Aquaculture 451 (2016) 353–356

Contents lists available at ScienceDirect

Aquaculture

journal homepage: www.elsevier.com/locate/aqua-online

Short Communication

Usefulness of a portable flow cytometer for sperm concentration and viability measurements of rainbow trout spermatozoa

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ARTICLE INFO

Article history:
Received 14 May 2015
Accepted 24 September 2015
Available online 28 September 2015

Keywords:
Sperm concentration
Viability
Flow cytometer
Rainbow trout

ABSTRACT

The aim of this study was to test the usefulness of a portable flow cytometer in relation to the measurement of sperm concentration and viability in rainbow trout (n = 12). This method was compared with the currently used methods, including the spectrophotometric method, computer-aided fluorescence microscopy and a haemocytometer. The mean concentration obtained using a spectrophotometer ($9.86 \pm 3.69 \times 10^9$ spermatozoa ml^{-1}) and a fluorescence microscope ($10.46 \pm 3.60 \times 10^9$ spermatozoa ml^{-1}) was significantly lower than that obtained with a flow cytometer ($12.35 \pm 3.88 \times 10^9$ spermatozoa ml^{-1}) and a haemocytometer ($11.73 \pm 4.78 \times 10^9$ spermatozoa ml^{-1}). Significant regressions ($P < 0.0001$) between sperm concentration obtained by using a flow cytometer, a spectrophotometer ($y = 0.93 \times -1.64$; $r^2 = 0.95$), a fluorescence microscope ($y = 0.90 \times -0.69$; $r^2 = 0.94$) and a haemocytometer ($y = 1.16 \times -2.57$; $r^2 = 0.87$) were found. The sperm viability determinations obtained using a flow cytometer were significantly higher ($97.00 \pm 0.99\%$) than the values obtained by using fluorescence microscopy ($86.22 \pm 1.16\%$). However, a significant regression was found between these two viability measurements ($r^2 = 0.26$, $P < 0.05$). To the best of our knowledge, this is the first study related to the usefulness of a portable flow cytometer in vertebrate sperm analysis. The flow cytometer provides fast measurement of sperm concentration and viability. The advantage of a compact flow cytometer is the ability to incorporate the examination of other sperm functions related to apoptosis, mitochondrial potential, oxidative stress and DNA fragmentation in future fish reproductive studies.

Statement of relevance

Study relevant to sperm concentration, viability monitoring.

1. Introduction

Sperm concentration is one of the major semen parameters that is evaluated as part of the standard fish semen analysis. However, accurate measurement of this parameter is quite challenging. Precise estimation of the number of sperm cells is necessary for fish reproductive studies including the determination of the optimal sperm-to-egg ratio in fertilization trials (Ciereszko et al., 2014; Nynca et al., 2014), the calibration of ultraviolet irradiation to induce gynogenesis, the optimization of staining with fluorescent dyes (Paniagua-Chávez et al., 2006) and nutritional and toxicological studies (Cuevas-Uribe and Tiersch, 2011). Different methods were developed for the measurement of fish sperm concentration; the most popular are based on counting a single sperm cell in a haemocytometer chamber and spectrophotometric measurement of absorbance caused by the turbidity of sperm suspensions (Suquet et al., 1992; Ciereszko and Dabrowski, 1993). Counting cells in a haemocytometer is reliable, but time-consuming and laborious, and thus cumbersome for experiments requiring fast measurement of sperm concentrations of numerous fish males. In the case of the spectrophotometric method, a standard species-specific curve is needed. Other techniques available to determine sperm concentration include fluorescence microscopy and flow cytometry. Nynca and Ciereszko (2009) demonstrated the usefulness of computer-aided fluorescence microscopy with the employment of a NucleoCounter SP-100 for accurate and fast measurement of the sperm concentration and viability of fish semen. Flow cytometry can be potentially used for counting cells, but until now it has been restricted to laboratory conditions (Hossain et al., 2011). However, recently a portable flow cytometer has been developed, which for the first time enables flow cytometry analysis to be carried out in field conditions.

The Muse Cell Analyser is a compact flow cytometer that uses miniaturized fluorescent detection and microcapillary technology to deliver quantitative cell analysis. The Muse Cell Analyser offers a variety of assays,

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http://dx.doi.org/10.1016/j.aquaculture.2015.09.027
0044-8486/

such as determination of cell concentration and viability, DNA fragmentation, detection of cells in various stages of apoptosis, counting cells undergoing oxidative stress, measurement of the changes in cell mitochondrial potential and cell cycle phase measurement. So far, the Muse flow cytometer has been mostly applied in studies related to the multiple aspects of cancer cells, including viability, apoptosis (Neri et al., 2014; Marusiak et al., 2014), cell cycle and cell signalling pathways (Guerriero et al., 2014). To our knowledge, there is no information regarding the usefulness of this instrument for the semen analysis of vertebrates.

The aim of this study was to test the usefulness of a portable Muse Cell Analyser in relation to the measurement of sperm concentration and viability in rainbow trout. This method was compared with the currently used methods, including the spectrophotometric method (Ciereszko and Dabrowski, 1993), computer-aided fluorescence microscopy (Nynca and Ciereszko, 2009) and a haemocytometer.

