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ORIGINAL RESEARCH ARTICLE

Preservation of honey bee spermatozoa using egg yolk and soybean lecithin-based semen extenders and a modified cryopreservation protocol

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Better protocols and suitable extenders for semen handling and cryopreservation are required for honey bees to enhance sperm quality and fertility similar to that of other species. The purpose of the present research is the improvement of honey bee sperm quality using different semen extenders and a modified cryopreservation protocol. Three different extenders were used: (1) buffer and egg yolk (EY); (2) buffer and 0.5% soybean lecithin (SL0.5); and (3) buffer and 2% soybean lecithin (SL2). Semen was collected and then diluted with the experimental extenders. The diluted semen was gradually cooled in a refrigerator to 5 °C and immediately loaded into straws and frozen with liquid nitrogen. Motility and viability data analyzed using the GENMOD and GLM procedure of SAS software. The results demonstrate that the mean fresh sperm motility in EY (4.75 ± 0.14) and SL2 (4.5 ± 0.2) were significantly ($p < 0.05$) higher than the SL0.5 (4.12 ± 0.12). Also, the mean of cooled motile spermatozoa in EY and SL2 were significantly higher than the SL0.5 (3.87 ± 0.24). Post-thawed sperm motility in EY (3.6 ± 0.24) was significantly ($p < 0.05$) higher than the other extenders. Furthermore, percentage of viable spermatozoa in EY ($69.75 \pm 2.32\%$) was significantly higher than SL0.5 and SL2 (38.5 ± 2.32 and $45 \pm 2.32\%$ respectively). Therefore, according to the results of this study, use of an egg yolk-based extender could better maintain honey bee semen motility and viability after freeze-thawing process, compared to SL-based extenders. However, more advanced tests and fertility evaluation are needed to reveal the exact effects of egg yolk and lecithin based semen extenders on post-thawed drone sperm quality.

Preservación de espermatozoides de la abeja de la miel usando extensores de semen basados en la yema de huevo y la lecitina de soja y un protocolo de criopreservación modificado

Se requieren mejores protocolos y diluyentes adecuados para el manejo del semen y la criopreservación en la abeja de la miel para mejorar la calidad del esperma y la fertilidad como en otras especies. El propósito de la presente investigación es la mejora de la calidad del esperma de la abeja de la miel utilizando diferentes diluyentes de semen y un protocolo de criopreservación modificado. Se utilizaron tres extensores diferentes: 1. tampón y yema de huevo (YH); 2. tampón y 0,5% de lecitina de soja (LS0.5); y 3. tampón y 2% de lecitina de soja (LS2). El semen se recogió y después se diluyó con los extensores experimentales. El semen diluido se enfrió gradualmente en un refrigerador a 5 °C e inmediatamente se cargó en canutillos que se congelaron con nitrógeno líquido. Los datos de motilidad y viabilidad se analizaron mediante el procedimiento GLM y GENMOD y el software SAS. Los resultados demuestran que la motilidad media fresca de espermatozoides en YH (4.75 ± 0.14) y LS2 (4.5 ± 0.2) fue significativamente ($p < 0.05$) más alta que la LS0.5 (4.12 ± 0.12). Además, la media de espermatozoides móviles enfriados en YH y LS2 fue significativamente más alta que la LS0.5 (3.87 ± 0.24). La movilidad posterior de los espermatozoides congelados en YH (3.6 ± 0.24) fue significativamente ($p < 0.05$) más alta que los otros extensores. Además, el porcentaje de espermatozoides viables en YH ($69.75 \pm 2.32\%$) fue significativamente mayor que en LS0.5 y LS2 ($38.5 \pm 2.32\%$ y $45 \pm 2.32\%$, respectivamente). Por lo tanto, de acuerdo con los resultados de este estudio, el uso de un extensor basado en yema de huevo podía mantener mejor la movilidad y la viabilidad del semen de la abeja de la miel después del proceso de congelación-descongelación, en comparación con los extensores basados en LS. Sin embargo, se necesitan pruebas más avanzadas y una evaluación de la fertilidad para revelar los efectos exactos de los diluyentes de semen basados en la yema de huevo y la lecitina en el la calidad del esperma descongelado de los zánganos.

