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Effects of Different Concentratıons of Proline, Cysteine and Glutamine on Some Sperm Quality Characterıstıcs and Fertility of Cryopreserved Bee Spermatozoa (*Apis Mellifera*)

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Abstract

Development of novel cryopreservation protocols is required to test novel chemical and non-chemical supplements in freezing extender for bee semen. Antioxidants demonstrate critical effects on post-thawed sperm quality, fertility, and subsequent embryo development. The purpose of the study was to determine of the effects of different concentrations of proline, cysteine and glutamine on some sperm quality and fertility of cryopreserved bee spermatozoa. The experiments were designed to compare the effects of 25mM, 50mM, 100mM proline, cysteine, glutamine and control II with no supplement on cryopreservation of honey bee semen based on sperm motility, viability, intact membrane (hipo osmotic swelling test) and fertility rates. Fresh semen was held as a control I in the study. 50mM proline amino acid in the cryopreserved groups had the most profound affirmative effect on sperm motility, viability, percentage of spermatozoa with intact membrane and fertility. Cryopreservation process in the tested groups caused gradually a decreasing of motility, viability, intact membrane and fertility rates when compared to fresh control group ($p<0.05$-0.01). However, proline, cysteine and glutamine did not provide any significant improvement in sperm quality and fertility compared to the cryopreserved control group. Our results indicate that successfully long-term storage of bee sperm cryopreservation and fertility are achievable when used appropriate sperm freezing protocol and antioxidant. Proline amino acid as an antioxidant in semen extender had more beneficial influence on sperm quality parameters and fertility. The success of cryopreservation with antioxidant is related with chosen antioxidant in a dose and type dependent manner.

Keywords: Apis mellifera; Cryopreservation; Antioxidant; Motility; Fertility

List of abbreviations: DMSO: Dimethyl Sulfoxide; ROS: Reactive Oxygen Species; MFI: The membrane function integrity; HOST: Hypo Osmotic Swelling Test

Introduction

Honey bees are important insects on the world and play a significant role in the pollination of many flowering food crops around the world. Globally there are more honey bees than other types of bee and pollinating insects, so it is the world's most important pollinator of food crops. It is estimated that one third of the food that people consume each day relies on pollination mainly by bees [1]. In addition to these, bee deaths, which have been increasing in the world in recent years and the cause of which cannot be determined, have become a phenomenon known as Colony Collapse Disorder [2,3].

To protect bees, gamete cryopreservation is significant tool for long term archiving with cryostorage. Previously, spermatozoa from mammals have been shown to remain viable for extended periods of time following cryogenic storage in liquid nitrogen [4,5]. However, Harbo [6] reported that honey bee queens produced a lower proportion of worker brood (compared to the proportion of bee brood) when inseminated with cryopreserved semen stored for different lengths of time. Queens inseminated with semen cryopreserved for 4 days produced 22% worker brood, while a sample frozen for two years resulted in only 8% worker brood. In additon, Harbo [7] tested different semen storage diluents and freezing conditions as a way to improve cryopreservation of honey bee semen in liquid nitrogen and reported that the best diluent contained 60% semen, 10% DMSO and 30% saline solution. The variation in fertilization rates following cryopreservation of bee semen have not been fully explained. Some scientific papers [8,9]

reported that the production of unfertilized eggs by queens that were inseminated with DMSO-treated semen may be due to DNA damage in frozen semen. DMSO has been reported to exhibit negative impacts on spermatozoa and queens when containing in semen used for inseminations [10]. Similar to the methods used in other farm animals, some methods are used for the storage of honey bee sperm. However, different extenders and chemicals used in cryo-storage solutions of honey bee spermatozoa have different effects on freezing success [11]. Many factors are effective in freezing sperm, the most important of these are cryoprotectant toxicity, oxidative stress, temperature, freezing rate and cold shock effects [12].

Cryopreservation process effects molecular functions of sperm cell and induces lipid peroxidation and enhances the levels of reactive oxygen species (ROS), that disrupt the balance between free radicals and antioxidant system of frozen-thawed spermatozoa, causing to oxidative stress [13]. Prolonged exposure of spermatozoa to ROS compromises plasma membrane integrity, causes a rapid loss of intracellular ATP and axonemal damage, decreases sperm motility, leads to the loss of intracellular enzymes and DNA fragmentation, impairs the fertilization ability of sperm cells, and decreases fertility rates [14,15]. Wegener *et al.* [16] reported that cryopreservation process increases the proportion of cells with DNA-nicks.

