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Effects of Hoechst33342 staining on the viability and flow cytometric sex-sorting of frozen-thawed ram sperm ☆

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ABSTRACT

Cytometric sorting of frozen-thawed sperm can overcome difficulties caused by the unavailability of sorting facilities on farms where semen is collected from male livestock. In order to optimize the cytometric sex-sorting procedure, effects of Hoechst33342 staining on the viability and cytometric sorting efficiency of frozen-thawed ram sperm were evaluated. The frozen-thawed sperm were stained with Hoechst33342 at various dye concentrations (80 μM, 120 μM, 160 μM, 200 μM, 240 μM, or 320 μM) for 45 min to evaluate effects of dye dose. The frozen-thawed sperm were stained with 160 μM Hoechst33342 for various durations (0 min, 15 min, 30 min, 45 min, 60 min, 75 min, or 90 min) to evaluate effects of staining duration. Sperm motility and moving velocity were analyzed using a computer-assisted sperm analysis system (CASAS). Acrosome status, membrane integrity, and distribution of phosphatidylserine (PS) in Hoechst33342-stained sperm were analyzed using flow cytometry after staining with fluorescein isothiocyanate-labeled lectin from pisum sativum (FITC-PSA), Annexin V, or propidium iodide (PI). The fertility of Hoechst33342-stained sperm was analyzed by *in vitro* fertilization (IVF). A high-speed cell sorter was used to evaluate effects of Hoechst33342 staining on cytometric sex-sorting of frozen-thawed sperm. The motility, moving velocity, membrane integrity, and PS distribution of Hoechst33342-stained sperm were significantly different from that of immediately thawed sperm ($P < 0.05$). However, there is no significant difference existing among the Hoechst33342-stained groups with respect to the above evaluated parameters. Additionally, along with the staining durations, the adverse effects of the staining procedure on sperm showed a steady increase. However, Hoechst33342 staining did not damage acrosome and *in vitro* fertilizing capability of frozen-thawed ram sperm. Results of cytometric sorting indicated that frozen-thawed sperm can be efficiently sorted into two sperm populations with X and Y chromosome when the Hoechst33342 concentration was 160 μM. Moreover, when the staining duration was equal to or longer than 45 min, the frozen-thawed sperm can be successfully sorted in the presence of 160 μM Hoechst33342. In conclusion, Hoechst33342 staining can detrimentally influence viability of frozen-thawed ram sperm except acrosome and *in vitro* fertilizing capability. Accordingly, the minimum values of Hoechst33342 concentration and staining duration can be set at 160 μM and 45 min respectively. However, the maximum values of Hoechst33342 concentration and staining duration were not determined based on the current study. Further research on how to reduce injuries caused by freezing, thawing, and Hoechst33342 staining on frozen-thawed ram sperm is needed.

Introduction

Currently, artificial insemination (AI) or IVF of sex-sorted sperm has been applied to produce sex-determined offspring in livestock industry in order to obtain economic traits like production of milk, meat, and wool, which are controlled by sex [21]. To successfully separate sperm with X or Y chromosome, investigators focused

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on sex selection according to presumed differences in weight, density, size, motility, and surface charge of mammalian sperm [10,21,40]. However, none of technique has proved as effective as cytometric sorting which is based on flow cytometry and differences in DNA content of X- and Y-sperm [39,42]. Beginning with the first report in rabbits [17], cytometric sorting of sperm with X or Y chromosome is considered as an effective method to obtain animals with expected sex [26]. Until now, this technique has been successfully applied to sort semen with good accuracy in more than 23 mammalian species, and normal offspring have been produced via AI or IVF with sex-sorted sperm in at least 7 species [9]. The accuracy of sex sorting by flow cytometry is approximately 85–95% [9,16,26,41,49]. Moreover, cytometric sorting has been commercially used in bovine industry [11,42].

To increase the viability of sorted X- or Y-sperm, stresses on sperm caused by cytometric sorting procedure must be considered, including the effect of dilution, the possible detrimental effect of Hoechst33342, high pressure in a flow cytometer, and exposition of laser [2]. Investigators have found detrimental effects of cytometric sorting on the structure and function of sperm [2]. Therefore, all stresses that mammalian sperm may face during the cytometric sorting procedure should be critically evaluated [27,36]. Researchers also concerned that Hoechst33342 staining may cause potentially genetic damage to sperm DNA, although Hoechst33342 staining had no apparent genotoxic effects on mammalian sperm according to most of the investigations [10]. The effects of Hoechst33342 staining on developmental capability of embryos after AI or IVF also need further elucidation [10]. According to recent reports, there were controversial results in different species. Guthrie et al. found a potential protective effect of Hoechst33342 staining on boar sperm during cytometric sorting [12]. On the contrary, in the investigation carried out by Vazquez et al. [47], the motility of boar sperm was significantly decreased in the presence of 60 μM Hoechst33342. Furthermore, all boar sperm stopped moving when the Hoechst33342 concentration was increased to 90 μM. Additionally, in bull, staining with 90 μM Hoechst33342 can decrease oxygen consumption of frozen-thawed sperm and subsequently result in lower sperm motility [7]. In human, sperm motility was fully lost after staining with 900 μM Hoechst33342. However, no adverse effect was observed when the concentration of Hoechst33342 was 90 μM [48]. In stallion, the detrimental effects of Hoechst33342 staining on sperm depended on the used media [2]. Sperm motility was significantly decreased after 45 min of incubation in the KMT extender containing 45 μM Hoechst33342. However, when the modified INRA96 was used, stallion sperm can tolerate greater concentrations of Hoechst33342 by observing unaffected sperm motility until the dye concentration was increased to 90 μM. It was found that Hoechst33342 staining had no detrimental effect on sperm membrane [2]. Additionally, a previous study carried out by Libbus et al. indicated that sperm chromatin might be damaged by staining with Hoechst 33342 and ultraviolet laser illumination during cytometric sorting [18].

