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Effectiveness of Glycerol, DMSO and Trehalose in the Process of Cryopreservation of Drone Semen from the Species *Apis Mellifera*

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ABSTRACT

Cryopreservation of economically important or endangered animal species is becoming an increasingly popular practice in the field of reproductive biotechnologies. The mass extinction of honey bee (Apis Mellifera) colonies in the last decade requires the use of new chemical components in cryopreservation media for the storage of male gametes. A number of studies demonstrate conflicting data regarding the success rate and cytotoxicity of glycerol and dimethyl sulfoxide and to a lesser extent Trehalose. Dimethyl sulfoxide is an organosulfur compound with low cryotoxicity which transforms into solids below room temperature and this property makes it suitable for cryoprotectant. The kosmotropic properties of glycerol allow it to create hydrogen bonds with water molecules, making it difficult to form intracellular ice crystals in the cryoprotective media. Due to its high-water retaining properties Trehalose can be used as cryoprotectant, but its effectiveness has not yet been thoroughly researched in relation to the cryopreservation of bee drone spermatozoa. The research aims to compare the effectiveness of each of the specified cryoprotectants regarding the preservation of spermatozoa from Apiss Mellifera drones.

Keywords: cryoprotectants, bee drones, trehalose, glycerol.

INTRODUCTION

Importance of honey bee drone spermatozoa cryopreservation

During the last decades the significant loss of bee colonies worldwide emerges as one of the mail problems for the ecology and the agricultural sector. The mass extinction of colonies can be caused by a number of environmental changes, as well as anthropogenic factors as a result of the policies for the treatment of plant crops. The improved methods for gamete cryopreservation could be a valuable tool for the conservation of the honey bee biodiversity. Already existing protocols for cryopreservation of drone spermatozoa preserve to certain extend the viability and motion characteristics of the gametes of frozen-thawed

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ejaculates. The introduction of cryoprotectants in the cryopreservation media in combination with other compounds can improve significantly the outcome not only of cryopreservation but of the subsequent artificial insemination as well [1]. The technology for preserving sperm cells is useful for disseminating valuable male genetic traits and is suitable for easily transporting spermatozoa as an alternative to the shipment of live animals. At the same time, there is a technical limitation of this process because the fertility of cryopreserved and cold stored sperm declines [2]. The process of cryopreservation is the freezing and long term storage of biological material at extremely low temperatures - (-196^{0}C - the boiling point of nitrogen). Cryoprotectants are molecules that prevent intracellular ice which is the main damaging agent during the process of cryopreservation [3]. Cold shock is the term used when cells are damaged by the low temperatures and the main reason for the unsatisfactory outcomes after thawing [4]. One of the goals of cryopreservation is to preserve the reproductive potential of gametes after a long period of storage and thawing. Cryopreservation of gametes in animal husbandry and agriculture makes it possible to carry out artificial insemination for the purpose of genetic improvement of certain breeds and selection of breeders with certain physical characteristics. Beekeeping has an important place in terms of nutritional value and human health when considering the final products as honey, pollen, propolis, royal jelly, beeswax, bee venom [5]. In the recent decades, the drastic death of honey bee colonies necessitates the use of modern methods for the storage of genetic material of bee hives and the subsequent successful artificial insemination of honey bee queens. Also, the cryopreservation of drone semen can be used to maintain and increase genetic diversity within populations of colonies. In addition, genetic diversity has been shown to enhance disease resistance in honey bees [6]. However, the process of cryopreservation exhibits a negative effect on spermatozoa because of the cold shock and the formation of ice crystals. It requires the use of cryoprotective agents that have minimal cytotoxic effects on the germ cells [7, 8]. In this review, some of the main cryoprotectants (Glycerol, Dimethyl sulfoxide (DMSO) and Trehalose) and their effect on Honey bee drone spermatozoa in the cryopreservation process are discussed.

Effect of Cryoprotectants

Effect of Dimethyl sulfoxide (DMSO)

The correct choice of cryoprotectant is a crucial factor in the cryopreservation process. In general, there are two types of cryoprotectant, permeating and non-permeating cryoprotectants. Dimethyl sulfoxide is common permeating cryoprotectant, DMSO is an organosulfur compound with the formula $(CH_3)_2SO$. Various cryoprotectants including DMSO reduce intracellular ice formation by slowly passing through the membrane, replacing intracellular water and allowing the cell to reach a new isotonic state that has less water available for freezing [9, 10]. The cryoprotectant has minor cytotoxicity, which makes it prominent candidate for cryopreservation. DMSO reduces the electrolytic concentration in the residual chilled contents in and around of a biological cell, during cryopreservation. However, rising of altered demarcated cells due to DNA methylation and histone alteration is a drawback of DMSO based cryopreservation. DMSO has typical property; it freezes within 18.5°C. This means, below room temperature DMSO transformed into solids, and this property makes it most suitable for cryoprotectant [11, 12]. DMSO is widely used in the freezing of sperm, and various concentrations are applied in different species (2 %, 5 %, 10 % and 15 %). The results indicate that the type and concentration of the penetrating cryoprotectant used can greatly affect the survival of spermatozoa after ultra-low temperature storage [13]. Recently, it was found that 5 % DMSO gave the best results and cryoprotectant

