fact, Dridi *et al.*(2007) proposed the food availability and the energy and protein intake ability of oysters, as one of main factors in the seasonal variations of reproductive activity. This argument would explain that breeding periods established in this work, and in coincidence with those reported by Dridi *et al.* (2007), coincided also with those described for the French atlantic coasts by

Chávez-Villalba (2002). Other authors (Enríquez-Díaz *et al.*, 2007) agreed on the food availability as an important effect on the *C. gigas* breeding periods, but they also detected that breeding periods also both significantly and directly depended on the variations of climatic conditions.

The percentage of selected males (with a minimum of 70% motile sperm) did not show significant differences depending on the *apparent gonad maturation degree* (Table 2), varying from 45-50 % in groups EA and BF to the 63-66 % in the groups classified as PD, D, TD. In the case of females, regarding to fertilizable oocytes, there were detected significant differences among groups (Table 2). However, a coherent pattern was not established, probably because the lower number of sampled specimens in some groups. Despite this, the major values regarding these fertilizable oocytes seemed to be finally ascribed to the group TD, being also of importance the fact that a minimum of 50% females from each group showed fertilizable oocytes.

Many technologic assays of interest require both sperm and oocytes availability. For example: cryopreservation of sperm, oocytes and embryos (Chao *et al.*, 1997; Dong *et al.*, 2005; Tervit *et al.*, 2005), embryo/larvae obtaining for toxicologic assays (da Cruz *et al.*, 2007; Stachowski-Haberkorn *et al.*, 2008) or embryo electrofusion, transfection and poliploidy induction studies (Buchanan *et al.*, 2001; Cadoret *et al.*, 1992; Guo *et al.*, 1993; Guo and Allen, 1994) among others. To this respect, in Table 3 the obtaining of both gametes could only be achieved in only 16 sessions from 29. Moreover, in only 11 sessions from the 16 mentioned there were obtained normal larvae (D-shaped larvae at 24 h) while the other 5 remaining sessions, the obtained larvae were abnormal. Abnormal larvae were detected essentially in March-June and all normal larvae were obtained from July to October. This result would reinforce previous observations reported by Massapina *et al.* (1999), which stated that oocytes quality determines also the quality of obtained larvae. Because of the reduced number of data from each annual period, statistical analysis was not performed. So their significance on the gametes availability from each session or in the obtaining of normal larvae when both sperm and oocytes were available could not be given.

The methodology of assessment proposed here is based in the *apparent gonad maturation degree*, the gametes presence or not in the gonad and their characteristics regarding to sperm movement and oocytes ability to rounding out after their contact

with seawater. Also, it was assessed their fertilization and further normal development to D-stage. All these parameters are of minor technical complexity in contrast with other methodologies (histological: Chávez-Villalba *et al.*, 2002; Dridi *et al.*, 2007; Enríquez-Díaz *et al.*, 2009; Lango-Reynoso *et al.*, 2000; Massapina *et al.*, 1999; biochemical: Dridi *et al.*, 2007; Lubet, 1959; Massapina *et al.*, 1999; Magnetic resonance imaging, MRI: Davenela *et al.*, 2006; Toussaint *et al.*, 2005). Moreover, the evaluation methodology do not impede the comparison of both gonad developmental degree and presence of gametes with their final quality assessment by IVF and their further obtaining of normal larvae in vitro.

Given results supported the possibility to assessing or foreseeing the either gametes or embryos availability for other type of studies, where the relevant objective is not the gametogenic evolution but the real gametes availability. In this sense, they also indicated that oysters from commercial cultures are an alternative to perform either laboratory studies or assays, but only during few months: from July to October. These results and methodologies could be of interest to labs which do not dispose of adequate installations or insuficient seawater of quality availability for the conditioning and maintaining of *C. gigas* (Chávez-Villalba *et al.*, 2002; Utting and Millican, 1997), although it is still indispensable the gametes cryopreservation if the gametes availability is required along the year.