Cryopreservation also causes damage at the molecular level including an increase in sperm DNA damage, loss of membrane-bound proteins, cytoplasmic proteins, and other cellular components [17,18] and reduction or elimination of certain gene transcripts [19]. Cryopreservation processes degrade specific mRNA of sperm by the cryopreservation procedure [20,21].

ROS can be deactivated by antioxidant enzymes such as, proline, cysteine, glutamine, superoxide dismutase, glutathione peroxidase and catalase. In sperm cells, the defense mechanism against lipid peroxidation also involves non-enzymatic molecules such as thioredoxin and glutathione [22]. Amino acids have antioxidant features and found in seminal plasma at high concentration (cysteine, glycine, proline and histidine e.g.). Amino acids have an important biological role for prevention of cell damage during cryopreservation. Thus far, conducted studies in mammalians have demonstrated that supplementation of amino acids (proline, cysteine, glutamine, taurine, hypo taurine, glycine and histidine e.g.) to extenders reduced sperm damage and DNA fragmentation and improved post-thaw motility [23,24]. In other word, motility, membrane stability, spermatozoa functionality and DNA integrity in sperm cell are affected by oxidative stress due to generation of ROS during dilution in the extender media, cryoprotectant exposure and cooling process [25-28]. Due to these reasons, usage of antioxidants in the cryopreservation is important for cryopreservation success.

To date, several researchers have studied regarding beneficial effects of different amino acids for semen cryopreservation in different mammalian sperm cells. Protective effects of the amino acids during cryopreservation of spermatozoa in stallions [29], rams [30,31], monkey [32], bulls [23,24,33], and human [34] have been reported. Proline, cysteine and glutamine have been reported to improve the motility and membrane integrity during freezing and thawing stages [31,34-37] probably by protecting free-radical-induced damage [38]. However, the exact mechanism of cryoprotection provided by amino acids is not completely understood and still some parts are unclear. To date, there is no any report regarding honey bee semen cryopreservation and fertility endpoint on effects of different amino acids in semen extender. Thus, it was aimed in this study to determine the effect of the different levels of proline, cysteine and glutamine amino acids on honey bee sperm cryosurvival and the fertility.

Material and Methods

Collection and Dilution of semen

This experiment was conducted at Hatay Mustafa Kemal University (HMKU), Hatay, Turkey, between May and August 2019. All bees and queens were collected from honey bees (*Apis mellifera anatoliaca*) reared at HMKU (Figure 1). Drones were collected (16 d or older) from

Figure 1: Bees and Queens Collection

colonies located in a single apiary during their daily flight period and held in a mixed flight cage. Semen was collected on the same days from mature drones by holding the head and thorax and squeezing the abdomen using standard techniques [39]. Experimental solutions were drawn into a Schley syringe tip (Schley Instrumental Insemination Equipment, Lich, Germany) to collect semen under a stereo microscope.

Collected semen was frozen by the methods which explained under experimental design title. All queens were reared inside queenless colonies and inseminated with frozen-thawed semen which was cryopreserved. Following insemination, each queen was clipped, tagged, and placed in a queen cage in nucleus hives containing three frames with young adult bees to determine the resulting brood patterns. Ten queens were inseminated with the frozen semen for each group.

Dilution was used in this study was modified from the Hopkins *et al.* [40]. The dilution composition was 14.20 mg sodium phosphate dibasic, 30 mg sodium citrate, 611.3 mg KCl, 490 mg NaCl, 42 mg, $NaHCO_3$, 687.8 mg TES buffer, 363.4 mg Tris base, 0.37 mg EDTA, 0.07 g penicillin and 0.05 g streptomycin in a final volume of 100 mL. The osmotic pressure and pH of the prepared solution were adjusted as 250 mOsmol and 7.8 pH, respectively.

In the study, unless otherwise stated, all chemicals were purchased from Sigma Chemical Company (St. Louis, MO, USA).

