Effect of Vitrification on Canine Sperm Parameters Using Coconut Water Extender and Egg Yolk as Cryoprotectant

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Abstract. *Our study was aimed to evaluate the effect of vitrification on canine sperm parameters using a coconut water extender with an addition of egg yolk as a cryoprotectant. Semen collection was done separately by manual stimulation from twelve healthy adult dogs. Only the second fraction of the ejaculate was used in this study, which was evaluated for volume, concentration, vitality, total and progressive motility, kinetic parameters and morphology. Semen was diluted with a coconut water extender (50% (v/v) coconut water, 25% (v/v) distilled water and 25% (v/v) 5% anhydrous monosodium citrate solution) with an addition of 20% (v/v) egg yolk and fructose at 1% until final concentration of 100 × 10^6 spermatozoa/mL. After equilibration at 5°C for 60 minutes, semen was vitrified by the "direct dropping method" into liquid nitrogen in spheres with a volume of 30 µL. After a week of storage, the spheres were warmed as three of them were dropped into 0.5 mL of CaniPlus AI (Minitüb, Germany) at 42°C for 2 minutes and evaluated about the same parameters. The results showed that vitrification produced a statistically lower percentage of vital sperms, normal morphology and total motility ($P < 0.05$), but progressive motility and most of velocity parameters (VCL, VSL, VAP, LIN, ALH and BCF) did not differ ($P > 0.05$) compared to fresh semen samples. In conclusion, our results demonstrate that vitrification with a coconut water extender with an addition of 20% egg yolk as a cryoprotectant affects the quality of canine sperms, but may be useful as a successful alternative to conventional cryopreservation. Further research on the spermatozoa vitrification technique on enhancement in cooling and warming should be conducted and investigated.*

Introduction

In recent years, there has been a demand on research for different methods for canine semen preservation. There have been many trials based on type of preservation, various component extenders, equilibration and warming procedures. Cryopreservation of spermatozoa is a method for assisted reproductive biotechnology, useful for extending their lifespan and viability, which increases reproductive capacity of male organisms (Gharajelar, 2016). Conventional cryopreservation uses a slow-gradual freezing method and has moderately poor post-thawed semen quality. Vitrification, on the other hand, uses an ultra-rapid freezing method for solidifying liquid into the glassy state by direct immersion into liquid nitrogen (LN_2) without ice crystallization. The method is widely used for embryo, oocyte or tissue storage (Isachenko, 2004; Rosato, 2013), and during the last decade, it has been successfully performed in different mammalian species as an option for sperm preservation; however, in dogs, there have been fewer investigations until now (Sánchez, 2011; Kim, 2012; Gharajelar, 2016; Caturla-Sánchez, 2018; Pipan, 2020; Galarza, 2021; Antonov & Ivanova, 2022). As a novel method, sperm vitrification protocols still require improvement and standardization for increasing post thaw sperm survival.

During the semen cryopreservation process, a cold shock phenomenon may occur, which can reduce the spermatozoa motility and viability (Enciso, 2006). Thus, the addition of cryoprotectants is mandatory to minimalize cryodamage of the spermatozoa. Most often glycerol is added to the extenders as a permeable cryoprotectant, which prevents intracellular ice crystals formation, but it has proven to be toxic for the cells (Curry, 2000; Holt, 2000). Egg yolk as a non-permeable cryoprotector, is also widely used in conventional semen cryopreservation. Since its discovery as a component of a cooling extender, egg yolk has been widely used in mammalian sperm cryopreservation to protect sperm from initial cold shock (Layek, 2016; Abdel-Aziz, 2019). Moreover, many studies have verified the benefits of egg yolk-based extenders for canine semen cryopreservation (Martinez-Rodriguez, 2020; Bencharif, 2020). Egg yolk contains LDLs, which can prevent cholesterol efflux and lower tyrosine-containing protein phosphorylation, thereby inhibiting sperm capacitation (Mahiddine & Kim, 2021). The addition of egg yolk greatly increases the viscosity of the solution, which prevents water precipitation and formation of intracellular and extracellular ice crystals (Isachenko, 2011). In the literature, there are limited

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data about the possible potential of egg yolk to preserve important physiological parameters of canine sperms during ultra-rapid cryopreservation.

Many researchers have focused on using antioxidants as preservatives in extenders to reduce the negative effect of oxidative stress on semen and to protect spermatological indices. A great number of studies have documented that adding antioxidants to the extender could be beneficial in preserving the sperm parameters after thawing in animals such as canines (Neagu, 2010; Lucio, 2016; Caturla-Sanchez, 2018). A viable alternative extender is the green coconut water based one. Coconut water as a whole seems to be suitable for a canine semen extender due to isotonic, not toxic, cheap, effective, and simple to use properties (Cardoso, 2003). It is a natural buffer and contains essential constituents with high antioxidant properties (Silva, 2009; Mantena, 2003; Cardoso, 2003, 2006).

The purpose of the current study was to evaluate the effect of vitrification on canine semen parameters using a green coconut water based extender with the addition of egg yolk and performing a simple cryopreservation method which can be applied for routine clinical use.