Keywords: honey bee; extender; soybean lecithin; sperm freezing

Introduction

Breeding programs and controlled crosses in the honey bee can be attainable through sperm preservation and artificial insemination (Cobey, 2007). The development of a practical means to store honey bee semen would enhance our ability to select and maintain superior honey bee stocks (Collins, 2000). The long-term storage of

human and livestock semen by means of deep-freezing techniques, known as cryopreservation, has been possible for many years. This technique, however, has not hitherto been successfully adopted for the preservation of honey bee sperm (Pegg, 2007). The composition of semen extenders and improved cryopreservation protocols plays a key role in maintenance or enhanced quality

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and fertility of preserved sperm (Aires et al., 2003; Cobey, Tarpy, & Woyke, 2013; Hopkins, Herr, & Shepard, 2012; Watson, 1981; Wegener, May, Kamp, & Bienefeld, 2014). Extenders that are used for semen cooling and freezing contain several components and additives that can provide a suitable milieu for sperm preservation (Curry, 2000; Holt, 2000).

Over the past 40 years, various extenders and protocols have been used to improve honey bee sperm quality in the cooled or frozen state (Hopkins & Herr, 2010; Kaftanoglu & Peng, 1984; Taylor, Guzmán-Novoa, Morfin, & Buhr, 2009; Wegener & Bienefeld, 2012). Also, utilization of mammalian sperm cryopreservation methods and techniques for the preservation of honey bee sperm have led to improvement in sperm quality (Taylor et al., 2009). Egg yolk based-semen extenders have been used for almost all farm animals and also for the honey bee (Emamverdi et al., 2015; Harbo, 1983; Hopkins et al., 2012; Watson, 1981). Soybean lecithin is a plant-derived product, often used as an emulsifier in human food production, which might possess cold shock protective qualities similar to the low density lipoprotein (LDL) in egg yolk. Soybean lecithin-based semen extenders have produced post-thaw sperm quality equal to or greater than traditional egg yolk extenders when used for semen cryopreservation of humans (Reed, Ezeh, Hamic, Thompson, & Caperton, 2009), cows (Aires et al., 2003) and sheep (Emamverdi, Zhandi, Zare-Shahneh, Sharafi, & Akbari-Sharif, 2013). So, Lecithin or phosphatidylcholine is one of the most important and effective component of egg yolk and soybeans during cryopreservation. It is the most potent cryoprotectant agent known, which preserves the membrane phospholipid integrity during the cooling process and cryopreservation (Bergeron & Manjunath, 2006; Moussa, Martinet, Trimeche, Tainturier, & Anton, 2002).

It has however been reported that egg yolk in semen extenders has problems such as cryoprotective antagonists, risk of microbial contamination and more (Aboagla & Terada, 2004; Emamverdi et al., 2013; Forouzanfar et al., 2010). Therefore, in recent years, the use of soybean lecithin-based semen extender has been developed for sperm cryopreservation in species such as humans (Reed et al., 2009), bulls (Aires et al., 2003), rams (Emamverdi et al., 2015), stallions (Papa et al., 2011), cats (Vick, Bateman, Lambo, & Swanson, 2012) and goats (Vidal et al., 2013). The use of this extender would be to decrease extender variability and the potential for disease transition compared to egg yolk-based extenders and in some cases, decrease extender cost and improve post-thawed sperm quality (Emamverdi et al., 2013; Vick et al., 2012). This is the first study using a new semen lab-made extender containing different levels of soybean lecithin for the cryopreservation of honey bee sperm. However, the goal of this study was comparison of the effect of egg yolk and lecithin based-semen extenders on drone sperm motility

and viability, and a modified cryopreservation protocol for the improvement of honey bee sperm quality.

Materials and methods

Chemicals

The chemicals used in this study were purchased from Sigma Chemical Co. (St. Louis, MO, USA) and Merck (Darmstadt, Germany).

Semen collection

Semen samples were collected from mature drone (aged 17–21 day, $n = 100$ /replicate) honey bees using a pulled pipette connected to an artificial insemination device (www.talghihe-malekeh.com). After collection, semen samples were pooled and diluted to three equal parts and each part was diluted by one of the following extenders. Semen collection was repeated five times on five different days.