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Table 1A. Effect of annual period on the percentage of recognised males and females from all specimens assessed.

	Annual period					
	J-F	M-A	M-J	J-A	S-O	N-D
Nº males from	1 %^{a}	14 %^{bc}	43 %^{d}	62 %^{e}	17 %^{c}	7 %^{b}
total specimens	1/127	20/146	61/141	68/110	44/254	8/109
Nº females from	0 %^{a}	12 %^{b}	13 %^{b}	32 %^{c}	6 %^{b}	0 %^{a}
total specimens	0/127	17/146	18/141	35/110	16/254	0/109

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

Table 1B. Effect of annual period on the percentage of males and females that showed maximum "*apparent gonad maturation degree*" from the total number of either recognised males or females, respectively.

	Annual period					
	J-F	M-A	M-J	J-A	S-O	N-D
Nº males fully mature	0 %^{abc}	35 %^{ab}	43 %^{a}	88 %^{c}	14 %^{b}	0 %^{ab}
(D+TD) from total males	0/1	7/20	26/61	60/68	6/44	0/8
Nº females fully mature	-	47 %^{a}	50 %^{a}	92 %^{b}	44 %^{a}	-
(D+TD) from total females	0/0	8/17	9/18	35/38	7/16	0/0

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

Table 1C. Effect of annual period on the percentage of selected males and females with quality gametes from the total number of either recognised males or females, respectively.

	Annual period					
	J-F	M-A	M-J	J-A	S-O	N-D
Selected males/	100 %	70 %	62 %	53 %	64 %	25 %
Total males	1/1	14/20	38/61	36/68	28/44	2/8
Selected females/	-	6 %[a]	72 %[b]	92 %[b]	100 %[b]	-
Total females	0/0	1/17	13/18	35/38	16/16	0/0

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

Table 2. Effect of *apparent gonad maturation degree* on the obtaining of both males and females with quality gametes from the total number of either recognised males or females, respectviely.

	Apparent gonad maturation degree				
	F	MP	PD	D	TD
Selected males/	45 %	50 %	63 %	66 %	63 %
Total males	18/40	11/22	26/41	35/53	29/46
Selected females/	60 %[ab]	83 %[ab]	47 %[a]	63 %[a]	97 %[b]
Total females	3/5	5/6	9/19	17/27	31/32

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

Table 3. Effect of annual period on the gametes availability from each session and in the obtaining of normal D-shaped larvae by IVF when both sperm and oocytes were obtained.

		Annual period						
		J-F	M-A	M-J	J-A	S-O	N-D	TOTAL
Gametes availability per session	Both gametes	0/4	1/4	4/6	5/5	6/10	0/4	16/29
	Only oocytes	0/4	0/4	0/6	0/5	0/10	0/4	0/29
	Only sperm	1/4	2/4	2/6	0/5	4/10	2/4	10/29
	None	3/4	1/4	0/6	0/5	0/10	2/4	3/29
Larvae (normal and abnormal) obtaining from fertilisation assays (0,1)	NOR	0/0	0/1	0/4	5/5	6/6	0/0	11/16
	ANOR	0/0	1/1	4/4	0/5	0/6	0/0	5/16

STUDY 16. Definition of fusion medium and electric parameters for efficient zygote electrofusion in the Pacific oyster (*Crassostrea gigas*).[*]

Abstract

Cell electrofusion has been widely used in the induction of tetraploidy in mammals, but little attention has been paid in molluscs. This work pursued the establishment of fusion medium (ionic vs non-ionic) and electric parameters in the electrofusion of Pacific oyster zygotes (prior to the completion of the first mitotic division), minimizing all deleterious effects possible to D-larval stage. The tested combinations of electric field intensity (Vcm^{-1}) and number of square DC pulses applied (for 50 µs each) were (Voltage x Nº pulses): 400x1, 400x2, 400x3 and 600x1, 600x2, 600x3.

