Доклади на Българската академия на науките
Comptes rendus de l'Académie bulgare des Sciences
Tome 77, No 7, 2024

BIOLOGY
Biotechnology

TREHALOSE, GLYCEROL AND DIMETHYL SULFOXIDE SUPPLEMENTATION FOR CRYOPRESERVATION MEDIA OF HONEY BEE DRONE SEMEN

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Received on March 26, 2024
Presented by H. Najdenski, Corresponding Member of BAS, on May 28, 2024

Abstract

The cryogenic storage of honey bee drone (*Apis mellifera*) semen is a rapidly developing practice, driven by the need to preserve bee colonies and the decline in gamete quality following cryopreservation. Advances in cryobiology, coupled with procedural refinements and the development of new methods for cryopreservation of male gametes at different temperatures, provide promising results for improving drone semen preservation. Innovative strategies involve the incorporation of additives within preservation media to enhance important spermatozoa parameters. Our study aimed to assess the effectiveness of cryoprotectants (CPAs), namely trehalose, glycerol, and dimethyl sulfoxide (DMSO), on the motion characteristics of honey bee drone sperm cells during cryopreservation and subsequent thawing. Using Sperm Class Analyzer (SCA) and Computer-Assisted Sperm Analysis (CASA), we evaluated the impact of these supplements on sperm motility and kinetic parameters. Our findings indicated significant differences in sperm motility (non-progressiveness and progressiveness) and kinetics (curvilinear velocity and linearity) across treatments with trehalose, DMSO, and glycerol. Trehalose supplementation retained significantly higher percentages of sperm motility and kinetic parameters post-cryopreservation compared to DMSO and glycerol. Nevertheless, despite the utilization of new protocols, media, and supplementation, the results obtained

This work is realized with the support of the Bulgarian National Science Fund in implementation of project No. KP-06 M66/6 of 15.12.2022.
https://doi.org/10.7546/CRABS.2024.07.06

are often unsatisfactory. The research provides valuable information for enhancing the efficacy of cryopreservation media to optimize the successful outcome of honey bee drone spermatozoa storage.

Key words: drones, spermatozoa, cryoprotectants

Introduction. The significant losses of *Apis mellifera* colonies in recent decades are a consequence of many factors, some of which result from human intervention and ongoing changes in the environment [1, 2]. The cryopreservation of drone spermatozoa is a vital technique for the conservation of the species but presents unique challenges due to the inherent fragility of spermatozoa and decrease in their viability after the freeze-thawing procedure [3]. Successful semen preservation is an important tool for preserving the honey bee biodiversity, nevertheless, there are not enough unified protocols available on this subject compared to other species [1, 4, 5]. During ultra-low-temperature storage, spermatozoa are exposed to stress, resulting in compromised cell structures, mechanical and oxidative damage to the phospholipids in the sperm plasma membrane and DNA, alterations in the gametes metabolism, and compromised cell function [6, 7]. To mitigate the mentioned risks, preservation media are enhanced with cryoprotective agents, which minimize the harmful effects of cold shock by stabilizing cellular structures, preventing ice crystal formation, and reducing morphological defects [2, 5, 7]. Various substances such as egg yolk, glycerol, DMSO, trehalose, ethylene glycol, polyvinylpyrrolidone, and others are commonly used CPAs with varying properties and effects on sperm viability [1, 8, 9]. However, recent studies demonstrate that combination of glycerol and centrifuged yolk give much better results compared with media containing egg yolk and DMSO [10]. Despite their cryoprotective action, most of these CPAs exhibit certain levels of cytotoxicity. Major disadvantage of DMSO is its toxicity to certain cell types, which can induce irreversible changes due to the denaturation of catalytic proteins, disruption of membrane structures within the cytoplasm and nucleus [11]. The unfavourable effects of glycerol are linked to a decrease in fertility, a reduction in motility of the sperm cells, and a decreased ability to penetrate the oocyte and achieve successful fertilization [12]. Other researchers report an improvement in the post thaw quality of drone spermatozoa with cryopreservation media containing trehalose as cryoprotectant which is the first step for a successful artificial insemination of the queen bee [13]. Accordingly, it is crucial to meticulously select the type of the CPAs, concentrations, equilibration time, and temperature, considering the permeability of the membrane and the sensitivity of the cell types.

