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Research Article

Effects of Various Cryoprotective Agents on Post-Thaw Drone Semen Quality

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Abstract

The aim of the present study was to evaluate the effect of different cryoprotectants on post-thaw semen motility and plasma membrane integrity of drone spermatozoa. Semen was obtained from mature drones (16 days or older) harvested from four colonies. Collected semen was diluted to a final concentration of 1/5 (semen/extender) in 0% cryoprotectant (control), 6% glycerol, 6% Ethylene Glycol, 6% 1,2 propanediol or 6% DMSO using a two-step dilution method. The equilibrated semen was frozen in 0.25-ml straws. The percentage of sperm motility and swollen tails (HOST) spermatozoa were evaluated following dilution with extender A (non-cryoprotectant), equilibration and post-thaw stages. In terms of post-thaw motility and plasma membrane integrity recovery we can rank the used cryoprotectant as DMSO, Ethylene Glycol, Glycerol and 1,2 Propanediol; respectively. In conclusion, post-thaw sperm motility and plasma membrane integrity of the present study was significantly better when sperm was frozen in DMSO with respect to control, glycerol, ethylene glycol, 1,2 propanediol ($P<0.05$).

Keywords: *Drone spermatozoa, Cryoprotectants, Cryopreservation*

Farklı Kriyoprotektanların Eritme Sonrası Arı Sperm Kalitesi Üzerine Etkileri

Özet

Bu çalışmanın amacı, farklı kriyoprotektanların eritme sonrası arı spermasının motilite ve plazma membran bütünlüğü üzerine etkisini değerlendirmektir. Sperma dört koloniden bulunan olgun arılardan (16 gün ve üzeri) alındı. Toplanan sperma final konsantrasyonu 1/5 olacak şekilde %0 kriyoprotektan içermeyen (kontrol), %6 gliserol, %6 etilen glikol ve %6 1,2 Propanediol ile iki aşamalı sulandırıldı. Ekilibrasyon sonrası sperma 0.25 ml'lik payetlerde donduruldu. Sulandırma sonrası, ekilibrasyon ve eritme sonrası aşamalarda motilite (%) ve HOS testi yapıldı. Eritme sonrası motilite ve plazma membran bütünlüğüne bakıldığında kullanım öncülüğüne göre kriyoprotektanları DMSO, Etilen Glikol, Gliserol ve 1,2 Propanediol olarak sıralayabiliriz. Sonuç olarak, eritme sonrası motilite ve plazma membran bütünlüğü bakımından; DMSO ile dondurulan sperma, kontrol, gliserol, etilen glikol, 1,2 propanediol gruplarıyla karşılaştırıldığında en iyi sonucu vermiştir ($P<0.05$).

Anahtar sözcükler: *Arı sperması, Kriyoprotektanlar, Dondurma*

INTRODUCTION

Honey bee breeder would like to obtain bee colonies that are tolerant or resistant to introduced or formerly unknown pathogens and parasites. Due to rapidly decreasing gene pool with many subspecies and ecotypes, *Apis mellifera* facing massive introgression of foreign genotypes [1].

Beekepers have little ability to control over the source of the semen that the queen stores in her spermatecha, unless they use isolated mating regimes or an area with desirable drone starins, ecotypes and races [2,3]. The development of the instrumentally insemination of the queen make it possible to control the number and genetic stock of drones involved in mating [4].

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The development of a practical means to store drone semen would enhance the bee breeder's ability to select and maintain superior honey bee stocks. Storing drone semen for a long time of particular colonies, strains, and races would allow bee breeders and scientists to retrieve, conserve, and spread valuable genetic traits [5]. Also, cryopreserving drone semen would be very useful for conserving germplasm that is being lost due to the recent and alarming mortality rates of honey bee colonies in many countries, often referred to as colony collapse disorder [6].

There have been successful attempts to store honey bee semen *in vitro* for various periods on time [4]. A number of storage techniques have been tried in drone semen with some success, using neat [5], cooled [7] and frozen [8] semen.

Neat and cooled semen have limited life span compared to frozen form. Maximum storage life at room temperature for fresh drone semen is approximately two weeks [5] and the cooled drone semen maintain fertilizing ability approximately for one year [7].

Deep-freezing techniques, known as cryopreservation, have been applied to mammalian semen for many years [9,10]. This technique, however, has not been successfully applied for honey bee *(Apis mellifera)* spermatozoa.