Materials and Methods

Experimental animals and initial semen quality

Twelve privately owned, clinically healthy male dogs, aged between 3 and- 6 years, were included in this study. They were presented at the University Veterinary Hospital of the Faculty of Veterinary medicine, Trakia university, Stara Zagora, Bulgaria. Semen samples from these dogs were previously conventionally cryopreserved and found to be with good cryotolerance. The experiment was conducted according to the recommendations of the Local Animal Ethics Committee and regulation for human attitude and animal protection. Dog owners were informed about the procedures and signed a written consent before semen collection. Inclusion criteria were that the ejaculates should have ⩾ 70% motile spermatozoa and ⩾ 70% morphologically normal spermatozoa.

Semen collection and evaluation

Semen was collected separately by digital manipulation into a pre-warmed sterile plastic vial. The procedure was performed by the same operator to eliminate variation due to the different collection technique and in the presence of a teaser bitch to provide stimulation. Only the second ejaculation fraction was used in the study, and immediately after collection, the vial was transferred to the laboratory for the preliminary analysis. Semen was evaluated for volume, concentration, vitality, kinematic parameters and morphology.

A pre-warmed graduated glass pipette was used to measure the semen volume.

Sperm concentration and velocity were analyzed by CASA System Sperm Class Analyser (SCA) (Microptic, S.L., Barcelona, Spain) with software analytical module Motility and Concentration, using a Makler counting chamber with volume of 10 µL samples. The CASA parameters were adjusted to accommodate canine semen according to protocols already in place. Examination was performed on a minimum of 30 optic fields. The assessed CASA parameters were: sperm concentration (× 10^6/mL), total motility (TM, %), progressive motility (PM, %), VCL (curvilinear velocity, µm/s), VAP (average path velocity, µm/s), VSL (straight line velocity, µm/s), LIN (linearity, %), STR (straightness rate, %), ALH (lateral head displacement amplitude, µm) and BCF (beat cross frequency, Hz).

Sperm morphology was evaluated by Sperm Class Analyser (SCA) (Microptic, S.L., Barcelona, Spain) with software analytical module Morphology. Smeared 10 µL semen samples were prepared on a clear glass slide and stained with SpermBlue (Microptic, Spain) for 2 minutes. The slide was analyzed by SCA with a minimum of 50 optic fields.

The sperm vitality was assessed by smearing on a slide a mixture of a 5 µL semen sample and 5 µL eosin-nigrosine stain. A minimum of 200 sperm cells were counted under a light microscope and oil immersion, magnified by 400×. Spermatozoa stained pink or red were identified as non- vital, and those unstained remaining white - as vital.

Extender preparation and semen dilution

After the initial processing, the second fraction was diluted with a green coconut water based extender until final concentration of 100 x 10^6/mL. Base vitrification media (BVM) was first prepared using 50% (v/v) coconut water, 25% (v/v) distilled water and 25% (v/v) solution of 5% anhydrous monosodium citrate. The extender consisted of BVM with an addition of 20% (v/v) egg yolk and 1% fructose. The extended semen was then equilibrated at 5°C for 1 hour.

Vitrifcation and thawing

The vitrification technique was performed according the description of Shah (2019) for human sperm. Sperm samples of 30 µL were dropped with micropipette from a 10 cm height upon a stainless steel strainer, which was previously placed into a styrofoam box and submerged in liquid nitrogen (LN_2). After solidification, the sperm pellets were transferred into pre-cooled cryotubes and placed in liquid nitrogen for a week.

The thawing process consisted of adding three sperm pellets to 0.5 mL of CaniPlus AI (Minitub, Germany) that had been pre-warmed in a water bath at 42°C for 2 minutes and then transferred at 37°C. After warming, the sperm parameters were immediately evaluated as described above.

Statistical analysis

The results were processed by statistical program Statistica version 7.0 (Stat-Soft., 1984-2000 Inc.,

Tulsa, OK, USA). All data are presented as mean ± standard deviation (SD) and were analyzed using ANOVA for repeated measures and compared using the Tukey's test. Value for $P < 0.05$ was considered significant.

Results

The volume of the second semen fraction was 0.95 ± 0.27 mL and the concentration was- 973 ± 175.04×10^6 spermatozoa/mL. The other evaluated parameters and the effect of vitrification on canine semen using the coconut water extender with the addition of egg yolk are presented in Table 1.

Fresh semen samples showed significantly higher ($P < 0.05$) vitality than those which were vitrified. A similar tendency was observed in total motility. Progressive motility was also improved by the vitrification process, but the levels were not significantly different ($P > 0.05$) with the fresh semen samples. After vitrification, there were also changes in most of the sperm velocity parameters; however, they were not significantly different ($P > 0.05$) than the fresh samples before cryopreservation.

The evaluation of sperm morphology showed significant variations ($P < 0.05$) between the fresh and vitrified samples. The major alterations found after vitrification were a detached head, a coiled and bent tail.