Experimental Design

The experiments were designed to compare different amounts of (25mM, 50 mM and 100mM) proline, cysteine and L-glutamine amino acids and control II group without addition on cryopreservation of honey bee semen based on sperm motility, viability, intact membrane and fertility rates. Therefore, bee semen was collected using standard methods as described above (Figure 2). Basic saline solution was loaded into the syringe tip for semen collection by Schley artificial insemination instrument. Fresh semen in the study was held as a control I in the study. Ten cryopreserved study groups were either Control II with no supplement, 25mM, 50mM and 100mM proline, 25mM, 50mM and 100mM cysteine, or 25mM, 50mM, and 100mM glutamine groups, respectively.

Figure 2: Semen Collection for Artificial Insemination in Honey Bees

Freezing and thawing of semen samples

Semen was collected from drones and frozen by the method developed by Hopkins *et al.* [40]. Semen was mixed with dilution (v/v, 5:1) after the collection process. Three parts of diluted semen were mixed with two parts of the cryoprotectant solution. The cryoprotectant solution consisted of 75% buffer (79.7 mM NaH_2PO_4 and 31.6 mM Na_2HPO_4 made up to a final volume of 25 mL using distilled water with pH adjusted to 7.2 using 6 M NaOH) and 25% DMSO. The cryoprotectant in the study was mixed and finalized with either 25mM, 50mM and 100mM proline (Molecular weight: 115.13 g/mol), 25mM, 50mM and 100mM cysteine (Molecular weight: 121.16 g/mol), or 25mM, 50mM, and 100mM glutamine (Molecular weight: 146.14 g/mol) amino acids, alternately. All mixed semen was loaded into 0.25 ml cryo-straws. Straws were heat-sealed on one side after filling and were placed in a water bath in a standard refrigerator for 2 hours as equilibration time until samples reached 4 °C. All straws were placed vertically into cryochambers of a controlled rate freezing system (Biogenics Crysalys Cryocontroller PTC-9500). The freezing program used in the present study was 3 °C/min from 5 °C to -40 °C in 23 min then held at -40 °C for 5 min. After the cooling process, all straws were placed into liquid nitrogen tank and stored about 10 days until insemination process. For thawing, all straws were removed from liquid nitrogen and plunged into a water bath at 35 °C for 30 sec. before sperm assessment. Following the sperm assessment, frozen-thawed semen artificially inseminated to queen bees on the same day to determine fertility rate.

Sperm assessment

Motility: The assesment of the motility was based on observations at three locations within the field of view, visually assessed by a single observer for each straw before insemination (Figure 3). A subjective motility scored from 0% to 100 %. The score of '100%' was assigned if motility was indiscernible from that of fresh semen and a score of '0%'was given if spermatozoa were only slightly moving or unmoving. An aliquot (10 µL) of the semen sample was immediately viewed under a light microscope at x 400 magnification [41]. To determine fertilization capacity, 32 virgin sister queens were reared, with 8 randomly chosen queens inseminated with 5 µl frozen-thawed semen for each method.