When pulses were applied for first time, there was detected that an ionic fusion medium (microfiltered seawater) offered better conditions than the non ionic fusion media previously used (0.6 M sucrose or 0.6 M mannitol) regarding embryo survival and lysis rates. In this fusion medium, two different combinations of electric parameters (3 square DC pulses of 400 Vcm^{-1} for 50 µs each at 26ºC and 1 square DC pulse of 600 Vcm^{-1} for 50 µs at 26ºC) offered the best technical results of fusion (57 and 79 % respectively) and survival until D-larva (44 and 41 % respectively). In conclusion, these electric parameters could be established, using seawater as electrofusion medium, for further approaches to evaluate individual ploidy and survival beyond spat.

Keywords: Cell electrofusion, Zygote, Embryo, Pacific oyster, *Crassostrea gigas.*

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INTRODUCTION

Triploid oysters are useful for aquaculture because of reduced gamete output (functional reproductive sterility) and improved meat quality and growth (Dong *et al.*, 2005). To accomplish this objective, different procedures have been assayed with low and variable efficiencies: Cytochalasin B treatments have been perfected over time so that success rate in producing triploids was about 90%, but it has been concern of repercussions from the public (García-Ximénez *et al.*, 1994) because this product is not only expensive but carcinogenic (Okumura *et al.*, 2007). Other procedures commonly used are thermal and pressure shocks but they present disadvantages in equipments and handling (Okumura *et al.*, 2007). To date, the only available method that achieves efficacies of 100% triploid production is mating tetraploid and diploid oysters (Helm and Bourne, 2004). Therefore, the obtaining of tetraploid oysters has an evident commercial interest for triploid seed production (Guo, 2004; Nell, 2002), and different approaches to tetraploidy induction have been made (Nell *et al.*, 1998), being the inhibition of polar body 1 in eggs from triploids the most effective option (Guo and Allen, 1994).

In mammals, the obtaining of tetraploid embryos by electrofusion of the two first blastomeres has been widely used (Elsheikh *et al.*, 1995; García-Ximénez *et al.*, 1994; Ozil and Modlinski, 1986; Rickords and White, 1992). This method reaches high fusion rates (90%, Ozil and Modlinski, 1986) and thus becomes the established method of tetraploid obtaining more efficient in mammals. From these observed results, the major vantage of electrofusion in Oyster would be the direct obtaining of tetraploid specimens from diploids unlike other processes which suppose more previous steps as it is the obtaining of triploids or the required use of chemicals as cytochalasin B (Guo and Allen, 1994). However, the extrapolation to molluscs has been only assayed in some detail by Cadoret (1992) in oyster and mussel. Unfortunately, results obtained from that work did not clearly validate their electrofusion technique in relation with the parameters and conditions employed. To this respect, one of difficulties arose was the definition of a non ionic electrofusion medium with a compatible osmolarity with embryo survival. This medium should also avoid embryo floatage in the fusion chamber. Moreover, that work did not raise the possibility of using ionic media, which are successfully used in mammalian fusion experiments (Rickords and White, 1992). However, non conductor media are the most habitual; perhaps it is because they permit

dielectrophoresis while ionic media cause electrohydrolysis and warming phenomena (Chang *et al.*, 1992).

It can be taken into account that in mollusc embryos at different developmental stages from zygotes (prior to completion of the first cell division) to even 4-cell stage could be present at the same moment after initial fecundation of high number of eggs, making it impossible to monitor at which developmental stages the embryos were pulsed. The reasons for this were their cleavage speed in the first cell cleavages, as well as the embryo asynchrony in fertilization (Lin *et al.*, 1999; Park *et al.*, 1989). Moreover, at 26 ºC, after the immediate observation of the 2-cell embryo, the second mitotic division was already taking place. This reason forced us to apply the electric fusion pulse few moments before the cytokinesis completion of the first mitotic division (Cadoret, 1992).