Cryopreservation of honey bee semen for later use would make it possible to maintain or increase genetic diversity in selected honey bee stocks. Higher intra-colony diversity has been shown to increase productivity, fitness and disease resistance and to decrease severity of infection and parasite loads in honey bees [11,12]. Additionally, there is an urgent need for the cryopreservation of honey bee spermatozoa to counter allelic losses caused by the population declines due to varroa mites, colony collapse disorder and other future threats [13].

With the aim of avoiding harmful effect of the cryopreservation, the addition of cryoprotectant to the extender is extremely important [14,15]. DMSO, Ethylene Glycol, Glycerol and 1,2 Propanenediol are the most used cryoprotectans in mammalian and drone semen using different concentrations and freeze-thaw procedures [14].

Using cryopreserverd semen is necessary to inseminate queens [13]. Because of the performance of instrumentally inseminated queens is related to semen quality, it is likely that poor results in previous studies may be consequence of low sperm viability after thawing [16].

The hypo-osmotic swelling (HOS) test which developed by Jeyendran et al.[17] has been effectively used to assess the functional integrity of mammalian and honey bee sperm plasma [18].

Honey bee queens can only be inseminated with fresh semen, when the drones are present [16]. The development of cryopreservation protocols for drone semen is a multi-factorial problem, involving the optimization of freezing rate, thawing rate, the nature and concentrations of cryoprotectants, and the extenders composition [8]. Therefore, the aim of the present study was to monitor the effect of different cryoprotectants on post-thaw semen motility and plasma membrane integrity of drone spermatozoa.

MATERIAL and METHODS

Experiments were performed in the Laboratory of Andrology in the Department of Reproduction and Artificial Insemination at the Faculty of Veterinary Medicine, Uludag University, Bursa, Turkey, in July.

Animals

Drones were reared in colonies established with a drone wax foundation. Mature drones (16 days or older) were collected from 4 colonies and brought to the laboratory.

Semen Collection and Dilution

Semen was collected from each drone by holding the head and thorax and gently squeezing the abdomen [4]. For semen collection at a 1/1 ratio, 0.8 mL of saline solution was drawn into a Schley syringe tip 1.10 (Schley Instrumental Insemination equipment, Lich, Germany) under a stereo microscope, followed by approximately 0.8 mL of semen (one drone). This process was replicated until a final volume of 10 mL of semen was obtained (approximately 12-14 drones) for each freezing groups.

Following semen collection, the samples were analyzed for sperm motility [16] and sperm plasma integrity using the water test [18].

Sperm Motility

Sperm motility was assessed by examining a drop of diluted ejaculate, covered with a cover slip under a phase-contrast microscope at 400x magnifications. Sperm motility was scored on a scale of 0 to 5 corresponding to 0%, 20%, 40%, 60%, 80% and ≥95% of the observed population being motile, respectively [16].

Water Test

A volume of 1.0 mL of semen was added to 250 mL of distilled water and incubated at room temperature for 5 min. Immediately after the incubation, one drop of semen was placed on a glass slide, covered and evaluated under a phase-contrast microscope (400x). Microscope fields were selected randomly. At least 100 spermatozoa were evaluated per slide, and the percentages of swollen tail spermatozoa were calculated [18].

Semen Dilution and Freezing

This study was adopted form one step dilution

mammalian semen freezing procedures. The composition of extenders was prepared according to Taylor et al.[16] diluents IV: (g/100 ml distillated water): Na Citrate 2.43, $NaHCO_3$ 0.21, KCl 0.04, Amoxicillin 0.03 and Catalase 200 mL). Prepared extender Ph' was adjusted to 8.1 and divided to five groups (Control group (cryoprotectant free), 6% Ethylene Glycol, 6% 1,2 6% Propanenediol and 6% DMSO)

Pooled semen (10 mL) was diluted with one of the extender at ratio of 1/5 semen/extender and cooled to 5°C within 1 h in water bath and than equilibrated at 5°C for 2 h. Equilibrated semen samples were filled into the center of 0.25 straws. The appropriate extender for each group was filled into one end of the straw, followed by an air space (~ 10 mm), then the equilibrated semen sample, followed by another similar-sized air space, and finally, the appropriate extender was added to the other end of the straw *(Fig. 1)*. Semen filled straws were sealed with different colors of the polyvinylalcohol (PVA) sealing powder for post-thaw straw identification *(Fig. 1)*. Semen filled straws were frozen in liquid nitrogen vapour at -110°C for 10 min and then plunged into liquid nitrogen at -196°C, where they were stored for at least one month.

At least three straws from each group were thawed at 37°C for 30 s in a water bath to evaluate post-thaw semen motility and plasma membrane integrity (5x3=15 straws). The procedure was repeated 5 times for each group.