treatment had significant effects on sperm motility and fertilization ($p < 0.05$). Reports have been published regarding DMSO maintaining the fertility and motility of sperm cells and also the enhanced levels of tyrosine phosphorylation of male gametes [2]. According to Stucky et al., queens inseminated with 50 % or greater viable spermatozoa after cryopresetvation, would have a good probability off laying normally throughout an entire beekeeping season using 25 % DMSO [14] However, in a different research Wagener et al. found that even during short-term cold storage toxicity appeared to be low for the media with included DMSO and significant reduction of sperm migration into the spermatheca of the bees is observed. In a later study it is proposed a mixture of two cryoprotectants for the purpose of reducing toxicity. The research is based on the premise that mixing DMSO with Trehalose will give promising results since the Trehalose is found in insects as a natural cryoprotectant and it is also present in the honey bee seminal plasma [15]. Using different concentrations (< 10 %) of the cryoprotectant some authors demonstrate a significant improving in viability of cryopreserved honey bee drone spermatozoa after thawing, proving that combination and correct ratio of the components and protective agents in the media have significant role in the successful outcome of the storage [16]. However, the cryoprotectant when used for drone sperm freezing, can harm the queen when reaching the spermatheca and reduce fertility after instrumental insemination [5]. A solution to the toxic effect of DMSO was reported as a method including centrifugation of cryopreserved sperm to remove the cryoprotectant which as a result does not affect queen survival, spermathecal sperm count, or sperm viability [17]. However, the effectiveness of DMSO as a component in the media for Honey bee drone spermatozoa cryopreservation demonstrate promising results, but the optimal concentrations and cytotoxicity of the cryoprotectant are yet to be fully determined.

Effect of Glycerol

Glycerol has good kosmotropic properties; it forms hydrogen bonds with water molecules. This condition makes difficult to form ice crystals by mixture (70 % glycerol and 30 % water), unless and until the temperature is very low such as -37.8°C. Compare to other cryoprotectant glycerol is less toxic in since the introduction of Glycerol as a permeating cryoprotective agent [18], many studies have been performed to determine the effectiveness of the cryoprotectant in the process of freezing and thawing of spermatozoa. The effectiveness of glycerol as cryoprotectant is associated with its protective role on the cell from freezing injury mainly by reducing intracellular ice crystal formation and osmotic pressure differences [19]. Glycerol one of the first cryoprotectors used for cryopreservation is preserving the viability of spermatozoa at -70^0C [20]. All the characteristics of glycerol is making it a very acceptable element even for the media for long-term storage of islet cells at subzero temperatures (typically less than -100°C [21]. Due to the cytotoxic effects that the glycerol exhibit during the freezing and thawing of male gametes, its appropriate concentrations in the media have been widely studied. Some authors include concentration of the cryoprotectant in the media ranging from 3 % to 11 % [22, 23]. No matter the exhibit cytotoxic effects, glycerol is still one of the most commonly used cryoprotectant for preservation of mammalian spermatozoa [24]. There have been some studies on its effects regarding different animal species: ram [25], boar [26] and bull [27]. In the case of cryopreservation of honey bee (*Apis mellifera*) drone spermatozoa one of the first researches on the matter demonstrate low percentage of viable and motile spermatozoa. Hopkins and Herr, after comparing the cytotoxic effect of different cryoprotectants determined that the use of glycerol in the cryopreservation media, especially in higher concentrations, causes massive reduce in motility and viability after

thawing of honey bee drone spermatozoa [28]. However, according to other studies it is concluded that the diluents based on glycerol on combination with centrifuged egg yolk are suitable for cryopreservation of sperm samples of drone [29]. By some authors, the degree of glycerol toxicity can be potentially eliminated by reduced exposure of glycerol to sperm cells during the process of equilibration. The addition of the cryoprotectant after equilibration just before starting the freezing procedure is a practical method, as its penetration ability into the sperm cell takes only several seconds [30]. The equilibration of the sperm samples lowers the risk of cell damage due to the penetration of glycerol into spermatozoa was reduced with a short period of glycerol exposure to sperm cells [31]. The effectiveness of glycerol as cryoprotectant in the case of preservation of bee drone spermatozoa demonstrates controversial results. Conclusive reports of glycerol's cytotoxicity and most adequate concentrations in the cryopreservation media are steel necessary in order to determine its efficiency.