Based on this, the present work aimed to evaluate different media and electrical parameters for two nuclei zygote electrofusion. In this way, the oyster embryos were used prior to their completion of the first cell cleavage and results were assessed by monitoring the percentage of fused embryos and their survival until the D-larval stage.

MATERIALS AND METHODS

Oyster source

The experiments were carried out in the middle of the breeding period (from June to September) of the oyster *Crassostrea gigas* in our climatic area.

Oysters were provided weekly by ACUIMA S.L. (Burriana, Comunidad Valenciana, Spain). They were transported to our lab, located at 60 Km from the company, where they were carefully cleaned and stored “dry” in a fridge at 4-5 ºC until use. Oysters remained refrigerated for a maximum of 5 days.

Obtaining of gametes

Gametes were obtained as recommended by the FAO (Helm and Bourne, 2004). Briefly, oysters were opened by removing the flatter shell valve and after validating adequate gonad maturation (Marteil, 1976), the gonad was biopsied with two sterile hypodermic needles. Gametes were removed by exerting gentle suction with a sterile

Pasteur pipette inserted beneath the overlying gonad epithelium under the maximum possible sterility conditions.

Males were only selected when sperm had a minimum of 70 % motility with a high degree of lineal motility. Motility was estimated visually at 200x magnification and was expressed as the percent of cells actively moving in a forward direction. Sperm vibrating in place were not considered to be motile (Dong *et al.*, 2005). On the other hand, the selected females had viable eggs (around 40-60 µm diameter; Lango-Reynoso *et al.*, 2000) and rounded out (activated) completely some minutes after the first contact in seawater, thus observing the germinal vesicle breakdown at 100x magnification (Dong *et al.*, 2005; Eudeline *et al.*, 2000a; Eudeline *et al.*, 2000b). In both males and females, the cleanliness of samples obtained was also a selection criterion.

For each daily experimental session, the lower number of oysters used to minimize individual variability was two males and two females (Dong *et al.*, 2005; Tervit *et al.*, 2005).

Recovered sperm was diluted with microfiltered seawater (0.22 µm Millex GP Filter Unit; Millipore Express) in order to make a "cloudy suspension" (Helm and Bourne, 2004). The procedure carried out to clean eggs consisted of introducing them into a 20 ml sterile vial with microfiltered seawater (0.22 µm Millex GP Filter Unit; Millipore Express) and replacing the supernatant with microfiltered seawater once the eggs dropped to the recipient bottom (after about 10 min). After 2-3 washes by this procedure, eggs looked clean and well rounded out.

Both sperm and eggs were stored separately at 4-5 ºC along each session (Dong *et al.*, 2005; Tervit *et al.*, 2005). The storage time did not spend more than two hours in any case, and no gamete quality alteration was detected (results not published).

Obtaining zygotes for electrofusion

Fertilization took place in 3.5 cm Petri dishes (corning, Sigma) with microfiltered seawater at room temperature. In each plate, around 1000 to 2000 eggs were deposited and sperm was added, achieving a final concentration of 1-10 spz/egg (Cadoret, 1992). Fertilization success was evaluated by the observation of the 1st and 2nd PBs expulsion. So, only gametes that showed a fertilization rate higher than 70 - 80 % after mixing them were used for experiments. Throughout each daily session, several fertilization groups (plates) were established sequentially to achieve a sufficient and continuous number of zygotes.

In all experiments, the embryos selected for electrofusion were zygotes conformed after the 1st and 2nd PBs expulsion (30 min post-fecundation at 26 ºC) but previous to the completion of the first mitotic division (45-50 min post-fecundation at 26 ºC). Embryos at 2-4 cell stage or higher were not used in any case. The control group was composed of a part of the same zygotes from each batch that would serve for electrofusion, but without treating them.