Since sorting facilities are often located at a distance from farms where male livestock are raised, the common strategy is to use sperm after liquid storage at low temperature (5 °C or 15 °C) [15]. The sorted X- or Y-sperm can be artificially inseminated or refrozen for later usage. An alternative strategy is to sort frozen-thawed sperm for immediate usage [14] or a second freezing-thawing cycle [6,13,44–46]. However, the cytometric sorting efficiency of frozen-thawed sperm is dependent on an optimized sperm cryopreservation method which can efficiently decrease cryoinjuries on structure and physiological function of sperm [15]. In certain animal species, the poor viability of frozen-thawed and sex-sorted sperm may limit the efficiency of utilizing this technology to assist livestock reproduction [28].

There are previous reports on cytometric sorting of ram sperm transported to a laboratory in a frozen state [6,13,14]. However, the low efficiency of transporting sperm using cryopreservation results in insufficient number of viable sperm available for cytometric sorting. In order to increase the sorting efficiency of frozen-thawed ram sperm, decreasing cryoinjuries caused by freezing and thawing is essential. The stresses associated with cytometric sorting should be considered as well. According to our knowledge, there is no report on effects of Hoechst33342 staining on frozen-thawed ram sperm, although offspring derived from frozen-thawed, cytometric sorted, and re-frozen-thawed ram sperm have been produced by AI [6] and IVF [13]. To further optimize the sorting procedure of frozen-thawed ram sperm, the effects of Hoechst33342 staining on viability and sorting efficiency of frozen-thawed ram sperm were systematically evaluated in this study. The objectives of this current study were: (1) evaluation of the effects of Hoechst33342 staining on parameters including motility, moving velocity, acrosome status, membrane integrity, PS distribution, and fertility of frozen-thawed ram sperm; (2) optimization of the cytometric sorting procedure based on a freezing and thawing procedure developed previously [34].

Materials and methods

Unless otherwise stated, all chemical reagents were purchased from Sigma–Aldrich (St. Louis, MO, USA).

Semen collection, freezing, and thawing

The study was performed in Yunnan Animal Science and Veterinary Institute (YASVI), which is located at Kunming city, Yunnan province, PR China (103°40′ E; 26°22′ N). Research protocols were reviewed and approved by the ethical committee of YASVI. Semen samples were collected from 6 fertility-proven Yunnan semi-fine wool rams (2–4 years old) using electric stimulation on the research farm of YASVI. Tubes containing ejaculates were placed in a 37 °C water bath and then transported to the laboratory within 15 min. The quality assessment of semen was performed immediately after collection. The semen for further usage should meet the following criteria: volume of 0.75–2 mL; minimum sperm concentration of $3x10^9$ sperm/mL; motility $\geqslant$ 75%.

The freezing and thawing procedure of ram sperm was described previously [34]. Briefly, the ram semen was diluted in the freezing extender (224.0 mM Tris, 66.6 mM citric acid, 55.5 mM glucose, 10% (v/v) egg yolk, 5% (v/v) glycerol, 100,000 IU penicillin, and 100,000 IU streptomycin). The pH value of the freezing extender was adjusted to 7.0 using Tris. The final sperm concentration in diluted samples was approximately 5×10^8/mL. The diluted semen were then loaded into the 0.25 mL plastic straws (IMV, France), cooled to 5 °C at 0.01 °C/s, and further equilibrated for 2 h at this temperature. Subsequently, the straws were frozen in liquid nitrogen vapor for 7 min. The freezing rate was approximately 1.5 °C/s. Finally, the straws were plunged into liquid nitrogen and preserved for six month. The cooling/freezing rate was measured using a thermometer (Testo 925, TESTO, Germany). The thermocouple was placed in the middle of the plastic straw. The measurement of the cooling rate was performed between 25 °C and 5 °C. The measurement of the freezing rate was performed between 5 °C and −50 °C. Prior to Hoechst33342 staining, the straws were directly thawed in a 37 °C water bath for 30 s.

Viability

The motility and moving velocity of ram sperm were analyzed using a CASAS (MaiLang, Songjingtianlun Co., Nanning, Guangxi