Extenders

Various preservation media or extenders (EY, SL0.5 and SL2) were used for this study and prepared as following: A. Tris-soybean lecithin-based (SL0.5) extender, prepared via addition of 0.5% (w/v) soybean lecithin (Sigma, P3644) to a Tris-based semen extender [1.91 g tris (Merck, 1083870500), 263 mg glucose (Sigma, G7021), 958 mg citric acid (Merck, 1002440500), 11% (v/v) glycerol (Merck, 1040940500), 10% (v/v) DMSO (Sigma, D8418), 32 mg streptomycin (Sigma, S9137) in 25 ml Milli-Q water]; B. Tris-soybean lecithin-based (SL2) extender prepared via addition of 2% (w/v) soybean lecithin to the Tris-based extender above; and C. Tris-egg yolk based (EY) extender prepared via addition of 20% (v/v) egg yolk to the Tris-based extender above. After preparation of tris buffer, soybean lecithin and egg yolk added to it and vortex for 20 min. Because this process and components of buffer, especially glycerol and DMSO caused better dissolvent of soybean lecithin and egg yolk in tris-based semen extender.

Semen dilution and freezing process

After semen collection in each replicate, semen samples were pooled in a micro-tube and then divided into three equal parts. Each parts was diluted with one of above mentioned extenders in ratio of 12:1 (extender and semen, respectively) that reported by Taylor et al. (2009). The micro-tube containing diluted semen samples were placed into falcon tube and then in a 100 ml beaker containing isotherm water and put in refrigerator for gradually cooling to 5 °C during a period of 2 h. After that, cooled semen samples were aspirated into 0.25 ml straws (IMV, L'Agile; France) that cut to a length of 6.5 cm, including the cotton/gelatin plug by modified

method of Hopkins et al. (2012). Straws were loaded with 20 μ l extender, an air space, 20 μ l semen–diluent mixture, air space and approximately 20 μ l extender or until the initial fluid sealed the plug. The open ends of the straws were sealed with bulb hematocrit. Before freezing, the straws were placed at 5 cm above of the liquid nitrogen vapor in a Styrofoam box with 3.6 cm thick walls (38 cm \times 25 cm \times 22 cm) for a period of 7 min.

Thawing process and semen evaluation

The straws were thawed for 10 s at 40 °C in a water bath, and the contents were poured into a glass tube and used for sperm evaluation. Motility of honey bee spermatozoa was assessed during fresh, cooled (after 5 °C) and post-thawed preservation. In addition, percentage of viable spermatozoa was evaluated after freeze-thawing process.

Sperm motility

Sperm motility was assessed using a phase-contrast inverted light microscope (CKX41; Olympus, Tokyo, Japan) with a warm stage maintained at 37 °C. Three different microscopic fields for each semen sample were assessed, and the mean of the three successive estimations were recorded as the final motility. Honey bee sperm motility reported as a ranking from 0 to 5 respectively that represent 0, 20, 40, 60, 80 and more than 90% motility (Taylor et al., 2009).

Sperm viability

Viable and non-viable spermatozoa were determined by the eosin–nigrosin staining (Lu, Huang, & Lü, 2010) by modified method. Sperm suspension smears were prepared by mixing a drop of sperm with two drops of stain on a warm slide and spreading the stain with a second slide. Viability was assessed by counting 200 cells at 400 \times magnification (CKX41; Olympus, Tokyo, Japan). Sperm displaying partial or complete purple staining were considered nonviable; only sperm showing strict exclusion of stain were counted as viable.

Statistical analysis

The SAS System (SAS version 9.12, SAS Institute Inc., Cary, NC, USA) was used to analysis of data. Data of motility and viability analyzed using the GENMOD and GLM procedure of SAS software, respectively. The significance level was set at $p < 0.05$. All values given are Lsmeans \pm SEM.