Electrofusion

The electrofusion equipment consisted of an Electro Cell Manipulator (ECM 2001; BTX) complemented with a digital oscilloscope from Tektronix. The chamber fusion used was model 450 from ECM.

The first step of this procedure consisted of the careful selection of 2 to 7 zygotes (for each fusion batch) which had already shown the expulsion of 1st and 2nd PBs (30 min post-fecundation at 26ºC) but in a previous stage to the completion of the first mitotic division (45-50 min post-fecundation at 26ºC). With the aid of a Pasteur pipette, previously stretched and fire polished, zygotes were pre-equilibrated in the electrofusion medium prior to transfer to the fusion chamber (sucrose, mannitol or seawater; see experimental design). Then, zygotes were carefully held in the fusion chamber. They were perpendicularly oriented to the electrodes with the aid of an alternate current (AC) pulse (dielectrophoresis) in non conductive fusion media or manually, in ionic medium, depending on the assay carried out. Then, the electric fusion pulse (see experimental design) was applied. This pulse was systematically verified through the oscilloscope. Finally, treated zygotes were transferred to Petri dishes (corning, Sigma) with microfiltered seawater at room temperature where they were cultured until D-larval stage at a density of less than 5 larvae per ml.

In all cases, fusion success and lysis were assessed by monitoring the zygote changes produced after applying the electric pulse. These changes were seen under microscope at 200x magnification as soon as a few minutes after applying the pulse as Cadoret described: Briefly, the resulting 1-cell embryos (fused embryos) first became oval and then almost completely rounded out (Cadoret, 1992).

The survival assessments took place at 10-15 min post-pulse, at 1 h and at 24 h post-fecundation (D-larva).

Experimental Design

Three experiments were performed sequentially:

Experiment I

In the first experiment, conditions and electrofusion parameters proposed by Cadoret (1992) were tested. In addition, an alternative fusion medium, based on the use of mannitol instead of sucrose, and a previously established AC pulse (dielectrophoresis: 6 V for 5 s) were also incorporated, in order to achieve a proper alignment of zygotes with the fusion chamber electrodes prior to the electric fusion pulse (DC).

The fusion media 0.6 M sucrose and 0.6 M mannitol both prepared in Milli-Q water. The fusion pulse consisted in all cases of a square DC pulse of 600 Vcm^{-1} for 50 µs at 26 ºC. The voltage applied with the fusion chamber employed was 30 V.

Experimental groups (zygotes pulsed in sucrose or in mannitol) were compared together and also with those control non-pulsed groups (zygotes 5 min in mannitol; zygotes 5 min in sucrose; zygotes in seawater). Lysis, fusion rates and further developmental rates at 1 h and at 24 h (D-larvae) were evaluated.

Experiment II

Results obtained in Experiment I proved, in our hands, that experimental conditions proposed by Cadoret (1992) and other authors cited in his work were not suitable for oyster (Crassostrea gigas) zygotes. The fusion medium composed of sucrose (or mannitol) penalized embryo survival at a high level. In consequence, sterilized seawater by microfiltration (0.22 µm Millex GP Filter Unit; Millipore Express) with an osmolarity of 1130 $mOsmKg^{-1}$ (measured with a Cryoscopic Osmometer, Osmomat 030) was tested as ionic fusion medium. In accordance with this, the parameters of the electric treatment employed in this experiment were as follows: The fusion pulse consisted of 1, 2 or 3 consecutive square DC pulses of 600 Vcm^{-1} or 400 Vcm^{-1} for 50 µs at 26 ºC. The voltage applied with the fusion chamber employed was 30 V vs 20 V. In this way, a total of 6 experimental groups were established in this experiment (Voltage x Pulses: 20x1, 20x2, 20x3 and 30x1, 30x2, 30x3).

Developmental rates of pulsed (fused or not) zygotes until D-larva (24 h) were compared with developmental rates of not pulsed zygotes group.