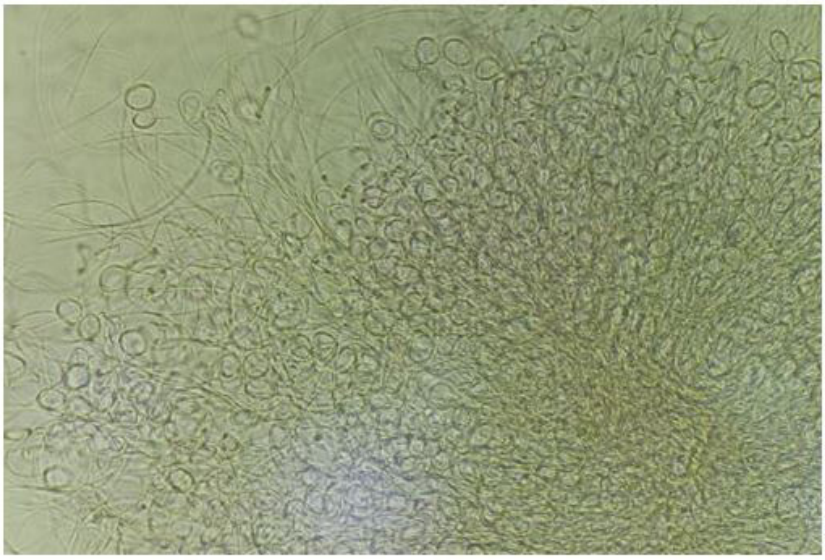

Figure 3: Sperm Motility Assessment in Honey Bee Drones

Viability Assessment: To assess live/dead sperm percentage, eosin-nigrosin preparations were made according to the method noted by Björndahl *et al.* [42]. Totally 300 sperm cells were counted on each slide at x1000 magnification under immersion oil.

Membrane Function Integrity (Hypo Osmotic Swelling Test: HOST): The membrane function integrity (MFI) was assessed by using the HOS test according to Jeyendran *et al.* [43] protocol with slight modifications. The hypoosmotic swelling test was used to evaluate the functional integrity of the sperm membrane, based on curled and swollen tails and was performed by incubating 10 µl of semen with 100 µl of 100 mOsm hypoosmotic solution (1.1 g fructose, 0.55 g sodium citrate per 100 ml of distilled water) at 37 °C for 60 min. After incubation total of 200 sperm cells was evaluated under x 1000 magnification with phase contrast microscope.

Fertility Assessment

All queens inseminated by a Schley instrument (Schley, Germany). Following insemination, each queen was clipped, tagged with plastic discs and placed in a 3-frame nucleus hives with young bees using queen cage. Twenty-four hours later, queens were treated with CO_2 to stimulate egg laying [44]. The queens were released from the cages 3 days later to determine brood pattern. Once larvae reached the pupal stage and cells were capped, images were taken of each colony and fertilization was determined from three different areas of the comb in all nucleus by counting worker and bee pupae using ImageJ [45]. Mean of measured worker bee brood area was recorded its worker brood ratio for each colony.

Statistical Analyses

All data were performed by SPSS (SPSS 21) using univariate analysis and all means were analyzed by DUNCAN test. Sperm cryopreservation experiments were repeated six times and the fertility checks of the best cryopreserved bee semen groups were repeated four times.

Results

Fresh semen which was control I, cryopreserved semen which was control II with no supplement, different amounts of proline (25mM, 50mM, and 100mM), cysteine (25mM, 50mM, and 100mM) and glutamine (25mM, 50mM, and 100mM) amino acids groups were tested and compared by using some spermatological quality parameters for bee semen (Table 1). Control I and Control II groups were held as control groups in the study (Table 1). When look at the motility parameters, except than fresh sperm, 50mM proline group had the highest motility rate compared to other tested groups including control II with no supplement. 100mM cysteine had the lowest motility rate when compared with control I, Control II, 25 mM or 50mM proline, and 50mM glutamine groups ($p<0.05$), which is presented in Table 1. The highest post-thawed motility rates among the different amino acid groups were 50mM proline 89.8 ± 2.1%,

50mM glutamine 88.1 ± 1.7%, and 25mM cysteine 81.6 ± 3.1%, respectively. There were significant statistical differences in motility among fresh semen control I and all other research groups ($p<0.05$).

Study Groups	Motility (%)	Viability Dead-Sperm (%)	Intact membrane (HOST) (%)
Control I - Fresh semen	99.6 ± 2.7^{a}	0.7 ± 1.3^{a}	99.1 ± 3.7^{a}
Control II (No supplement)	86.1 ± 2.6^{b}	10.8 ± 1.4^{b}	88.7 ± 2.6^{b}
Proline 25 mM	85.7 ± 3.1^{b}	11.3 ± 1.6^{b}	89.3 ± 1.1^{b}
Proline 50 mM	89.8 ± 2.1^{b}	9.6 ± 2.3^{b}	90.6 ± 1.7^{b}
Proline 100 mM	83.9 ± 1.9^{bc}	12.9 ± 1.3^{bc}	87.5 ± 3.2^{b}
Cysteine 25 mM	81.6 ± 3.1^{bc}	15.7 ± 2.6^{bc}	88.6 ± 2.9^{b}
Cysteine 50 mM	80.9 ± 2.2^{bc}	15.1 ± 3.1^{bc}	85.8 ± 1.4^{bc}
Cysteine 100 mM	75.1 ± 3.7^{c}	21.3 ± 2.9^{c}	77.9 ± 1.8^{c}
Glutamine 25 mM	80.3 ± 3.1^{bc}	11.6 ± 1.9^{b}	81.1 ± 3.6^{bc}
Glutamine 50 mM	88.1 ± 1.7^{b}	10.2 ± 1.7^{b}	88.6 ± 2.3^{b}
Glutamine 100 mM	79.4 ± 2.9^{bc}	17.9 ± 2.3^{bc}	80.8 ± 1.7^{bc}

