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Improvement of single-cell gel electrophoresis (SCGE) alkaline comet assay

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ABSTRACT: Although the single-cell gel electrophoresis (SCGE) alkaline comet assay can detect DNA damage quickly and with high sensitivity, it does not work well for cryopreserved sperm of some marine teleosts. Using large yellow croaker *Pseudosciaena crocea* sperm that were cryopreserved in increasing concentrations (5 to 30%) of DMSO, we made modifications to the classic SCGE method that included using common microscopic slides without rough surfaces, pretreatment of sperm before gel spread, and a single layer of gel. Electrophoresis conditions were 130 mA, 15 V, and 60 min, providing a high SCGE sensitivity and definition of the comet image. The improved method is useful for detecting cryopreserved sperm DNA damage of some marine teleosts, especially for testing the quality of genetic resources.

KEY WORDS: *Pseudosciaena crocea* · Sperm · Cryopreservation · SCGE · DNA damage

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INTRODUCTION

Single-cell gel electrophoresis (SCGE), or the comet assay, is a classical method for the detection of DNA damage (Singh et al. 1988, Rojas et al. 1999). This assay follows the process of cell lysis and DNA unwinding; the DNA fragments flow from the nucleus and move to the positive pole, generating a comet-like band. After fluorescent staining, DNA damage of the cells can be observed under the microscope. The fluorescence intensity of the comet tail depends on the degree of DNA damage. SCGE is widely used for detection of DNA damage in plant cells (Kim & Richard 2004), mice cells (Giovannelli et al. 2003), lymphocytes (Stavreva et al. 1998), human sperm (Lu et al. 2002), and fish sperm (Labbe et al. 2001, Xu et al. 2005, Ye et al. 2009). Although SCGE alkaline comet assay can detect DNA damage fast and with high sensitivity, it does not work well for cryopreserved sperm of marine teleosts. In order to obtain higher sensitivity, we improve the classic SCGE method by using large yellow croaker *Pseudosciaena crocea* sperm cryopreserved in increasing concentrations (5 to 30%) of DMSO.

MATERIALS AND METHODS

Samples and materials. Male yellow croakers *Pseudosciaena crocea* of 500 to 650 g were obtained from Haiwang Hatchery, Xiangshan, Zhejiang Province, China, in March 2008.

Low melting-point agarose (LMA), EDTA, sodium sarcosinate, Triton X-100, proteinase K, Tris, DMSO and ethidium bromide (EB) staining solution were purchased from Shanghai Santa Bio-Techonology. All reagents were analytically pure grade. A DYY2C

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electrophoresis apparatus and a DYCP-33A electrophoresis bath were purchased from Liuyi Instrument Company. A Nikon ECLIPSE 80i fluorescence microscope was purchased from Nikon Corporation; and CometScore 1.5 image analysis software was from TriTek Corporation.

Sperm cryopreservation. Fresh semen was collected from 5 anesthetized male fish. Semen was mixed with the extender, which contained Cortland solution and 5 to 30% DMSO with a volume proportion of 1:3. The suspension was put on ice for 10 to 15 min and then injected into 500 µl straws. The straws were then placed horizontally 3 to 5 cm above the liquid nitrogen surface. After 5 to 8 min freezing, the straws were plugged into nitrogen for storage. Each DMSO concentration treatment was repeated 4 times.

SCGE procedure. Straws were thawed in a water bath at 40°C, centrifuged (344 × *g*) and washed twice with phosphate buffered saline (PBS; pH 7.4) at 4°C. The sperm suspension (50 µl, with concentration of approximately 8×10^6 sperm ml^{-1}) was diluted with 350 µl 1% LMA in a 5 ml centrifuge tube; the final sperm concentration was 10^6 sperm ml^{-1}. The sperm suspension was then placed in a lysis solution (2.5 M NaCl, 100 mM EDTA, 10 g l^{-1} sodium sarcosinate, 10% DMSO, 1% Triton X-100, pH10) for 1 h at 10°C. This step and the following steps (except staining and observation) were performed in the dark. The sperm suspension was placed in a digestive solution (2.5 M NaCl, 5 mM Tris, 0.5 g l^{-1} sodium sarcosinate, 0.5 g l^{-1} proteinase K, pH 7.4) in a water bath at 55°C for 3 h. After washing with PBS twice, the mixture was melted at 70°C in a water bath for 3 min, then a 100 µl drop of the mixture was pipetted on the slide, covered with a coverslip and allowed to solidify at 10°C for 10 min. The slides were placed in a horizontal electrophoresis bath with a fresh alkaline electrophoresis solution (300 mM sodium acetate, 100 mM Tris, pH 10.0) for 30 min to allow the DNA to unwind. Electrophoresis was carried out using a current of 130 mA and 15 V for 1 h. The slides were then neutralized in a freshly prepared Tris-HCl buffer (0.4 mM, pH 7.0) for 15 min.