Results

All sperm parameters evaluated decreased with increasing storage time. The mean fresh sperm motility in extenders containing egg yolk (4.75 ± 0.14) and 2%

soybean lecithin (4.5 ± 0.2) were significantly ($p < 0.05$) higher than the 0.5% soybean lecithin based-extender (4.12 ± 0.12) (Figure 1). Also, cooled spermatozoa motile in egg yolk and 2% soybean lecithin based-extendents (4.5 ± 0.2 and 4.62 ± 0.12 , respectively) were significantly higher than the 0.5% soybean lecithin based-extender (3.87 ± 0.24) (Figure 2). Post-thawed sperm motility in extender containing egg yolk (3.6 ± 0.24) significantly ($p < 0.05$) was higher than the other extenders (Figure 3). Furthermore, percentage of viable spermatozoa in egg yolk based-extender ($69.75 \pm 2.32\%$) was significantly higher than extenders containing 0.5 and 2% soybean lecithin, but the two extenders (38.5 ± 2.32 and $45 \pm 2.32\%$ respectively) were not significant different (Table 1).

Discussion

Successful cryopreservation of honey bee sperm is affected by many factors, including the size and age of the drone, quality of the semen, extender and protective substances contained in it, the ratio of semen and extender dilution and the cooling, freezing, and thawing techniques. It is also worth noting that the field of cryopreservation of honey bee sperm is very difficult and has limitations such as small semen volumes, lack of adult drone bees and problems for semen collection. Therefore, during recent years there have been few studies on honey bee sperm preservation. The major problem of honey bee semen preservation is declines in sperm quality such as viability, motility and low sperm fertility during long-term storage. So, one of the main strategies that many researchers have followed has been the use of appropriate extenders and finding suitable technique for preservation of honey bee sperm quality and fertility (Hopkins & Herr, 2010; Taylor et al., 2009).

In the current study, the effect of a modified new semen extender containing egg yolk or different levels of soybean lecithin to preserve honey bee sperm quality

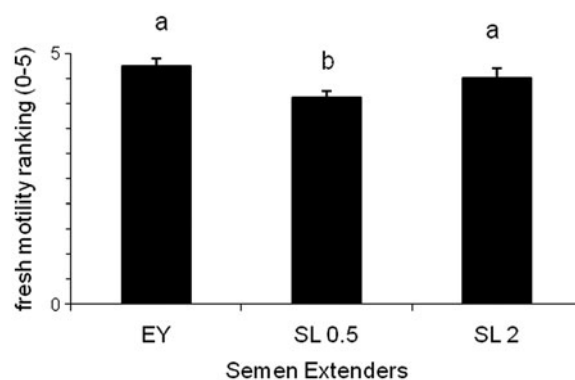


Figure 1. The effect of different semen extenders on fresh honey bee motile spermatozoa (Lsmean \pm SEM). Note: a and b indicate differences ($p < 0.05$). EY (egg yolk-based extender), SL0.5 (0.5% soybean lecithin-based extender) and SL2 (2% soybean lecithin-based extender).

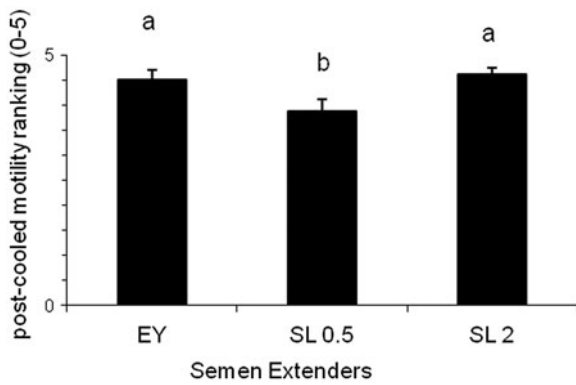


Figure 2. The effect of different semen extenders on post-cooled honey bee motile spermatozoa (Lsmean ± SEM). Note: *a* and *b* indicate differences ($p < 0.05$). EY (egg yolk-based extender), SL0.5 (0.5% soybean lecithin-based extender) and SL2 (2% soybean lecithin-based extender).

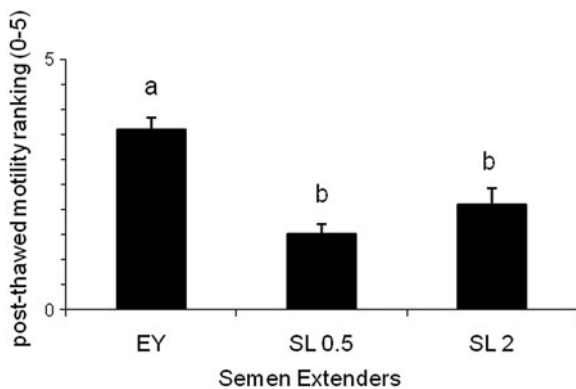


Figure 3. The effect of different semen extenders on post-thawed honey bee motile spermatozoa (Lsmean ± SEM). Note: *a* and *b* indicate differences ($p < 0.05$). EY (egg yolk-based extender), SL0.5 (0.5% soybean lecithin-based extender) and SL2 (2% soybean lecithin-based extender).