Experiment III

III-a

Fusion rates and timings of the first mitotic cleavages of pulsed and non-pulsed synchronous zygotes were evaluated. Applied pulses were those established as the more suitable in Exp. II. Cleavage timings were assessed in each group by counting the number of embryos with four or more counted cells about 15-20 min after applying the pulse.

Development until D-larvae was compared between non-pulsed control groups and zygote pulsed following the 30x1 and 20x3 pulse sequences.

III-b

With the aim of achieving more accuracy in terms of the possible treatment effect on cell division dynamics, this experiment only compared the cleavage timings of the control (not pulsed) with those embryos that clearly and completely rounded out (fused) after pulsing them. Cleavage timings were assessed in each group by counting the number of embryos with four or more counted cells about 15-20 min after applying the pulse.

Given its complementary condition with experiment *III-a*, the survival rates were not assessed in this case.

Statistical analysis

In all experimental groups from each experiment, a minimum of three replicates were done. In each experiment, the rates of zygote fusion and/or zygote lysis and/or embryo development were compared between experimental groups by the Chi-square test. When a single degree of freedom was involved, Yates' correction for continuity was performed.

RESULTS

From Experiment I:

The two fusion media used in this first experiment (0.6 M sucrose, 750 $mOsmKg^{-1}$; 0.6 M mannitol, 650 $mOsmKg^{-1}$) were hyposmolar with seawater (1130

$mOsmKg^{-1}$). However, due to their high density, the embryos floated in both fusion media, thus making difficult their handling (Cadoret, 1992).

Results are presented in Table 1. Significant differences in the lysis rate post-pulse were not observed between sucrose and mannitol, but in the cell-fusion rate was in favour of sucrose. As was observed, the media osmolarity conditioned the embryo survival, even after short exposures (5 min). In fact, the scarce difference in osmolarity between 0.6 M sucrose (750 $mOsmKg^{-1}$) and 0.6 M mannitol (650 $mOsmKg^{-1}$) involved significant differences in normal developmental rates at 1 h between them and when compared with the fresh seawater control group.

Based in the results obtained in this experiment, it was concluded that the conditions and fusion medium proposed by Cadoret were not at all appropriate. In his work, Cadoret assured that survivals of 90 % or higher could be achieved with 0.6 M sucrose. However, in our hands the use of this medium (or 0.6 M mannitol) involved a total failure, so microfiltered seawater was proposed as an ionic fusion medium (despite the fact that it is a conductor medium) because it maintained isosmolar conditions with the embryo culture medium, the seawater.

From Experiment II:

The final fusion medium used (microfiltered seawater of 1130 $mOsmKg^{-1}$) ruled out the use of dielectrophoresis (by AC) to achieve a correct alignment of embryos with the fusion chamber electrodes. The reason was, as expected, the generation of an instantaneous warming effect of the fusion medium due to its ionic (and thus current conductor) properties and favouring subsequent damage to embryos. On the other hand, the generation of electrohydrolysis with this fusion medium should be assumed in the rest of experiments.

Different sequences were tested with this fusion medium (1, 2 and 3 square DC pulses of 20 V or 30 V for 50 µs). The results are presented in Table 2. The fusion treatments of 2 and 3 square DC pulses of 30 V were discarded because they generated an excessive electrohydrolysis phenomenon in electrodes of the fusion chamber with their subsequent liberation of oxygen into the fusion medium. The 20x1, 20x2 and 20x3 experimental groups presented similar results among them and with the non-pulsed control group. Because the fusion rates expected are higher when the electric stimuli increase (Chang *et al.*, 1992), the 20x3 and 30x1 groups were selected for the subsequent assays.

It is important to point out that in all experimental groups assayed with this conductor medium, a chorion detachment was observed in opposite embryo poles after applying the pulses (zygotes were manually oriented perpendicularly to the electrodes of the fusion chamber). Another relevant aspect was the low lysis rate detected when results of this experiment were compared with those of Exp. I (only 3 embryos from 368 lysed; results not shown in tables).