The slides were stained with EB solution (50 µg ml^{-1}) for 10 min, and then photographed on an epifluorescence microscope with an excitation wavelength of 580 nm. Each sample was measured 4 times, and 100 sperm from each slide were observed.

Image analysis. The comet image was analyzed with CometScore 1.5 software (Ye et al. 2009) to obtain parameters such as comet length, tail length, and DNA percentage in the tail.

Comet rate and damage coefficient calculation. According to the method of Singh et al. (1988) and Rojas et al. (1999), DNA damage was divided into 5 grades, depending on tail length as a proportion of total length.

Grade 0: no damage, normal cell, tail length <5%, nucleus was intact (Fig. 1a).

Grade I: slightly damaged, tail length 5 to 20% (Fig. 1b).

Grade II: moderately damaged, tail length 20 to 40%, obvious tail observed (Fig. 1c).

Grade III: heavily damaged, tail length 40 to 95%, fluorescence signal becomes strong, nucleus reduced significantly (Fig. 1d).

Grade IV: totally damaged, tail length >95%, nucleus becomes dim or disappears altogether, fluorescence signal becomes extremely strong (Fig. 1e).

Comet rate and damage coefficient were calculated as follows:

Comet rate = (comet cells/total cells) × 100;

Damage coefficient = [(Grade 0 cell number × 0) + (Grade I cell number × 1) + (Grade II cell number ×2) + (Grade III cell number × 3) + (Grade IV cell number × 4)] (Chen 1998).

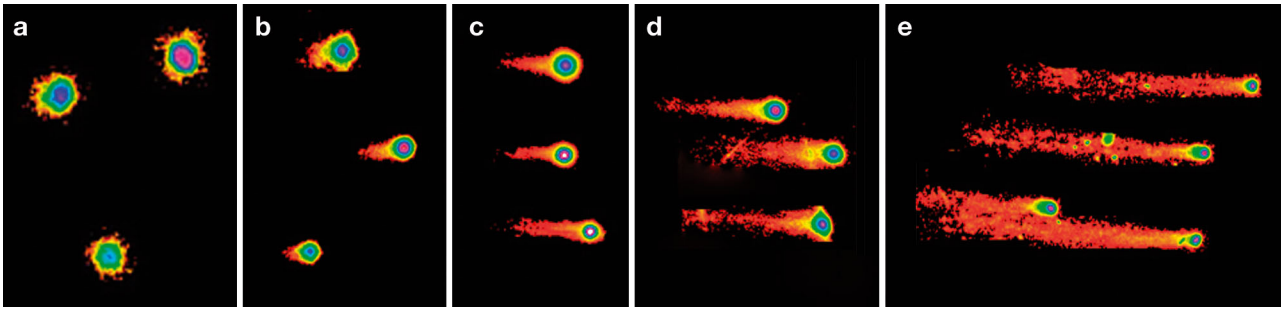

Fig. 1. Single-cell alkaline comet assay gel electrophoresis of fresh and cryopreserved sperm. (a) Grade 0, normal cell; (b) Grade I, slightly damaged, comet tail short; (c) Grade II, weakly damaged, nucleus reduced, fluorescence signal of tail stronger; (d) Grade III, strongly damaged, nucleus reduced significantly, strong fluorescence signal of tail; (e) Grade IV, totally damaged, very long comet tail. Magnification: ca. 2000×

RESULTS AND DISCUSSION

In fresh and cryopreserved sperm, comet and tail length showed an increasing correlation with concentration of the cryoprotectant DMSO (Fig. 1). We set 6 different DMSO dilutions, which were marked as D1–D6, with concentrations increasing from 5 to 30% DMSO in 5% increments. Compared with fresh sperm, comet length and tail length of D1–D4 were similar to that of fresh sperm (Fig. 1a–c), but the comet length and tail length of D5 and D6 were obviously longer than that of fresh sperm (Fig. 1d,e). A detailed analysis is in preparation

Since the alkaline comet assay was reported by Singh et al. (1988), continuous improvement has made it feasible to detect not only double-strand breaks but also single-strand breaks at alkaline fragile sites quickly and with high sensitivity (Yang et al. 2004). We made some modifications to the classic SCGE method used in our previous investigations on *Sparus macrocephalus* (Ye et al. 2009). The improvements include: (1) common microscopic slides without rough (frosted) surfaces were used because common slides have a better light transmittance and definition and lower price than manufactured SCGE slides; (2) pretreatment of sperm was carried out before gel spread, which simplified the procedure of SCGE and reduced the degelatinization and dosage of Protease K rapidly; (3) in contrast to 2 or 3 layers of gel, the spread of a single layer of gel turned out to be more convenient, with less degelatinization occurring; (4) electrophoresis conditions are different between sperm from different species, and for the yellow croaker sperm, 130 mA, 15 V, and 60 min provided a high SCGE sensitivity and definition of the comet image (Ye et al. 2009).

In our SCGE analysis, we employed both manual index analysis and computer image analysis. Manual index analysis refers to the measurement of comet rate and distance index (head length, tail length, etc.), which are easy to measure but provide relatively rough estimates.

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