Protocol G - Inactivation of sperm DNA by UV-light [Pratical guide of protocols: chromosome set manipulation]

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I - Introduction

Gynogenesis and androgenesis are terms that describe uniparental reproduction with exclusively maternal and paternal inheritance, respectively. The production of uniparental inheritance individuals under experimental conditions requires genome inactivation of sperm, in the case of the gynogenesis, or the destruction of egg DNA, in the case of the androgenesis, previously to the fertilization.

Genome inactivation can be achieved by ionizing irradiation (i.e., gamma irradiation or X-rays), UV-irradiation or by chemical treatments (Thorgaard, 1983; Ihssen *et al*., 1990; Felip *et al*., 2001; Piferrer *et al*., 2007). Ionizing irradiations have a high power of penetration and effectively fragment the DNA. However, the use of this type of treatment requires specific radioactive facilities that are only available in a few laboratories. Although UV-irradiation shows a lower power of penetration in contrast to ionizing irradiation, it is cheaper and easier to be used in any laboratory (Komen and Thorgaard, 2007).

The scaling-up of this UV irradiation is simple. It is based on the distance between the germicidal bulbs emitting UV light at 254 nm and the sample. The intensity of irradiation is optimized by varying their duration. For better results, eggs are commonly kept in a synthetic ovarian fluid while sperm has to be diluted in adequate extenders. These diluents prevent precocious gamete activation. During the irradiation treatment, the samples are usually stirred and kept on ice. In the case of species with thick sperm, an adequate ratio of extender and sperm is crucial in order to optimize genome inactivation conditions (Carrillo *et al.*, 1995; Felip *et al*., 2001).

The UV-light causes the formation of pyrimidin dimmers on DNA that provokes changes in the conformation of DNA, thus impairing its genomic contribution in the egg during the fertilization (Thorgaard, 1983; Komen and Thorgaard, 2007). Although ionizing irradiation continues being used for irradiating eggs, UV-irradiation has become the most common method used for sperm DNA inactivation. Optimal UV-irradiation avoids producing chromosome fragments (minichromosomes), thus if adequately applied, high yields of truly gynogenetic fish can be obtained. To establish the appropriate UV dose to use for complete sperm DNA inactivation, the Hertwig effect needs to be initially elicited in each species (Ijiri and Egami, 1980).

Hertwig effect refers to the improved early survival of gynogenetic haploids, which are free of dominant lethal mutations by the complete inactivation of the sperm genome. The Hertwig effect is frequently reported in the induction of haploid gynogenetic fish and has also been demonstrated with UV-irradiated sperm in molluscs (Pan *et al.*, 2004).

II - Purpose

Previously to induction of gynogenesis, sperm DNA has to be inactivated in order to asses the production of fish with exclusively maternal inheritance. The UV-irradiation is the common procedure in order to inactivate sperm DNA. However, the optimization of the intensity of UV-irradiation requires a careful analysis based on the induction of the Hertwig effect. A detailed analysis of treatment variables and UV irradiation conditions are discussed in the present Protocol in order to completely inactivate sperm DNA in sea bass.

III - Procedure

(i) Anaesthetize a male with MS-222 or 2-phenoxyethanol. Clove oil can be also used. Induction of anaesthesia is rapid (1-3 min) in small fish although it is slower in larger fish. The above doses may be lethal after about 20 min, thus animals must be carefully handled as soon as possible. The use of gloves is recommended during this process.

(ii) After cleaning the genital area with fresh water and drying, collect total expressible milt by gentle abdominal massage pressure in a 15 ml glass vial and store on ice.

(iii) Dilute sperm 1:10 with an appropriate diluent for sea bass sperm.

(iv) Take 2 ml of diluted sperm and place them in a glass Petri dish (9 cm diameter), forming a film aprox. 0.3 mm thick.

(v) Expose the petri dish with the diluted sperm to UV light at the optimal time previously optimized according to the Hertwig effect results. The petri dish must be maintained on ice and gently agitated with an orbital shaker during irradiation.

(vi) After irradiation, transfer irradiated sperm to a glass vial and protect it from ambient light. Keep sperm on ice.

(vii) Microscopically monitor sperm motility and duration of the movement according to Chambeyron and Zohar (1990) and Sorbera *et al*. (1996). Motility duration is determined to be the time period from sperm activation to cessation of cell displacement with only <5% of the sperm population beating the flagella. The percentage of motility is characterized using an arbitrary scale, based on five categories, in which category I represents 0% of the sperm population motile and category V represents 90-100% of the sperm population vigorously motile.