Discussion and Conclusion

In recent years, there has been a demand for research focused on canine sperm cryopreservation in order to increase the reproductive capacity of stud dogs. Conventional freezing methods usually result in high percent sperm mortality and morphological damage (Falah, 2020). However, cryopreservation by direct plunging of cells into liquid nitrogen (vitrification) has its own unique characteristics. The decisive factor in successful cryopreservation is avoiding intracellular crystallization, which is incompatible with living systems. A "popular" point of view holds that vitrification is the solidification without formation of hexagonal (big, lethal) intracellular crystals by extreme increase in viscosity during cooling (Merino, 2011).

Extenders tend to be extremely important for successful cryopreservation and choosing the proper one is an important part of semen processing (Peterson, 2007; Ogbu, 2014). A lot of commercial extenders for dog semen preservation, which consist of different chemical combinations, are available, but most of them could be replaced using alternative sources, including such as those of animal or plant origin (Bustani & Baiee, 2021). Our choice to use a coconut water based extender relies on the cheap, easy to find ingredient, which is also an excellent antioxidant and is in accordance with the "green trend" of recent years. The quality of preserved canine semen after devitrification in the present study exceeded any previously reported results (Sánchez, 2011; Kim, 2012; Gharajelar, 2016; Caturla-Sánchez, 2018; Pipan, 2020; Galarza, 2021; Antonov & Ivanova, 2022).

According to Gharajelar (2016), egg yolk is a common part of semen diluents with a protective effect on spermatozoa against cold shock during freezing and thawing, which also acts as an energy source and protectant at the level of the cell membrane (Sánchez, 2011). In the scientific literature, the best canine sperm vitality and total motility reported after vitrification were with a TRIS based extender (Pipan, 2020), and a previous study of our team using a coconut water extender, which showed even better results (Antonov & Ivanova, 2022). In both investigations, the extenders contained 1% soy lecithin and 0.25M sucrose as cryoprotectants. In the present investigation, we replaced them with egg yolk and found improved quality of preserved semen after devitrification, so it might be concluded that it can effectively preserve important physiological parameters of canine sperm during ultra-rapid cryopreservation.

Motility is one of the most important features of a fertile spermatozoa (Partyka, 2012). In previously reported results for conventional freezing, wide variability is observed, with studies reporting sperm motility ranging between 33% (Peña, 1998) and 70% (Ström, 1997). Concannon and Battista (1989) suggest that 30–50% sperm motility in frozen semen is considered acceptable and motility above 50% is ideal for artificial insemination with canine frozen semen. Our result showed that despite the significant difference in total motility between the fresh and vitrified sample, the mean average percent of sperm motility after devitrification is above mentioned

Table 1. Parameters of fresh and vitrified canine semen samples (n = 12) using the coconut water extender

	Vitality, %	Total motility, %	Progressive motility, %	VCL, µm/s	VSL, µm/s	VAP, µm/s	LIN, %	STR, %	ALH, µm	BCF, Hz	Normal morphology, %
Fresh semen	94.45 ± 1.59	87.76 ± 1.94	52.11 ± 2.89	191.1 ± 26.60	129.2 ± 16.60	146.3 ± 15.10	68.50 ± 8.36	88.93 ± 4.31	5.11 ± 0.71	25.1 ± 3.60	84.08 ± 5.56
Vitrified semen	67.22 ± 4.02 a	58.13 ± 5.61 a	48.98 ± 1.59	179.4 ± 17.3	123.8 ± 19.7	144.3 ± 13.3	67.97 ± 5.54	87.01 ± 3.57 a	5.01 ± 0.89	21.1 ± 2.8	73.67 ± 6.11 a

Data are expressed as mean ± SD. The values in a row marked with a superscript differ at $P < 0.05$.

levels and thus can be successfully used for artificial insemination.

In our research we found that there were no significant differences ($P > 0.05$) between the velocity parameters (VAP, VSL, VCL) and BCF of fresh and vitrified samples, which is in agreement with previously reported results of our team (Antonov & Ivanova, 2022). Their evaluation is the most useful method for comparing semen from fertile and infertile dogs (Domoslawska, 2013), because they are important for the progression of sperms into cervical mucus and penetration of zona pellucida of oocytes (Verstegen, 2002). According to the reported results, sperm vitrification yields a high survival rate of fertile canine spermatozoa after warming.

Our study demonstrates that canine sperm vitrification in a coconut water extender with an addition of 20% egg yolk could be successful for routine clinical use as alternative to conventional cryopreservation. This ultra-rapid freezing method is a much faster, simpler and cheaper method, which could prevent the high spermatozoa mortality rate observed in conventional freezing. Additionally, we believe that coconut water could successfully replace some of the expensive chemical ingredients of semen extenders and has a positive impact on the environment. Therefore, further research on fertility studies should be conducted and investigated to detect true measure of successful dog sperm vitrification with a coconut water extender.

Our results demonstrate that when a coconut water extender with an addition of 20% egg yolk as a cryoprotectant is used, vitrification affects the quality of canine spermatozoa, but could provide quality results near the conventional freezing method.

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