HOST; Hypo Osmotic Swelling Test (Membrane Function Integrity)

[a,b,c] Within a column, means without a common superscript differ ($P< 0.05$)

Table 1: Post-thaw spermatological results of cryopreserved bee sperm by using different amino acids (n:6)

Bee sperm viability rates were determined after cryopreservation that is presented in Table 1. Control I group was the lowest dead sperm rate (0.7±1.3%) when compared to all cryopreserved study groups ($p<0.05$). There were no statistical significances among Control II and other cryopreserved amino acid groups except than 100mM cysteine for dead sperm rates. 50mM proline group had the lowest dead sperm rate when compared to other cryopreserved and control II groups (Table 1).

When compared to intact membrane rate among groups, 50mM proline was the greater than other cryopreserved research groups but not fresh sperm group. Control I was the highest intact membrane rate compared with all tested groups ($p<0.05$). Except than 100mM cysteine, there were no statistical significances among control II and other cryopreserved amino acid groups for intact membrane rates. Cryopreservation process caused gradually a decreasing of motility, viability and intact membrane rates in all tested research groups when compared to fresh semen control I group ($p<0.05$).

Fertility results of fresh semen and other cryopreserved study groups are presented in Table 2. There were no statistical differences between proline 50mM and control II with no supplement groups. However, proline 50mM and control II groups were greater compared to 25mM cysteine and 50mM glutamine groups ($p>0.05$). Fertilization rate of Control I fresh semen group had the highest score compared with all cryopreserved control II and other study groups ($p<0.05$; Table 2). However, cryopreservation process in all tested groups decreased sharply infertility rates compared to control I group in the study. In addition, proline, cysteine, and glutamine did not provide any significant improvement in sperm quality and fertility compared to the cryopreserved control group.

Study Groups	No. Inseminated queens	No. Fertilization (%)
Fresh Control I - Fresh semen	8	97.3 ± 3.76^{a}
Control II (No supplement)	8	28.4 ± 3.47^{b}
Proline 50 mM	8	31.7 ± 2.81^{b}
Cysteine 25 mM	8	27.3 ± 1.52^{b}
Glutamine 50 mM	8	24.6 ± 2.59^{b}

[a,b] Within a column, means without a common superscript differ ($P<0.01$)

Table 2: Post-thaw fertility results of cryopreserved bee sperm by using different amino acids (n:4)

Discussion

Cryopreservation process causes dramatically a decreasing of total sperm assessment values (motility, viability and functional cell membrane integrity), DNA integrity and fertility rates because of cold shock, oxidative damage and changing of cell osmotic pressures. During freezing and thawing process, excessive formation of ROS in the semen causes changes in the antioxidant defense system and damages the sperm membranes [27,28]. These changes in the cryopreserved semen cause poor sperm motility and viability in the female reproductive system and lower fertility rates [46].

The membrane lipids of mammalian spermatozoa are oxidized by partially reduced oxygen molecules such as superoxide, hydrogen peroxide and hydroxyl radicals, and highly sensitive to lipid peroxidation [47]. Be able to reduce these side effects, many researchers have been tested by using naturel and non-naturel antioxidants. Proline, cysteine, and glutamine amino acids are natural antioxidants. Amino acids interact with the phosphate group of the sperm plasma membrane phospholipids [48], forming

a layer on the surface of the sperm, which act as a cushion against ice crystal damage and protects against thermal shock [30]. The effects of semen extender supplementation with different concentrations of proline, cysteine and glutamine amino acids on selected quality parameters of frozen-thawed bee spermatozoa were conducted in this study. In the study, obviously fresh semen parameters regarding sperm assessment and fertility were pretty high compared to cryopreserved semen as similarly reported several researchers for different specious like mouse [49,50], boar [51,52], ram [30], dog [53,54], stallion [55], monkey [32] and also bee sperm [11,16,56].