IV - Materials and equipment

- 15-ml glass vials to collect sperm
- 9 cm diameter glass petri dishes
- 15 cm diameter glass petri dishes
- Plastic pasteur pipettes
- Aluminum foil to protect irradiated sperm from ambient light
- 500-ml glass vial to collect eggs
- Plastic tray for artificial fertilization
- Feathers
- Graduated cylinders
- Plastic trays for cold shock
- Glass vials to put fertilized eggs to be shocked
- Timer
- Thermometer
- UV-light source
- UV-meter
- Orbital shaker

- Safety glasses for UV light protection

- Plastic containers to anaesthetize fish

- An incubation system

- Gloves, lab coat and boots

- Kitchen clothes

V - Reagents and solutions

- Anaesthetic: MS-222 (0.1 g l^{-1} of seawater) or 2-phenoxyethanol (0.5 ml l^{-1} of seawater). Alternatively, the induction of anaesthesia can be carried out using clove oil. Clove oil has been evaluated as an effective anaesthetic in sea bass and it can be used at almost 10-fold lower doses than 2-phenoxyethanol.

- Ice for UV-light irradiation and storage

- Sperm diluent: 100 mM NaCl, 13.4 mM KCl, 26.2 mM $NaHCO_3$ and 83.2 mM glycine, pH 7.35.

VI - Results and discussion

The procedure used to irradiate the sperm is important for the complete DNA inactivation (Fig. G.1). Thus, sperm usually needs to be diluted to form a thin film in a glass petri dish before irradiation treatment. During exposure to UV light the petri dish is maintained on ice and gently agitated using an orbital shaker (Fig. G.1 A, B, C). Finally, the induction of the Hertwig effect is a key experiment to determine the intensity of UV light required to completely inactivate the DNA of the sperm of any particular species while it retains the ability to activate egg development. A typical Hertwig effect is observed in the sea bass (Fig. G.1 D). Results from this experiment demonstrate that the inactivation of the DNA of the sperm in this species is established at doses of 35.000-40.000 erg mm^{-2} when the sperm is diluted 1:10 (Felip *et al*., 1999).

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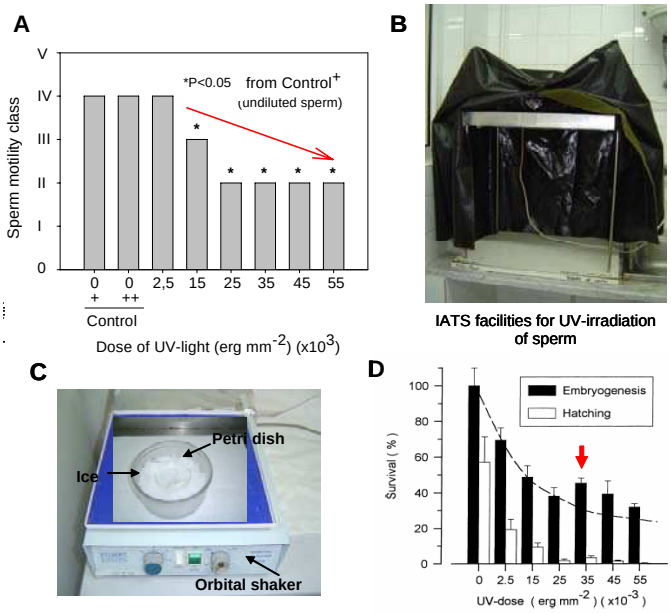

Fig. G.1. Inactivation of sea bass DNA sperm by UV-irradiation. (A) Representation of motility of sea bass spermatozoa after exposure to different doses of UV light according to Chambeyron and Zohar (1990) (*) = significantly different from Control^{+}, fertilized with undiluted sperm. Control^{++} indicates eggs fertilized with diluted sperm, (B) UV-light unit for UV-irradiation of sea bass sperm, (C) a petri dish maintained on ice and agitated with an orbital shaker for sperm irradiation and (D) a typical Hertwig effect curve in the sea bass. Survival of fertilized eggs (in D) at embryogenesis (48 hours postfertilization, HPF) and hatching (72 HPF) is shown as a function of the dose of UV-irradiation applied to different aliquots of the sperm (diluted 1:10) used for fertilization. The dotted line indicates expected survival at 48 HPF when extrapolated from lower (≤ 25000 erg mm^{-2}) to higher (≥35000 erg mm^{-2}) doses of UV light. Differences between expected and observed survival with doses ≥35000 erg mm^{-2} evidence a typical Hertwig effect. Modified from Felip *et al.* (1999).