Cryopreservation of Honey Bee Spermatozoa

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Abstract

Our project investigated a new method for the cryopreservation of honey bee (*Apis mellifera*) spermatozoa. Few methods have been developed and none achieve normal sex ratios in progeny. Recently honey bee colonies have been decimated by colony collapse disorder and infestation by *Varroa* bee mites. A bank of preserved spermatozoa might enable the creation of a seed stock for restoration of genetic diversity through artificial insemination. We investigated two freezing rates using two diluents and their effect on post thaw survival of the spermatozoa. The slower freezing rate was chosen from a report with the highest success to date (Harbo JR 1983 Ann. Entomol. Soc. Am. **76**, 890-891). The rapid freezing rate was a method developed us. The sperm cells were frozen either in 40% Harbo's DMSO diluent containing: 25% DMSO, 25% egg yolk, 50% buffer (1.1% $NaH_2PO_4 \bullet H_20$ (w/v) and 0.845% $Na_2HP0_4 \bullet 2H_20$ (w/v)) and 60% semen; or in 50% glycerol-based diluent containing: 9% glycerol, 24% egg yolk, 67% buffer (5.9% Tris (w/v), 0.8% glucose (w/v) and 3.2% citric acid (w/v)), and 50% semen. A Freeze Control Cryochamber (CryoLogic PL, Australia) was used to cool samples slowly from 5 °C to -40 °C at 3 °C/min. At -40 °C the loaded Cassou straws were plunged in liquid nitrogen (LN_2). Rapid freezing was done by plunging micro-glass cryostraws into a LN_2 vortex, created using a magnetic stir bar. The micro-glass cryostraws were thawed by removal from the Cassou straws and immediate immersion in a 35 °C H_2O bath. The samples with the largest portion of live staining cells (93.18%) were treated with DMSO diluent using the rapid freezing. The remaining treatments ranked as follows: slow freezing with DMSO (78.84%), rapid freezing with glycerol (38.9%), and slow freezing with glycerol (26%). All treatments differed significantly ($P<0.01$). Studies state queens inseminated with greater than 50% viable spermatozoa have a good probability of producing normally throughout a season. Therefore, our technique of rapid freezing in DMSO diluent might be useful to apiculturists.

Introduction

The development of cryopreservation techniques for honey bee spermatozoa would enable the storage of its genetic material to preserve genetic diversity in the event of a catastrophe and provide seed stock for selective breeding efforts (Harbo and Harris 1999). The preservation of honey bee genetic material is particularly important because all estimates to date indicate that honey bees make an enormous contribution to pollination for crop production (Goulson 2003). In the United States, estimates of the economic value of honey bees range from $1.6-40 billion (Goulson 2003). Fortunately, honey bees are particularly suitable for genetic preservation because they are among the few insect species capable of reproducing via artificial insemination (Collins 2003).

Materials & Methods

Semen Collection From Bees

We obtained drones from colonies managed by the Department of Entomology at Washington State University and collected semen using a technique similar to existing methods (Woyke, 1955; Woyke *et al.*, 2001). Applying pressure bilaterally to the abdomen of the drones caused the endophallus to evert, and we extracted the ejaculate into 20-µL gel-loading tips using a 10-µL Hamilton pipettor. We sealed the tips with Critoseal (VWR, cat# 18000-298) and placed them in a styrofoam container with a frozen block of glacial acetic acid to maintain the temperature at 17 °C during transportation and storage. The following day, essentially all of the spermatozoa were motile prior to mixing the semen with diluents and freezing it. For a second collection from a local hive in Cheney, WA, we improved the collection procedure by replacing the gel-loading tips with siliconized 50-µL capillary tubes fitted to a Hamilton threaded-plunger syringe preloaded with Fluorinert (cat# F4758 Sigma, USA) to act as a hydraulic fluid. The spermatozoa were visible through the capillary tubes under white light using a Zeiss IM-35 inverted microscope (Carl Zeiss, Inc., Germany), which was advantageous for selecting samples to freeze.

Diluents

The freezing experiments involved the use of two diluents. We prepared the first diluent containing 25% DMSO (ICN, cat# 191418), 25% egg yolk, and 50% buffer as described in a previous study (Harbo, 1983). The buffer (pH 7.2) contained:

- 1.1% (w/v) $NaH_2PO_4 \cdot H_2O$ (Sigma, cat# S-8282)
- 0.845% (w/v) $Na_2HPO_4 \cdot 2H_2O$ (Fluka, cat# 71643)
- 0.25% (w/v) streptomycin (Sigma, cat# S-1277)

Diluting semen in the first diluent using a 3:2 semen:diluent ratio produced final concentrations of 10% (v/v) DMSO and 10% (v/v) egg yolk for freezing. We adapted the second diluent from a modern cryopreservation protocol for ram semen (Evans and Maxwell, 1987) by increasing the glycerol concentration to 9% (v/v).

