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Effect of different cryoprotectants, equilibration time, and warming regimens on canine spermatozoa after vitrification using coconut water extender

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Abstract

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The aim of the present investigation was to evaluate the effect of different cryoprotectants, equilibration time and warming regimens on canine sperm quality after vitrification using coconut water extender. Ten ejaculates were collected separately by digital manipulation from 10 adult dogs. Only the second fraction of the ejaculate was used in this study. It was evaluated about volume, concetration, viability, total and progressive motility, kinetic parameters and morphology, divided into two aliquots and diluted with 2 types of coconut water based extender until final concentration of 100×10^6 spermatozoa/ml. Base vitrification media (BVM) was prepared using 50% (v/v) coconut water, 25% (v/v) distilled water and 25% (v/v) 5% anhydrous monosodium citrate solution. Extender A consisted of BVM with addition of soy lecithin and fructose at 1% and 0.25 M sucrose and Extender B consisted of BVM with 20% (v/v) egg yolk and 1% fructose. Both of the extended samples were divided into three aliquots and each of them was processed at different regimens: without equilibration (E0), 5°C for 30 min (E30) and 5°C for 60 min (E60) and then vitrified by dropping 33 µl of sperm suspension directly into liquid nitrogen. Sperm pellets were devitrified at least one week later and warming was done at 37°C or 42°C for 2 min. Sperm motility parameters were assayed using a computer-aided sperm analysis (CASA) system, viability-by supravital staining technique and morphology parameters were evaluated in Haemacolor® stained semen samples. In conclusion, our results demonstrate that when vitrification and coconut water extender were used, egg yolk as a cryoprotectant, presence of equilibration time of 60 min and warming at 42°C for 2 min provided the best canine sperm quality results.

Keywords: dog; semen; vitrification; coconut water

Introduction

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 et al., 2016). Two types of sperm cryopreservation are developed until now-conventional cryopreservation or vitrification. The first one is a slow-gradual freezing process accompanied by dehydration in order to reduce intracellular ice crystallization (Amirat-Briand et al., 2010), resulting in cryodamage and poor post-thawed semen quality (Falah et al., 2020). Vitrification is an ultra-rapid cooling method for solidifying liquid into glassy state by direct immersion into liquid nitrogen (LN_2) without ice crystal formation in fast and inexpensive manner (Isachenkoet al., 2004; Amirat-Briand et al., 2010; Magnotti et al., 2018). The method is widely used for embryo, oocyte or tissue storage (Isachenko et al., 2004; Rosato et al., 2013). In last few years

sperm vitrification has been successfully developed in different mammalian species (Isachenko et al., 2011; Merino et al., 2011; Figueroa et al., 2015; Pradiee et al., 2015; 2017, Swanson et al., 2017; Diaz-Jimenez et al., 2018; Hidalgo et al., 2018) and, recently in dogs (Sánchez et al., 2011; Kim et al., 2012; Gharajelar et al., 2016; Caturla-Sánchez et al., 2018; Pipan et al., 2020; Galarza et al., 2021), but conventional freezing methods are still prefered for cryopreservation of canine semen (Sánchez et al., 2011).

The addition of cryoprotectors to semen extenders is mandatory for providing cell survival after the freezing process. Two groups of cryoprotectors are available. First are permeable cryoprotectants, e. g. glycerol, which prevents ice crystals formation inside the cells, but it has high toxic effects on spermatozoa (Curry, 2000; Holt, 2000). The second group consists of nonpermeable ones such as different combinations of carbohydrates (sucrose, lactose and trehalose) and proteins (bovine serum albumin, milk, lecitin or egg yolk) (England, 2000; Isachenkoet al., 2004). They prevent water precipitation and formation of intracellular or extracellular ice crystals, because viscosity greatly increases (Isachenko et al., 2011).

During the conventional cryopreservation process, canine semen is first diluted with a suitable extender, equilibrated with a cryoprotector by cooling at 4-5°C for a relatively long time (1-3 h) and stored frozen into liquid nitrogen (Okano et al., 2004). Equilibration process is necessary to permit membrane changes or ionic flux which increases the membrane resistance during the cooling process (Igna et al., 2008). The cooling time before vitrification of dog sperms in the previously described investigations was not performed (Sánchez et al., 2011) or within 30 minutes at 5°C (Caturla-Sánchez et al., 2018; Pipan et al., 2020; Galarza et al., 2021), but there are not any comparative results between different equilibration protocols until now.

Another important factor that affects the sperm vitrification outcome is the warming procedure, called devitrification (Mazur & Seki, 2011; Pradiee et al., 2017; Caturla-Sánchez et al., 2018). Slow (37°C for 1–2 min) and fast (65°C for 2–5 s) warming regimens are used for vitrified dog spermatozoa (Sánchez et al., 2011; Caturla-Sánchez et al., 2018) and it was concluded that the warming rate is more critical than the cooling one in kinetic vitrification (Mazur & Seki, 2011). It was also suggested that slow warming helps prevent damage to vitrified dog spermatozoa (Caturla-Sánchez et al., 2018). The effect of different slow-warming temperatures to vitrified dog spermatozoa has not been yet reported.