Effect of Trehalose

The trehalose is a compound comprised with two molecules of glucose. Trehalose is otherwise called as mycose or tremalose. Due to its high-water retaining properties, it can be used as cryoprotectant. Trehalose is less soluble than sucrose, except at high temperatures (> 80°C). Trehalose forms rhomboid crystals of 90 % calorific sucrose. The anhydrous forms of the cryoprotectant readily regain moisture to form dehydrate. Trehalose improves cell survival after thawing compared with the standard freezing procedure. The compound is a non-reducing disaccharide composed by two glucose moieties joined by an alpha-1, 1-glucosidic bond [32]. In insects, trehalose is used as a common blood sugar [33] and plays a crucial role as an instant source of energy and in dealing with abiotic stresses [34] but it exists naturally also in some plants, fungi and bacteria [35]. Insect trehalose has been shown to exist in two distinct forms, Tre-1 as the soluble form, while Tre-2 being the membrane bound form. Tre-1 has been purified from hemolymph, cavity of goblet cells in midgut and from egg homogenates of some insects [36, 37]. The effect of trehalose is associated with stabilization of the cellular membrane. During dehydration conditions trehalose helps maintaining the membrane integrity by the formation of a hydrogen bond between the sugar hydroxyl group and the phospholipid polar head group [38]. Some animal species undergoing extreme environmental are able to go through a state of suspended animation known as "cryptobiosis" in order to survive [39]. The cryoptobisis can be categorized on the basis of the stressor that is causing it: cryobiosis (low temperatures; cold stress), anoxybiosis (aerobic stress) or chemobiosis (chemical stress). Cryptobiotic organisms often owe their survival to the accumulation of compatible solutes, often times trehalose [34]. In the context of cryopreservation, the effect of the trehalose is often associated with stabilization of the cellular membranes and the protective effect on proteins by replacing water molecules and facilitating cytosolar vitrification. However, in experiments using Raman scattering is proven that the disruption of the tetrahedral network of water molecules occurs on addition of trehalose [40]. This "destructuring" of the water network by trehalose and ordering the water molecules around itself (as a kosmotrope) does not allow the formation of ice and makes trehalose one of the best cryoprotectants known [41]. Trehalose supplementation in semen extender for cryopreservation enhances the membrane integrity in many animal species: ram [42], buffalos [43] and boars [44]. Resent researches in humans provide information about the beneficial effects of trehalose as stabilizing factor influencing the plasma membrane of the male gametes during cryopreservation and thawing

[45]. The premise that trehalose is naturally present in the honey bee drones seminal plasma [46], gives the opportunity at first to be used as cryoprotectant in a mixture with DMSO [47]. Most resent researches demonstrate that a diluent containing DMSO and trehalose improve both post-thaw cell motility and plasma membrane integrity in drone spermatozoa [48]. In other reports it is described an increase in longevity of spermatozoa from *Apis mellifera* after dilution of the samples with trehalose medium for a prolonged period of time. The authors attribute this effect to the protective role of trehalose in cell membrane and protein scaffolding [49]. However, the effectiveness of the cyoprotectant is supposed to be due to the fact that it occurs naturally in the plasma of insects and demonstrates protective effects during low seasonal temperatures. Being an extracellular cryoprotectant trehalose demonstrates lower levels of cytotoxicity, and the possibility of being combined with intracellular protectant makes it a promising candidate for an optimal cryopreservation agent of Honey bee drone spermatozoa.

CONCLUSIONS

The extinction of honey bee colonies in a global scale requires a chemical and biotechnological approach for preserving the genetic diversity of the species. The cryopreservation of honey bee drone spermatozoa is a target to many researches and can provide adequate solution for long term storage of genetic material from endangered subspecies valuable for the agricultural sector. Glycerol and DMSO are some of the most commonly used cryoprotectants for preserving semen material from vast number of species, and demonstrate relatively satisfactory results for preserving drone sperm cells. Tehalose being a disaccharide that accumulates naturally in honey bees provides a promising alternative, not only as an extracellular cryoprotectant but also by its compatibility with other intracellular cryoprotectants in the preservation media. However, further research in this area is needed in order to determine the toxicity and effectiveness of the cryoprotectants in the process of low temperature storage of drone spermatozoa. The aim of this article is to summarize the chemical characteristics and effectiveness of one of the most commonly used cryoprotectants in the process of low temperature storage of drone spermatozoa.

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