From Experiment III

III-a

The rate of fused embryos was significantly favourable to the sequence 30x1 (30X1: 79 %; 33/42 vs 20X3: 57 %; 25/44. $p<0.05$). Otherwise, as shown in Table 3, when the cleavage timings were assessed simultaneously in pulsed (fused and non-fused) and in control (non-pulsed zygotes), no significant differences were detected with neither the 30x1 sequence nor the case of 20x3 sequence.

The observed differences in the percentage of pulsed zygotes that reached D-larva stage at the same time compared with their respective controls (non-pulsed zygotes) did not reach levels of significance.

III-b

Likewise, in this last assay no statistical differences were observed between fusion rates of the 30x1 (79 %, 34/43) and 20x3 (64 %, 30/47) experimental groups, although these differences were in favour of the 30x1 group, according to the results observed in the *III-a* experiment in which given differences reached levels of significance.

When the cell-division timings were compared between fused zygotes and control (non-pulsed) groups at the same given time, no significant differences were detected neither in the 30x1 group nor the 20x3 group. In fact, these results reinforce the comments in experiment *III-a*, where the application of electric pulses do not involve an alteration in the cell-division ability of fused embryos.

DISCUSSION

This work pursued the establishment of conditions and parameters in the electrofusion of oyster zygotes, minimizing all deleterious effects possible to D-larval stage.

As described, the pulses finally applied and which provided the best results were done in an ionic fusion medium: microfiltered seawater. This innovation was introduced after observing the lethal effects occasioned by the use of non-ionic fusion media (sucrose and mannitol) in contrast with the results obtained by Cadoret. Although the application of electric pulses in an ionic media is possible it is not the usual method (Chang *et al.*, 1992). In this respect, ionic media have been used successfully for cell electrofusion in mouse embryos, obtaining similar results to non-ionic media (Elsheikh *et al.*, 1995; Rickords and White, 1992). Certainly, the main drawback of the ionic fusion medium was related with the impossibility of using dielectrophoresis (AC), requiring the manual alignment of embryos.

It is important to point out that the present work avoided the strategy of large-scale pulses followed by Cadoret. The cleavage speed in the first cell divisions as well as the embryo asynchrony in fertilization made it impossible to take control of the different developmental stages where embryos were pulsed and so, assessment on the ploidy condition at an individualized level was not possible. Therefore, these treated embryos could present mixoploidy with diploid, triploid and tetraploid cells.

It is known that the cell fate in molluscs is highly specified, even from earlier embryonic stages (Lambert, 2008) and so, the fusion of the two first cells (with the subsequent 1-cell embryo formation) could affect embryo morphology and its immediate or subsequent survival. However, in the present work, the fused embryos obtained were of good morphology, at least at the D-larval stage.

Conclusions

Once the fusion medium and electric parameters that provide, in principle, the best technical results (both 20x3 and 30x1 groups in seawater as fusion medium) for the electrofusion of oyster zygotes were defined in this work, it is important to assess in future steps the survival ability to adult stage of fused embryos or, at least, the percentage that overcome the metamorphosis stage before the evaluation of their ploidy condition, evaluated individually.

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Table1. Experiment 1. Survival and normal developmental rates of treated embryos, using two different non-ionic fusion media.

	10 min		1 h		24 h (D-larva)	
Fusion Medium	Lysed	Fused	Norm	Abnorm	Norm	Abnorm
Pulsed in mannitol	27%[a] (7/26)	15%[a] (4/26)	0%[a] (0/4)	(2/4)	(0)	(0)
Pulsed in sucrose	24%[a] (10/42)	31%[a] (13/42)	31%[a] (4/13)	(8/13)	(0)	(3)
5' in mannitol	-	-	28%[a] (20/71)	(27/71)	(0)	(3)
5' in sucrose	-	-	69%[b] (24/35)	(11/35)	(0)	(2)
Seawater	-	-	97%[c] (76/78)	(2/78)	49% (38/78)	(3/78)

Values in rows with different superscripts are statistically different ($p<0.05$).