Materials & Methods CONTINUED

Our modified diluent (pH 7.2) contained the following:

- 5.814% (w/v) Tris Base (Invitrogen, cat# 15504-020)
- 0.8% (w/v) glucose (Sigma, cat# G-5000)
- 3.184% (w/v) citric acid (Sigma, cat# C-2404)
- 24% egg yolk
- 9% glycerol (v/v) (BDH, cat# 101186M)
- 100k units penicillin per 100 mL (Sigma, cat# P-4687)
- 0.1% streptomycin (Sigma, cat# S-1277)

Diluting the semen in the modified buffer using a 1:1 semen:diluent ratio produced final concentrations of 4.5% (v/v) glycerol and 12% (v/v) egg yolk for freezing.

Micro-glass Cryostraw Freezing Containers

Figure 1 - Micro-glass cryostraw shown outside Cassou straw for freezing/thawing (right). The micro-glass cryostraws were inserted into Cassou straws (left) under liquid nitrogen for storage in tank.

We constructed the micro-glass cryostraws shown in Figure 1 by heating and pulling Pasteur pipets (VWR, cat# 14672-380) to fine diameters (265-µm OD; 230-µm ID). To facilitate fluid delivery to and from the micro-glass cryostraws, we slid a piece of Silastic® tubing (0.062" ID; 0.095" OD; Dow Corning cat# 602-265) over the large end. The tubing provided two options to load and unload the micro-glass cryostraws. First, the tubing could connect the micro-glass cryostraws to a threaded-plunger syringe. Our preferred alternative was to squeeze the tubing like an eye-dropper, roll the fingers toward the open end, and release before the fluid level reached the height where the micro-glass cryostraws began to widen. Fire polishing the large end of the micro-glass cryostraws before sliding them into the tubing seemed to prevent tears in the tubing and cracks in the micro-glass cryostraws.

Freezing Protocols

Figure 2 - Programmable Freezing Equipment

We loaded three micro-glass cryostraws per treatment with approximately 5 µL of diluted semen, and sealed the micro-glass cryostraws with Critoseal. Inserting the micro-glass cryostraws into 500-µL Cassou straws, we placed them in a water bath and cooled the semen-diluent mixture from room temperature to 5 °C over a two-hour period to allow the cryoprotectants to equilibrate. After equilibration, the freezing experiment followed a 2 x 2 factorial design using two diluents for freezing at slow and fast rates. For the slow freezing scheme, we loaded the cryostraws into a Freeze Control Cryochamber (CryoLogic PL, Australia) to cool the samples from 5 °C to −40 °C at 3 °C/min as Harbo (1983) described. The cryochamber is shown in Figure 3. When the samples reached −40 °C, we removed the cryostraws from the programmable freezer and plunged them immediately into liquid nitrogen.

Figure 3 - Cryochamber, Top View

To promote rapid cooling, we sought to maximize the surface area:volume ratio of the samples and minimize the formation of a vapor envelope between the liquid nitrogen and the micro-glass cryostraws (Libermann and Tucker, 2005). The small diameter of the micro-glass cryostraws maximized the surface area: volume ratio. Stirring the liquid nitrogen during cooling with a magnetic stir bar minimized the formation of a vapor envelope between the liquid nitrogen and the micro-glass cryostraws. We removed the Cassou straws from the 5 °C water bath, removed the micro-glass cryostraws from the Cassou straws, and plunged the micro-glass cryostraws into a liquid nitrogen vortex created using the stir bar. Submerging the empty Cassou straws in liquid nitrogen, we reinserted the micro-glass cryostraws into the Cassou straws and transferred the loaded cryostraws to a liquid nitrogen tank. Samples from the first and second collections remained stored in liquid nitrogen for 49 weeks and 6 days, respectively.