When look at the post-thawed motility and intact membrane results in the study, 50mM proline amino acid group, had the highest motility and intact membrane values compared to other tested cryopreserved groups ($p>0.05$), but not fresh semen ($p<0.05$). Beneficial effects on the post-thawed motility of proline antioxidant to against cryopreservation damage were stated previously with different researchers as we found similar effects on proline group in the presented study [30,32,55]. Likewise, Li *et al.* [32] previously reported that adding a low amount of proline, glutamine and glycine amino acids to the freezing media had beneficial on motility for freezing of monkey sperm. In addition, recently the frozen-thawed motility values of bee semen with different diluent rates were reported between 25.0% and 62% [11,16,56,57] and membrane integrity as 68.9% [56], previously. In the presented study, the best cryopreserved 50mM proline amino acid group demonstrated higher motility (89.8%) and membrane integrity rate (90.6%) compared to previously limited reported results regarding bee sperm above. These disagreements could be due to different freezing extender, using of different bee strains, freezing technique, and protective effects of antioxidant in the study. General beneficial effects during cryopreservation process of proline amino acid in the study are also in agreement with previous confirmations [31,35,58,59]. Proline protected sperm cells against free-radical-induced damage, which is in agreement with Smirnoff and Cumbes [38], indicating an overall protective effect on the spermatozoa during cryopreservation. It has been reported that proline could have stabilized membrane structure and function by interacting with phospholipid bilayers during freezing [60].

In the presented study, 50mM glutamine after proline group was also better motility, viability and membrane integrity rates as 88.1%, 10.2%, and 88.6%, respectively. Similar beneficial effects on cryopreservation and mechanism of glutamine were stated previously. Glutamine acts at the extracellular level, improving the motility [34,36,37] and ability to fertilize the spermatozoa [34]. Glutamine is also known to have important roles in various cell specific processes such as metabolism, cell integrity, protein synthesis and degradation, redox potential, gene expression [61].

In the presented study, cysteine amino acid did not show beneficial effects due to using of inappropriate antioxidant dose for bee sperm cryopreservation. We assume that it could be due to high amount using of cysteine in the freezing extender. Because, higher dose using (50mM or 100mM) of cysteine caused poor spermatological values in the study (Table 1). Li *et al.* [32] also reported that adding a high amount of proline, glutamine or glycine amino acids to the freezing extender caused lower motility rate in freezing of monkey sperm as we obtained similar low results in the presented study.

Post-thaw fertility results of cryopreserved bee sperm by using different amino acids were presented in Table 2. Fertilization rate of the fresh semen was the best compared with all cryopreserved tested groups ($p<0.01$; Table 2). However, cryopreservation process decreased sharply infertility rates compared to fresh semen in the study. There were no statistical differences for fertility between proline 50 mM and control II (no supplement) groups that found 31.7% and %28.4 fertility rates, respectively. Wegener *et al.* [16] reported that 80.0% fertility rate when used cryopreserved bee sperm with 21.0% DMSO. Rajamohan *et al.* [62] reported 75.0% fertility rate with conventional Tris diluent combination for bee semen cryopreservation. In the study, obtained lower fertility rates can be due to used different cryopreservation protocols, type of used bee strain, and different semen extender. In addition, it is known that cryopreservation process, ice crystal formation and recrystallisation during freezing-thawing procedure, of spermatozoa enhances oxidative stress which not only disrupts the motility and fertilizing ability of spermatozoa, but also increases DNA damage that can cause low fertility and subsequent embryo development rate, as similar obtained low fertility results in the study can be occurred probably due to high DNA damage in the study [49,63,64].

Conclusions

In conclusion, our results confirm that long term storage of bee sperm cryopreservation and successful fertility are achievable when used appropriate sperm freezing protocol and antioxidant. The success of cryopreservation is related with chosen antioxidant in a dose and type dependent manner. Proline as an antioxidant in semen extender had more beneficial influence on sperm quality parameters and fertility.

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