2. Materials and methods

2.1. Source of milt

Mature rainbow trout males (3 years of age, n = 12) were cultivated at the Rutki Salmonid Research Laboratory at the Institute of Inland Fisheries in Olsztyn, Poland. Rainbow trout males with a mean weight of 1188 ± 236 g and length of 44.4 ± 3.5 cm were kept in concrete ponds supplied with water from the Radunia river with oxygen saturation levels maintained at 85–95% and temperatures of 7–10 °C during spawning. Prior to milt collections, the fish were anaesthetized with Propiscin (1 ppm IFI, Żabieniec, Poland). Milt was collected during spring spawning in April by abdominal massage, with special care to avoid blood, urine or faeces contamination. Approval by the Animal Experiments Committee in Olsztyn, Poland, was obtained before starting any of the experiments.

2.2. Measurement of sperm concentration

Sperm concentration was measured independently using four methods. All measurements were made in duplicate. Semen was diluted 100 times with a phosphate-buffered saline (PBS) and this suspension was used for all measurements. Three sperm concentration measurements were performed on the day of analysis and the sperm concentration was measured with a haemocytometer on the next day (diluted sperm samples were stored at 4 °C until analysis).

2.2.1. Spectrophotometric method

Sperm concentrations were estimated by measuring the absorbance of sperm suspensions at 600 nm diluted 2000 times in 0.7% NaCl (Ciereszko and Dabrowski, 1993) using a portable spectrophotometer (Eppendorf, Hamburg, Germany) and a standard curve established previously in our laboratory.

2.2.2. Fluorescence microscopy

Sperm concentrations were measured using the computer-aided fluorescent microscopy NucleoCounter SP-100 (Chemometec, Allerød, Denmark) as described by Nynca and Ciereszko (2009). Briefly, semen was diluted 100 times with PBS then 51 times with Reagent S100 and subsequently loaded into a disposable cassette containing propidium iodide. Data were processed and documented using SemenView software (Chemometec, Denmark).

2.2.3. Flow cytometry

The sperm concentration and viability were measured using a Muse Cell Analyser (Millipore, Billerica, MA, USA) according to the instructions supplied by the manufacturer. Semen samples were first diluted 100 times with PBS, then 3000 times with PBS. Twenty microliters of diluted sample was mixed with 380 μl of Muse Count and Viability Assay Reagent (Millipore, USA) in 1.5 ml screw-cap microfuge tubes and incubated for 5 min in the dark at room temperature. Samples were introduced to the system by microcapillary. Data were generated with the Muse™ Count and Viability Software Module (Millipore, USA), providing at the same time a viable cell count, total cell count and sample viability (%).

2.2.4. Haemocytometer

A 100 μm-deep Bürker haemocytometer (BT, Brand, Wertheim, Germany) was used for sperm counting. Approximately 10 μl of sample diluted 2000 times with 0.7% NaCl was loaded into each side of the haemocytometer. After loading, the chamber was left for 2 min to allow all the sperm to settle. Counts were performed under the microscope using 40× magnification and phase contrast.

2.3. Measurement of sperm viability

2.3.1. Fluorescent microscopy

The semen of 12 males was diluted 100 times with PBS, then 51 times with either Reagent S100 (total count) or immobilizing solution containing 100 mM NaCl, 40 mM KCl, 3 mM $CaCl_2$, 1.5 mM $MgCl_2$ and 50 mM Tris, pH 8.5 (Morisawa and Morisawa, 1988) (non-viable count). When immobilizing solution is used for sperm dilution only non-viable spermatozoa are counted. Samples were loaded into the cassettes containing propidium iodide — under such conditions non-viable spermatozoa are stained, but viable spermatozoa are not stained. The viability was calculated as 100% × (total − non-viable)/total.

2.3.2. Flow cytometry

Viability was analysed using the Muse Count and Viability kit as described above. Both viable and non-viable cells are differentially stained based on their permeability to the mix of two fluorescent DNA-binding dyes in the reagent.

2.4. Statistical analysis

All the results are presented as mean ± SD. All analyses were performed at a significance level of 0.05 using GraphPad Prism software (GraphPad Software Inc. San Diego, CA, USA). For statistical procedures, data percentages were transformed by arcsine square root transformation. Data were subjected to repeated measures one-way ANOVA followed by Tukey's post hoc test. Additionally, regression equations and correlation coefficients were calculated between estimated parameters.

3. Results

3.1. The comparison of sperm concentration measurements

Significant differences between values of sperm concentration evaluated by the applied methods were noticed (Table 1). The mean concentration obtained using the spectrophotometer and NucleoCounter SP-100 was significantly lower (by 15–20%) than that obtained by the flow cytometer and haemocytometer. The flow cytometer produced concentration outcomes similar to the outcomes obtained by the haemocytometer (Table 1). The sperm concentration calculated by the haemocytometer was characterized by the highest standard deviation and range in comparison to the other methods.

Significant regressions ($P < 0.0001$) between sperm concentrations obtained by the Muse flow cytometer, the Eppendorf spectrophotometer ($y = 0.93 \times -1.64$; $r^2 = 0.95$), the NucleoCounter SP-100 ($y = 0.90 \times -0.69$; $r^2 = 0.94$) and the Bürker haemocytometer ($y = 1.16 \times -2.57$; $r^2 = 0.87$) were found (Fig. 1).

3.2. Sperm viability measurements

The sperm viability determinations obtained using the NucleoCounter SP-100 were significantly lower (by about 12%) than the values obtained by using the Muse flow cytometer (Table 1). A significant correlation was found between the percentage of sperm viability measurements