Statistical Analyses

The Kruskal Wallis–Mann Whitney U test was used to compare the mean percentages of motile spermatozoa, and swollen spermatozoa obtained with water tests. All data were analyzed with the SPSS statistical package (SPSS 10.0 for Windows; SPSS, Chicago, IL, U.S.A), and a difference was considered significant at the $P<0.05$ level.

RESULTS

The mean observed semen motility and plasma membrane integrity in function of cryoprotectant at three stage were presented in *Table 1*. Sperm motility was progressively reduced through cooling and the freeze-thaw process ($P<0.001$). The motility of diluted semen in control group was significantly lower than those in the glycerol, Etilene glychol, 1,2 propanediol and DMSO ($P<0.05$). There are no significant differences among groups in terms of sperm plasma membrane integrity of diluted and equilibrated semen ($P>0.05$).

The sperm motility and plasma membrane integrity of the equilibration stage were lower than after dilution stage ($P<0.01$). The mean of sperm motility of equilibrated spermatozoa was higher in DMSO supplemented group as compared to control group ($P<0.05$).

Post-thaw semen motility and sperm plasma membrane integrity were lower than the equilibration and after dilution stages ($P<0.001$). All used cryoprotectants support post thaw semen motility and plasma membrane integrity to some degree. Post-Thaw semen motility and plasma membrane integrity was better in DMSO supplemented group than the glycerol, Etilene glychol, 1,2 propanediol and control groups ($P<0.05$).

DISCUSSION

The freeze-thaw process is detrimental to mammalian sperm motility and functional integrity [14,19,20], and to drone semen viability and motility [16]. Cryopreservation of honey bee semen has potential for long-term preservation of germplasm, however several factors need to be studied further to optimize post-thaw survival rates. Various extenders and cryoprotective agents have been developed for the cryopreservation of mammalian [14,21,22] and drone [16]

Fig 1. Straws filled and sealed semen with different colors of the polyvinylalcohol (PVA) powder for identification on freezing rack

Şekil 1. İçinde sperma doldurularak farklı renkli polyvinyl-alcohol (PVA) ile kapatılmış payetlerin dondurma tarağı üzerindeki görüntüsü

Table 1. *The mean (x±Sx) of studied sperm parameters in the function of cryoprotectant*

Tablo 1. *Kriyoprotektanlara göre elde edilen spermatolojik bulguların ortalamaları (x±Sx)*

Stage	Cryoprotectant	n	Motility (%) x±Sx	HOST (%) x±Sx
After dilution	Control	5	3.3±0.3^{a}	88.4±2.5
	Glycerol	5	3.9±1.9^{ab}	86.0±3.2
	Ethylene Glycol	5	4.1±0.1^{b}	89.4±2.4
	1,2 Propanediol	5	3.8±0.1^{ab}	87.0±3.2
	DMSO	5	4.0±0.0^{b}	92.8±0.7
Equilibrated	Control	5	2.2±0.6^{a}	81.0±4.3
	Glycerol	5	3.0±0.3^{ab}	74.6±5.4
	Ethylene Glycol	5	3.7±0.1^{b}	87.0±2.0
	1,2 Propanediol	5	3.4±0.3^{ab}	85.8±2.4
	DMSO	5	3.8±0.2^{b}	89.2±1.2
Post-thaw	Control	15	0.5±0.0^{a}	30.7±2.1^{a}
	Glycerol	15	2.0±0.2^{bc}	51.8±3.6^{b}
	Ethylene Glycol	15	2.3±0.2^{b}	55.9±4.0^{b}
	1,2 Propanediol	15	1.5±0.2^{c}	49.3±5.4^{b}
	DMSO	15	3.1±0.2^{d}	69.5±3.2^{c}

semen. In the present study, we evaluated the effects of different cryoprotectants on post-thaw semen motility and plasma membrane integrity of drone spermatozoa frozen in a Na citrate based extender.

The motility of semen diluted in Na citrate based extender in control, glycerol, Etilene glychol, 1,2 propanediol and DMSO group were 3.3, 3.9, 4.1, 3.8 and 4.0 and the percentage of intact sperm with plasma membrane functional integrity 88.4%, 86.0%, 89.4%, 87.0% and 92.8%; respectively. The sperm motility and plasma membrane integrity are similar to Nur at al.[18] used neat drone semen.

