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Flow cytometric and near-infrared Raman spectroscopic investigation of quality in stained, sorted, and frozen-thawed buffalo sperm

Xiao-xia Li[a,b], Meng Wang[a], Huan-hua Chen[a], Qing-yang Li[a], Huan Yang[a], Hui-yan Xu[a], Yang-qing Lu[a], Ming Zhang[a], Xiao-gan Yang[a,*], Sheng-sheng Lu[a], Ke-huan Lu[a,*]

[a] State Key Laboratory for Conservation and Utilization of Subtropical Agro-bioresources, Guangxi High Education Key Laboratory for Animal Reproduction and Biotechnology, Guangxi University, Nanning, Guangxi 530004, PR China
[b] College of Animal Science and Technology, Henan University of Science and Technology, Luoyang, Henan 471003, PR China

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ABSTRACT

Flow cytometry and Laser Tweezers Raman spectroscopy have been used to investigate Nili-Ravi buffalo (Bubalus bubalis) sperm from different samples (fresh, stained, sorted and frozen-thawed) of the flow-sorting process to optimize sperm sex sorting procedures. During the sorting and freezing-thawing processes, the two detection methods both indicated there were differences in mitochondrial activity and membrane integrity. Moreover, a dispersive-type NIR (Near Infrared Reflection) use of the Raman system resulted in the ability to detect a variety of sperm components, including relative DNA, lipid, carbohydrates and protein contents. The use of the Raman system allowed for PCA (principal components analysis) and DFA (discriminant function analysis) of fresh, stained, sorted and frozen-thawed sperm. The methodology, therefore, allows for distinguishing sperm from different samples (fresh, stained, sorted and frozen-thawed), and demonstrated the great discriminative power of ANN (artificial neural network) classification models for the differentiating sperm from different phases of the flow-sorting process. In conclusion, the damage induced by sperm sorting and freezing-thawing procedures can be quantified, and in the present research it is demonstrated that Raman spectroscopy is a valuable technology for assessing sperm quality.

1. Introduction

In recent years, flow cytometric separation of sperm based on the difference in the relative DNA contents of X-and Y-bearing sperm has been an effective method for preselecting the sex of animals. Use of this method has resulted in the birth of healthy piglets (Rath et al., 2003), lambs (Hollinshead et al., 2002), foals (Buchanan et al., 2000) and calves (Tubman et al., 2004). This previous research provided evidence as to the feasibility of sperm sorting by flow cytometry, and the *in vitro* production of sex-preselected embryos and offspring in buffalo (Lu et al., 2006, 2007). Nevertheless, the sorting and

* Corresponding authors.
E-mail addresses: xgyang@gxu.edu.cn (X.-g. Yang), kehuanlu@163.com (K.-h. Lu).

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freezing-thawing procedures can induce sperm damage as a possible consequence of the greater than typical dilution of sperm during processing, Hoechst nuclear staining and incubation, mechanical forces, centrifugation, freezing and thawing. These external factors can contribute to an increase in the number of damaged sperm in the sample, which may produce reactive oxygen species (ROS; Chatterjee and Gagnon, 2001). The ROS can contribute to oxidative damage of sperm cell membranes that are rich in highly unsaturated fatty acids (Sikka, 1996), and may also interact with DNA to cause strand breaks and base damage (Novotny et al., 2013). The production of frozen sex-sorted sperm results in stress to sperm and may cause an increase in lipid peroxidation (LPO; Chen et al., 1997; Kadirvel et al., 2009), and LPO in sperm may be one of the mechanisms responsible for the negative biochemical and physiological changes that occur during sperm storage.

In this present study, two detection methods, namely flow cytometry and Raman spectroscopy were used to optimize the procedures for sexing semen and preserving the functional properties of frozen sex-sorted sperm. Mitochondria in the sperm are arranged in a helical form in the midpiece, and generate energy for the flagellar beat. The JC-1 (5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolyl-carbocyanine iodide) has been used as an indicator of mitochondrial membrane potential (MMP; Garner et al., 1997). The externalization of phosphatidylserine from the inner to outer leaflet of the cell plasma membrane can be used as a marker for disturbed membrane function of bull (Anzar et al., 2002) sperm after thawing. This early sign of apoptosis can be monitored by the calcium-dependent binding of Annexin-V, which in combination with propidium iodide (PI, supravital fluorescent dye) allows the simultaneous detection by flow cytometry of apoptotic and necrotic cells, or cells with compromised plasma membranes. The integrity of the sperm plasma membrane is essential for cell survival and fertilizing ability, it can be assessed by dual staining of the membrane with the permeable nucleic acid stain, SYBR-14 combined with propidium iodide (Garner and Johnson, 1995). Using this approach in the present study, buffalo sperm were examined for viability during the sorting and freezing-thawing processes.