Table 1. Improvement of post-thawed honey bee sperm viability using different semen extenders (Lsmean ± SEM).

Parameter	Semen extenders ^{a,b}			SEM
	EY	SL0.5	SL2	
Viable sperm (%)	69.75 ^a	38.5 ^b	45 ^b	2.32

Notes: Different superscripts within rows are significantly differences ($p < 0.05$). EY (egg yolk-based extender), SL0.5 (0.5% soybean lecithin-based extender) and SL2 (2% soybean lecithin-based extender).

has been investigated. Many studies reported that phosphatidylcholine of egg yolk and soybean lecithin has beneficial effects on preservation of mammalian sperm function and improves the microscopic parameters of spermatozoa (Emamverdi et al., 2013; Forouzanfar et al., 2010; Vick et al., 2012). Our results showed that honey bee sperm motility was higher in fresh semen with extenders containing egg yolk and 2% soybean lecithin. Furthermore, during the cooled storage, sperm motility was higher in extenders containing egg yolk and 2%

soybean lecithin. Also, after freeze-thawing process, sperm motility was higher in the extender containing egg yolk than soybean lecithin based-extendere. Better preservation of honey bee sperm motility using egg yolk based-extender than extenders containing soybean lecithin, possibly could due to the protective mechanism of egg yolk LDL on sperm membrane, which provided a suitable environment for sperm quality preservation (Bergeron & Manjunath, 2006; Moussa et al., 2002).

Our results also revealed that the percentage of post-thawed viable spermatozoa in egg yolk based-extender was significantly higher than extenders containing 0.5 and 2% soybean lecithin. Also, according to experiments conducted by Hopkins and Herr (2010) on the factors in the success of honey bee sperm freezing, the highest percentage of sperm viability after 6 days storage in liquid nitrogen, contained egg yolk. Taylor et al. (2009) reported that the use of mammalian sperm cryopreservation techniques to preserve honey bee sperm led to improvements in sperm quality, so that 63% live sperm was obtained after the freeze-thawing process. Also, it is reported that sperm viability higher than 46% can be used for successful artificial insemination of honey bees (Collins, 2000).

The success of cryopreservation could be affected by extender components, such as the buffer system, osmotic pressure, and concentration of glycerol, dimethyl sulfoxide, and soybean lecithin or egg yolk. The stabilization of sperm membrane plays an important role in the improvement of post-thawed sperm quality (Barbas & Mascarenhas, 2009; Maxwell & Watson, 1996). The disruption of plasma membrane integrity caused by disarrangement of lipids within the membrane during cryopreservation may induce further cellular damage and consequently led to sperm death (Emamverdi et al., 2013; Holt & North, 1994). It has been suggested that soybean and egg yolk lecithin protect phospholipids in sperm membranes by occupying the surface of sperm plasma membrane and increases freezing tolerance (Bergeron & Manjunath, 2006; Quinn, Chow, & White, 1980; Watson, 1981). Therefore, egg yolk based-extender might have provided better protection for the plasma membrane integrity that led to enhanced post-thawed sperm motility and viability. Of course, due to the problems of egg yolk in animal or honey bee semen extender and the acceptable results obtained with soybean lecithin extenders, this research demonstrated the suitability of soybean lecithin as an appropriate extender when egg yolk is undesirable.

In conclusion, it seems that semen preservation of honey bee sperm using egg yolk and perhaps 2% soybean lecithin based-extendere would be appropriated for artificial insemination in order to accelerate breeding programs. So, this study was the first research that used soybean lecithin as a protective extracellular with a mixture of glycerol and dimethyl sulfoxide as a protective intracellular for honey bee sperm cryopreservation. Of course, further studies using more advance tests and

fertility assessment are needed to improve and modified this extender for reveal exact effects of egg yolk and soybean lecithin-based semen extenders on post-thawed sperm quality.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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