Table 2. Experiment 2. Survival of (fused and not fused) embryos obtained after pulsing zygotes under different electric fusion parameters (Voltage x Pulses), using microfiltered seawater as ionic fusion medium.

	No pulse (Control)	Experimental groups: Pulse parameters (Voltage x Nº pulses)					
		20x1	20x2	20x3	30x1	30x2	30x3
Survival at 24 h	57%[a] (76/133)	58%[ac] (32/55)	47%[ad] (34/72)	44%[ae] (22/50)	41%[bcde] (26/64)	33%[bdeg] (21/63)	19%[fg] (12/64)

Values in columns with different superscripts are statistically different ($p<0.05$).

Table 3. Effects of electrofusion 30x1 and 20x3 pulse sequences on cleavage timings of pulsed (fused and non-fused) and control (non-pulsed) zygotes, as well as the percentage of D-larval stage achieved. Cleavage timings were assessed in each group by counting the number of embryos with four or more counted cells about 15-20 min after applying the pulse.

	Cell division timings		D-larva stage	
Exp. groups	Pulsed (fused or not)	Non-pulsed	Pulsed (fused or not)	Non-pulsed
30x1	41% 17/42	44% 18/41	29% 12/42	51% 21/41
20x3	25% 11/44	42% 19/45	39% 17/44	51% 23/45

For each parameter, statistical analysis was performed between values in columns. No statistical differences were observed ($p \geq 0.05$).

Table 4. Effects of electrofusion 30x1 and 20x3 pulse sequences on cleavage timings. Cleavage timings were assessed in each group by counting the number of embryos with four or more counted cells about 15-20 min after applying the pulse.

	Cell division timings	
Experimental groups	Pulsed (only fused)	non-pulsed (control)
30x1	38% 13/34	38% 11/29
20x3	20% 6/30	27% 9/33

Values in columns did not differ statistically ($p \geq 0.05$).

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Conclusions.

Cell Cryopreservation in zebrafish:

- The vitrification of caudal fin explants allows their preservation and further establishment of primary cell cultures of fibroblasts.
- It is possible to vitrify blastomeres from MBT zebrafish embryos, although their larger size after thawing make difficult to perform germ-line chimaerism.
- The vitrification of testicular tissue pieces is more efficient than isolated cells cryopreservation. Despite this, their further integration in germ-line hosts was not achieved.

Chimaerism in zebrafish:

- The both embryo penalization by ultraviolet radiation and the handling medium osmolarity of 300mOsm/kg during micromanipulation are determinant for a 50% germ-line chimaerism obtaining.
- A strain effect was detected regarding UV sensitivity bethween the two strains used (*wild* or *gold*) in experiments.

Nuclear transplant in zebrafish:

- The previously reported ageing patterns in both eggs and sperm are inappropriate. They are excessive in the case of eggs and lower in the case of sperm.
- There have been developed three cell nuclear transplant strategies: before, during and after egg activation.
- The most efficient egg activating stimulus is provided by non-genetically inactivated sperm. It is followed by inactivated sperm and the lower activating stimulus is provided by the only water contact.
- The egg electroactivation is possible in ionic medium, although when eggs are previously micromanipulated; this electroactivation treatment induces their total lysis.
- The reconstruction of heteroparental gynogenetic diploid condition by haploid cell nuclear transplant into non enucleated eggs is feasible.

Zygotic electrofusion in Pacific oyster:

- The normal larvae obtaining by *in vitro* fertilization of gametes from commercially cultured oysters can only be achieved in the period comprised from July to October.
- It has been developed an efficient zygotic electrofusion technique in highly ionic medium (seawater), and achieving both high fusion and larval survival rates to D-stage.