Raman spectroscopy is an optical technique that provides an objective method of diagnosis based on the molecular composition of cells or tissues, and optical tweezers have become a useful tool with which to capture and manipulate biological particles, including cells, bacteria, viruses, and chromosomes (Ashkin et al., 1987; Visscher et al., 1999). Raman spectroscopy has been used to investigate the efficiency of DNA-packaging for each sperm cell (Huser et al., 2009), the characteristics of the mitochondria-rich middle piece of a human sperm (Meister et al., 2010), and morphological and biochemical alterations of bull sperm (Ferrara et al., 2015), but its application to the sorting and freezing-thawing processes with buffalo sperm has not yet been described in detail.

The overall objective of the present research was to evaluate the effects of staining, sorting, and freezing-thawing on buffalo sperm quality as estimated by apoptosis, mitochondrial activity and viability by flow cytometry, and at the same time examine single buffalo sperm by Raman spectroscopy to determine if the sorting and freezing-thawing processes produce changes in the DNA, protein, and carbohydrate components. The multivariate statistical techniques, including principal components analysis (PCA), discriminant function analysis (DFA) and artificial neural network (ANN), were employed to develop effective diagnostic algorithms for differentiation between fresh and stained, sorted, and frozen-thawed sperm.

2. Materials and methods

2.1. Semen collection and sperm sorting

All experimental procedures with three Nili-Ravi buffalo bulls used in this study received prior approval from the Experimental Animal Management Committee of Livestock and Poultry Breeding Station of Guangxi.

Semen was collected from three fertility tested Nili-Ravi buffalo bulls using an artificial vagina at the Livestock and Poultry Breeding Station of Guangxi, China. The quality of the ejaculates was assessed in fresh semen by determining the sperm concentration (6×10^8/mL) and progressive motility (>60%). Each semen sample with added antibiotics (Gentamicin, 100 μg/mL, G-1397; Sigma Chemical Co., St. Louis, MO, USA) was aliquoted into two fractions for conventional (fresh) treatment and for sex sorting using flow cytometry.

For the experiments, one fraction of raw semen was extended in DPBS (Dulbecco's Phosphate-Buffered Solution) to a final concentration of 10×10^6 sperm/mL, and another in modified TALP (Tyrodes, Bovine Serum Albumin, Lactate and Pyruvate; Sigma-Aldrich Chemical Co, St. Louis, MO, USA) solution to 100×10^6 sperm/mL. Hoechst 33342 (Sigma-Aldrich Chemical Co, St. Louis, MO, USA) was subsequently added to produce a concentration of 40 μg/mL, and the samples were incubated at 34 °C for 45 min. After incubation, food dye (FD&C 40, 0.01305%) solution was added to stain the dead sperm, and semen samples were filtered through a 50-μm cell strainer prior to sorting. Each stained semen sample was aliquoted into two fractions. In detail, one fraction of stained semen was extended in DPBS solution to a final concentration of 10×10^6 sperm/mL for analytical measurement (Stained treatment), and another sample was used for sex sorting as subsequently described.

Flow-sorting was performed using a flow sorter (Dako MoFlo® SX, DakoCytomation, Fort Collins, CO, USA) with an argon laser (wavelength 351 nm at 150 mW). The stained sperm were sorted into 50-mL tubes containing 2 mL of 20% (v/v) Tris-egg yolk buffer. After collection of 10×10^6 sperm per tube, the Y chromosome bearing sperm was divided into two fractions. One (Sorted treatment) was re-suspended with DPBS and the other chilled for 60 min at 4 °C, followed by centrifugation at 850*g* for 20 min at 4 °C. After centrifugation, sorted sperm that were loaded into 0.25 mL straws were frozen in Tris extender supplemented with 20% (v/v) egg-yolk and 6% (v/v) glycerol. Cryopreserved sperm were rapidly warmed at approximately 1000 °C/min by placing straws into a 37 °C water bath for 15 s. The frozen-thawed semen was then re-suspended in DPBS for measurement.