The development of drone semen cryopreservation protocols is a multi factorial problem, involving the optimization of cooling and freezing rate, thawing rate, the kind and concentrations of cryoprotectants, and extender composition. Like this study, the drone semen freezing protocols of many studies have been adopted from mammalian semen freezing procedures. Semen extension procedure of the present study was adopted form one step dilution mammalian semen freezing procedures [15]. Equilibrated semen quality is a sign of cryosurvivability in farm animals and included in many study related on semen freezing. Unfortunately there are no documented results related to equilibrate drone semen. Equilibrated semen motility and plasma membrane integrity in control, glycerol, Etilene glychol, 1,2 propanediol and DMSO group were 2.2, 3.0, 3.7, 3.4 and 3.8, and 81.0, 74.6, 87.0, 85.8 and 89.2; respectively.

The extender composition, extension temperatures, dilution rate, cooling rates to 5°C and presence of cryoprotectant have an effect on ram [14,15,18] bull [20] and drone [16] semen quality. As expected, the sperm motility and plasma membrane integrity of the equilibration stage were lower than after dilution stage (P<0.01). The mean of sperm motility of equilibrated spermatozoa was higher in DMSO supplemented group as compared to control group (P<0.05). It was observed that DMSO is good choice for drone semen cooling.

The equilibrated semen motility was better in all cryoprotectant supplemented groups compared to control group. This difference was observed in percentage of swollen tail spermatozoa generally except glycerol supplemented group. Glycerol containing diluents lead to cell death prior freezing stage [13]. For the criyoprotectant supplemented groups, it was observed that the lowest sperm motility and plasma membrane integrity were obtained in glycerol supplemented group.

The success of cryopreservation depends on many factors other than the freezing rate, such as species, breed, or variation among individual animals. Also the nature of the cryoprotectant [14,16], thawing temperature [18], sperm concentration [16] and variations in methodology [15] effect the post-thaw sperm recovery. Different animal species exhibit different sperm membrane compositions, such as different cholesterol/phospholipid ratios and degrees of hydrocarbon chain saturation, which can affect how the sperm responds to cooling and, subsequently, confer different sperm cryosensitivities across various species [23]. Despite the advances in cryopreservation techniques for mammalian spermatozoa, the success achieved with the cryosurvival of farm animal sperm has not been obtained from drone spermatozoa at the same success rate.

The main cryoprotective effect of cyoprotectant is visible at post-thaw stage. Post-thaw semen motility and plasma membrane integrity in control, glycerol, Etilene glychol, 1,2 propanediol and DMSO group were 0.5, 2.0, 2.3, 1.5 and 3.1, and 30.7, 51.8, 55.9, 49.3 and 69.5; respectively. All cryoprotectant supplemented groups were yield better motility and plasma membrane integrity. Post-thaw semen motility and sperm plasma membrane integrity were lower than the equilibration and after dilution stages (P<0.001).

The extenders composition assists in stabilizing the cell during the freezing and thawing process [14,19]. Post-thaw plasma integrity was unbroken for some degree in control group. These findings indicated that used extender has a protecting capability on drone plasma membrane integrity. Also the better survivability of post-thaw plasma membrane integrity than motility means that drone sperm plasma membrane integrity is more resistant to the cryopreservation related damages.

In terms of post-thaw motility and plasma membrane integrity recovery we can rank the used cryoprotectant as

DMSO, Ethylene Glycol, Glycerol and 1,2 Propanenediol; respectively. Although the presence of glycerol and 1,2 propanedidiol in freezing extenders protect sperm motility and plasma membrane integrity, they were incapable of preserving sperm plasma functional integrity. 1,3 Propanediol is a very poor glass-former [24] and a low dose of 1-3 Propanediol is likely to be insufficient for the efficient cryopreservation of drone sperm. It is unlikely to be suitable as single-component CPA for honey bee drone semen, but could be interesting as one component of a CPA cocktail [25]. It was observed that 6% 1,3 propanediol supplemented group post-thaw motility and plasma membrane integrity was the lowest in cryoprotectant supplemented groups.

The cryoprotective agent most frequently used with drone semen freezing is dimethyl sulfoxide (DMSO), although this substance has been suspected of causing genetic damage in sperm [25]. Due to the DMSO led to higher post-thaw survival [16], different concentration of DMSO has became the main choice for many studies on drone semen preservation up to the present [8,16]. Post-thaw sperm motility and plasma integrity of the present study were significantly better when sperm was frozen in DMSO with respect to control, glycerol, Ethylene glycol, 1,2 propanediol.

It could be concluded that DMSO supplemented extenders are a better choice for drone semen freezing compared to Glycerol, Ethylene Glycol, and cryoprotectant free control group for post-thaw motility and plasma membrane integrity. Further studies on combination of CPA with some sugars should be done for the overcome the detrimental effect of CPA and cryopreservation related damages on motility and plasma membrane integrity.

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