Materials and methods. ***Collection of drone semen.*** For the purpose of the study, 160 honey bee drones of the species *Apis mellifera macedonica* were used, and animal welfare standards were followed. In order to obtain fresh ejaculates the drones were selected to be at least 20 days old from hatching or older. The ejaculation is induced by applying pressure on the thorax and abdomen of

the drone manually. Semen is collected directly from the tip of the everted endophallus. The extraction of the ejaculate was performed using a workstation with Nexius zoom trinocular microscope (7-45x) and SCHLEY-syringe.

Computer-Assisted Sperm Analysis (CASA) of drone spermatozoa. Initial sperm motility and kinetics were assessed using SCA (Microptic, Spain). The following parameters were observed: sperm motility (static (%), non-progressive (%), progressive (%)) and sperm kinetic parameters (VCL –curvilinear velocity (µm/s), VSL – straight-line (rectilinear) velocity (µm/s), VAP – average path velocity (µm/s), ALH – amplitude of lateral head displacement (µm), LIN – linearity (%), STR – straightness (%), BCF – beat cross frequency (Hz), WOB – wobble (%).

Cryopreservation of drone semen. The obtained semen samples were diluted with three different modified cryoprotective diluents containing each of the CPAs DMSO, glycerol and trehalose, and divided into three groups: 1st group – DMSO based diluted with modified Harbo medium [14]: 20% DMSO, 30% egg yolk, 50% buffer (1.5% NaH_2 $PO_4 \cdot H_2O$ (w/v); 0.950% Na_2 $HPO_4 \cdot 2H_2O$ (w/v)); 2nd group – Glycerol based diluent: 10% glycerol, 20% egg yolk, 70% buffer (3% citric acid (w/v) 1% glucose (w/v), 6% tris (w/v); 3rd group – Trehalose based diluent: 20% trehalose, 20% egg yolk, 60% buffer (3% citric acid (w/v), 1% glucose (w/v), 6% tris (w/v). A control (C) group with extender without CPAs was used 20% egg yolk and 80% buffer (6% tris (w/v), 1% glucose (w/v), 3% citric acid (w/v). The control and treated groups were equilibrated at 4 °C for 1 hour, followed by subsequent equilibration at −20 °C for 1 hour. All samples were loaded into cryo straws (300 µl, Cryo Bio Systems High security straws) and cryopreserved in liquid nitrogen vapour. The changes in motility and kinetic parameters of drone spermatozoa were observed at the 24th and 48th hour after cryopreservation, with emphasis placed on the percentage of non-progressive and progressive sperm cells in samples. Thawing of cryopreserved samples was performed at 35 °C in a water bath for 15 s, and sperm quality was assessed for motility and kinetics via SCA.

Statistical analysis. Statistical analysis of the results was conducted using Excel's *t*-Test. The software programs Motility & Concentration (Microptic, Spain) were used.

Results. After obtaining the ejaculates, initial sperm parameters were measured as follows: motility (%) < 70, non-progressive motility 71.83 ± 5.18, progressive spermatozoa 23.76 ± 3.51, static spermatozoa 6.21 ± 1.01, VCL – 61.73 ± 4.11; VSL – 28.86 ± 1.03, VAP – 40.33 ± 1.06, STR – 52.81 ± 3.71, LIN – 41.18 ± 5.05; WOB – 50.00 ± 1.85, BCF – 8.00 ± 1.00, ALH – 3.82 ± 1.00.

The results indicate significant variations in sperm parameters among the supplementation of different CPAs. Changes in sperm motility and kinetics were detected at the 24th and 48th hour after cryopreservation and are presented in Tables 1–4.

The addition of the three CPAs showed specific effects on sperm motility

Table 1

Motility parameters of ejaculates from drones cryopreserved with the cryoprotectants glycerol, DMSO, and trehalose, at the 24th hour

	Control	Glycerol	DMSO	Trehalose
Static	74.67 ± 3.10^{c}	50.76 ± 5.83^{b}	55.01 ± 2.30^{b}	40.88 ± 2.51^{a}
Non-progressive	21.88 ± 3.67^{c}	45.13 ± 5.23^{b}	38.77 ± 3.33^{c}	53.05 ± 4.44^{a}
Progressive	2.65 ± 0.98	2.44 ± 0.88	5.05 ± 1.01	5.83 ± 1.11

$\pm$ the significant difference between a and b $p < 0.05$ and a and c $p < 0.01$, $n = 40$

parameters. Sperm progressiveness remained low in all samples and controls, with no significant differences observed between the examined groups.