Materials & Methods CONTINUED

Thawing and Staining

We thawed the cells by removing the micro-glass cryostraws from the Cassou straws and transferring them immediately from liquid nitrogen to a recirculating water bath (VWR, model 1167) at 35 °C. Using a dual fluorescent nucleic acid staining system, (catalog #L7011, Molecular Probes, Eugene, OR), we evaluated the survival rates according to a technique similar to the one Collins and Donoghue (1999) described. Increasing the concentration of the SYBR-14 stain by a factor of 50 from the manufacturer's recommendations alleviated difficulty with photobleaching and poor fluorescent intensity. Additionally, reducing the concentration of the propidium iodide by half provided similar fluorescent intensity from the live and dead cells. We expelled the frozen-thawed semen into 0.5-µL microcentrifuge tubes containing 1 µL of 50X SYBR-14, 1 µL of 5X propidium iodide, and 5 µL of IVC-ONE medium (InVitroCare, cat# 2006). After a 6-minute incubation period at 37 °C, we placed 2-µL drops on Petri dishes and counted cells using the Zeiss inverted microscope and epifluorescence via a xenon light source.

Statistical Analysis

Our analysis of post-thaw viability ratios involved the large-sample statistical test for estimating differences between binomial parameters of two binomial populations (Mendenhall, 1979). The *z* statistics calculated according to Mendenhall formed the basis for a two-tailed test for significant difference between the proportions of viable cells measured for each treatment.

Results

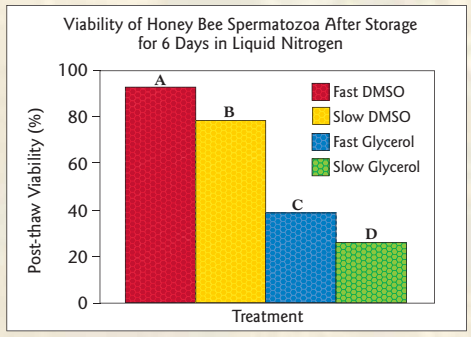

Figure 4 - Bee cell viability calculated as (# viable cells)/(total # cells) using dual fluorescent staining system after storage for 6 days. Different letters for each treatment indicate significant difference.

Figure 4 shows the proportions of viable honey bee spermatozoa after storage for 6 days in liquid nitrogen. The samples with the largest proportion of viable cells (93.18%) received the treatment with fast cooling and the DMSO diluent. The proportions of viable cells in the remaining treatments ranked as follows: slow cooling with DMSO (78.84%), fast cooling with glycerol (38.90%), and slow cooling with glycerol (26%), respectively.

Discussion

Consistent with previous studies (Harbo, 1983; Kaftanoglu and Peng, 1984), we observed that honey bee spermatozoa appear to survive short-term and long-term storage more effectively in the presence of DMSO than in glycerol. Although the measure of success for cryopreservation protocols has traditionally been the production of adequate numbers of workers to support the hive, Collins (2000b) reported that queens receiving 42.6% live spermatozoa measured via dual fluorescent staining produced brood composed entirely of workers. Subsequently, Collins (2003) reported that queens inseminated with 50% or greater viable spermatozoa would have a good probability of laying normally throughout an entire beekeeping season. Consequently, Collins concluded that a cryopreservation technique capable of preserving at least 50% viable spermatozoa would enable breeders to rear daughter queens and incorporate desirable genotypes into their breeding programs.

Using the same staining system in our experiments, our results demonstrate that exceeding the 50% viability threshold after short-term and long-term storage in liquid nitrogen is possible for honey bee spermatozoa using DMSO. Particularly encouraging is the marginal difference between the survival rate after 49 weeks using our fast freezing technique (41.46%) and the proportion Collins (2002b) reported for producing 100% workers (42.6%). Following optimization, our fast freezing technique may provide long-term post-thaw viability greater than 50%.

In addition to their favorable heat transfer characteristics, the micro-glass cryostraws in our technique serve as an excellent fluid delivery system similar to an eye dropper; however, the presence of two open ends simplifies the control of the fluid level while loading diluted semen. Releasing the tubing from between the fingers stops the flow of fluid almost immediately, which is not always possible while using a syringe. This insures that the fluid level remains below the part of the micro-glass cryostraw where it begins to widen near the tubing. Keeping the fluid in the narrow part of the micro-glass cryostraw maximizes the surface area:volume ratio and therefore the rate of cooling.

Our micro-glass cryostraw may also prove particularly useful for instrumental insemination. We observed during our experience with semen collection from the drones that the volume of semen varies between individuals from < 1 µL to 2-3 µL. The volume of our micro-glass cryostraws was approximately 5 µL, which would be sufficient to hold a volume of diluted semen from a single drone. Following warming, the micro-glass cryostraws may serve as a convenient insemination instrument and eliminate the need for additional fluid delivery equipment (Harbo, 1979) for insemination.

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