Supplementation of glycerol and DMSO leads to an increase in static spermatozoa, and a decrease in non-progressiveness, with significant reductions observed at the 48th hour after cryopreservation. Drones spermatozoa with added trehalose, as a cryoprotectant, exhibited significantly lower values in static spermatozoa (45.88 ± 5.77), and a higher percentage of non-progressive spermatozoa (49.00 ± 3.18) compared to DMSO (static 64.01 ± 3.99 and non-progressive 34.03 ± 3.67), glycerol (static 58.17 ± 1.88 and non-progressive 37.89 ± 2.24) and the control (static 82.56 ± 3.51 and non-progressive 16.18 ± 1.98) at the 48th hour after cryopresevation.

Table 2

Motility parameters of ejaculates from drones cryopreserved with the cryoprotectants glycerol, DMSO and trehalose at the 48th hour

	Control	Glycerol	DMSO	Trehalose
Static	82.56 ± 3.51^{c}	58.17 ± 1.88^{b}	64.01 ± 3.99^{c}	45.98 ± 5.77^{a}
Non-progressive	16.18 ± 1.98^{c}	37.89 ± 2.24^{b}	34.03 ± 3.67^{c}	49.99 ± 3.18^{a}
Progressive	2.65 ± 0.99	1.82 ± 1.00	2.78 ± 0.99	3.71 ± 1.00

$\pm$ the significant difference between a and b $p < 0.05$ and a and c $p < 0.01$, $n = 40$

Statistically significant differences were observed regarding the kinetic sperm parameter, after treatment with trehalose, DMSO and glycerol at the 24th and 48th hour after cryopreservation (Tables 3, 4).

The results of CASA kinetic parameters demonstrated a decrease in the VCL parameter in all samples, with added different CPAs, and the control. Samples exhibiting highest VCL values at the 48th hour after cryopreservation were observed in spermatozoa supplemented with trehalose (45.65 ± 3.44), compared to the group with DMSO (36.66 ± 2.11) and the control (18.56 ± 2.56).

Significant differences were observed in several sperm parameters, including VSL, VAP, LIN, and WOB. The highest values of these CASA parameters were es-

Table 3

Changes in CASA kinetic parameters of drone spermatozoa cryopreserved with glycerol, DMSO, and trehalose at 24 hours

	Control	Glycerol	DMSO	Trehalose
VCL	44.91 ± 5.11^{b}	45.88 ± 2.77^{b}	40.22 ± 1.11^{b}	48.03 ± 3.33^{a}
VSL	22.56 ± 4.81^{c}	30.71 ± 3.93^{b}	29.831 ± 1.45^{b}	33.85 ± 2.19^{a}
VAP	30.39 ± 5.66^{c}	37.24 ± 3.89	29.73 ± 2.61^{c}	42.03 ± 1.67^{a}
STR	4.99 ± 1.00	3.83 ± 1.01	3.53 ± 0.97	4.18 ± 1.00
LIN	44.44 ± 3.17^{c}	63.82 ± 6.35	60.00 ± 3.62^{b}	68.15 ± 2.45^{a}
BCF	8.01 ± 2.03	7.76 ± 1.23	8.21 ± 0.75	11.43 ± 1.55
WOB	66.11 ± 2.6^{c}	68.92 ± 3.66^{c}	71.02 ± 3.94^{b}	81.18 ± 4.23^{a}
ALH	3.99 ± 0.82	2.73 ± 1.00	3.29 ± 1.00	3.86 ± 1.11

$\pm$ the significant difference between a and b $p < 0.05$ and a and c $p < 0.01$, $n = 40$

Table 4

Changes in CASA kinetic parameters of drone spermatozoa cryopreserved with glycerol, DMSO, and trehalose at 48 hours

	Control	Glycerol	DMSO	Trehalose
VCL	18.56 ± 2.56^{c}	39.11 ± 3.49	36.66 ± 2.11^{b}	45.65 ± 3.44^{a}
VSL	19.601 ± 1.77^{c}	26.55 ± 4.06^{b}	27.03 ± 3.15^{b}	32.63 ± 1.00^{a}
VAP	28.32 ± 2.32^{b}	31.15 ± 3.74^{b}	26.87 ± 3.52^{c}	38.88 ± 2.22^{a}
STR	2.65 ± 1.00	3.33 ± 0.99	3.76 ± 1.00	3.97 ± 0.95
LIN	41.68 ± 1.70^{c}	59.23 ± 4.67	52.21 ± 2.3^{b}	60.66 ± 4.67^{a}
BCF	5.72 ± 1.00	7.89 ± 0.89	7.01 ± 1.00	8.88 ± 1.01
WOB	55.64 ± 3.66^{c}	65.91 ± 3.33^{b}	68.56 ± 1.65^{b}	78.32 ± 5.87^{a}
ALH	2.20 ± 0.73	2.58 ± 1.01	2.91 ± 1.50	2.76 ± 1.05

$\pm$ the significant difference between a and b $p < 0.0$ and a and c $p < 0.01$, $n = 40$

tablished at the 48th hour after cryopreservation in spermatozoa with supplementation of trehalose. In contrast, spermatozoa with added DMSO demonstrated a significant decrease in VAP, LIN, and WOB over the same period. No statistically significant differences were found in the ALH parameter in all treated groups.

Discussion. Preserving sperm quality is one of the most important factors in cryopreservation. Sperm cells are very sensitive to alterations during ultra-low temperature storage, and cryodamage during this process cannot be disregarded [15]. The use of different CPAs has allowed cells to be preserved at very low temperatures, furthermore, advancements in enhancing the supplements of cryoprotective extenders will represent a significant stride in the field of cryobiology [16, 17]. In our research, trehalose supplementation demonstrates promising results in maintaining drone sperm motility and kinetics post-thaw, compared to

DMSO and glycerol, which adversely affect the gametes parameters after cryopreservation. It was observed that in media with added trehalose, preserved sperm progressive motility and non-progressiveness reached higher values compared to glycerol and DMSO supplementation. Trehalose has beneficial effects on several kinetic parameters – VSL, VAP, LIN, BCF, WOB, and ALH. According to some data, glycerol and DMSO supplementation has distinct effects on honey bee drone sperm cryopreservation [1,3,12,17]. One of the most commonly used CPA-glycerol, is widely used to protect the male gametes from cold shock by stabilizing the lipid bilayer [16]. Glycerol is the preferred cryoprotectant for several mammalian species, but regarding the preservation of drone spermatozoa, it is proven that supplementation with higher concentrations of glycerol has high cytotoxic effects on the gametes [1, 18]. Glycerol shows potential but requires further optimization of concentrations to mitigate concentration-dependent effects. DMSO, although one of the most common CPAs, exhibits variable outcomes and may necessitate cautious selection of concentrations during cryopreservation. The obtained data have indicated that employing DMSO as a CPA can lead to an increase in the incidence of sperm exhibiting damage freezing [9, 17]. However, these two cryoprotectants show promising results, but require further optimization to maximize their efficacy and minimize adverse effects on spermatozoa. Using trehalose emerges as a promising cryoprotectant for maintaining sperm viability and motility, possibly due to its ability to stabilize cellular membranes and inhibit ice crystal formation. Trehalose in cryoprotectant extenders mitigates cryopreservation-induced changes in sperm motility and morphology, thereby highlighting its promising role in preserving the gametes quality under cryogenic conditions [13, 19, 20]. Our research contributes to the development of optimized cryopreservation techniques for honey bee spermatozoa, thereby supporting conservation efforts and selective breeding programmes for honey bee populations. Further research is warranted to elucidate the underlying mechanisms and optimize the supplementation for cryopreservation protocols regarding honey bee drone sperm. This highlights the importance of CPAs selectivity in drones sperm cell cryopreservation.

Conclusion. The optimization of protocols for the cryopreservation of honey bee drone spermatozoa, utilizing cryoprotectants with low cytotoxicity, would provide an opportunity to preserve the genetic diversity of the species. The establishment of cryobanks with genetic material from different subspecies would contribute to the creation of more resilient hybrids through artificial insemination, better equipped to withstand environmental conditions.

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