Semen extenders were discovered and developed in order to protect spermatozoa from different harmful factors (Bustani & Baiee, 2021) and choosing the right one is an important part of semen processing (Peterson et al., 2007; Ogbu et al., 2014). Commercial extenders for dog semen preservation are available and they differ in content and complexity. Most of them consist of chemical combinations, but there is an international demand for using alternative sources in semen extenders of different animals, including such as those of animal or plant origin (Bustani & Baiee, 2021). One of the natural buffer solutions, which has been successfully used for canine semen preservation, even in cooled or cryopreserved type is coconut water (Cardoso et al., 2003, 2005, 2006; Gunawan et al., 2016; Puja et al., 2018). As a biological ingredient it contains essential constituents (Silva & Bamunuarachchi et al., 2009) with high antioxidant properties (Mantena et al., 2019), which is suitable as a canine semen extender due to isotonic, not toxic, cheap, effective, and simple to be used (Cardoso et al., 2003). In the literature there are no data about the possible potential of using coconut water extender for dog sperm vitrification.

Therefore, this study demonstrated the successful use of a natural ingredient like coconut water and the effect of different cryoprotectants, equilibration time and warming regimens on canine sperm quality after vitrification.

Materials and Methods

Experimental animals and management

Ten ejaculates were collected by digital manipulation from 10 adult kennel-owned German Shepherds, aged 3–7 years and weighted 31–36 kg, which were presented at the Small animal clinic of the Faculty of Veterinary Medicine, Trakia University, Stara Zagora, Bulgaria. The dogs were previously used for conventional semen freezing and were found cryotolerant. The experiment was conducted according to the recommendations of the Local Animal Ethics Committee and regulations for human attitude and animal protection. All the owners signed informed consent form that there are no risks associated with the procedures and the research will be published.

Semen collection and evaluation

The collection was performed separately for the three fractions in sterile plastic tubes. It was done by the same operator in a presence of a teaser bitch in order to provide stimulation and immediately after the semen was transferred to the laboratory for analysis. Only the second fraction of the ejaculate was used in this study. It was evaluated about volume, concetration, viability, motility parameters and morphology.

The volume was measured by a graduated pipette. Sperm concentration ($x10^6$/mL) was determined by a Photometer SpermaCue® (Minitüb, Germany).

The sperm viability was assessed by mixing 5 µL of semen with 5 µL of eosin-nigrosin and allowed to air dry. At least 200 cells were counted under a light microscope and oil immersion at magnification of 400×. Sperm cells that were unstained (white) were accepted as alive, whereas stained (pink or red coloration) were considered to be dead.

Sperm motility parameters were assayed by Computer-Assisted Sperm Analysis (CASA) and Sperm Class Analyser (SCA) (Microptic, S.L., Barcelona, Spain) with a Makler counting chamber of 10 µL semen samples. A minimum of 10 fields were examined. The evaluated parameters included total motility (TM), progressive motility (PM), curvilinear velocity (VCL), straight line (VSL), average path velocities (VAP), linearity (LIN), straightness rate (STR), lateral head displacement amplitude (ALH) and beat cross frequency (BCF).

To evaluate the morphology, at least 200 sperm cells were evaluated in semen samples after Haemacolor® staining (Merck KGaA). A 5 µL aliquot of canine semen was placed on the slide, smeared, fixed with methanol, stained with the two solutions of stain, rinsed with distilled water and allowed to air-dry. Slides were examined by a light microscope at magnification of 400× and the sperm cells were assessed for their normality (normal shape and normal structure).

Preparation of extenders and semen processing

After the initial evaluation, the sperm rich fraction was divided into two aliquots and diluted with 2 types of coconut water extender until final concentration of 100×10^6 spermatozoa/ml. Base vitrification media (BVM) was first prepared using 50% (v/v) water from green coconut, 25% (v/v) distilled water and 25% (v/v) 5% anhydrous monosodium citrate solution. Extender A consisted of BVM with addition of soy lecithin and fructose at 1% and 0.25 M sucrose and Extender B consisted of BVM with 20% (v/v) egg yolk and 1% fructose. Both of the extended samples were divided into three aliquots and each of them was processed at different regimens: without equilibration (E0), 5°C for 30 min (E30) and 5ºC for 60 min (E60).

Vitrification and warming

Vitrification was based on the methodology previously described by Shah et al. (2019) for human sperm. Aliquots of 33 µl of the sperm suspension were directly dropped with a micropipette into styrofoam box filled with liquid nitrogen (LN_2) and contained a stainless steel strainer from a height of 10 cm. After solidification process the droplets settled down into the strainer (Figure 1 A), transferred into pre-cooled cryotubes and stored in LN_2 for a week until devitrification for evaluation (Figure 1 B).

The devitrification process was performed as three of the sperm pellets were dropped into 0.5 mL of CaniPlus AI (Minitüb, Germany), which was previously warmed in a water bath at 37°C or 42°C for 2 min. Sperm viability, motility parameters and morphology were evaluated as previously described.

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 the mean ± SD and were

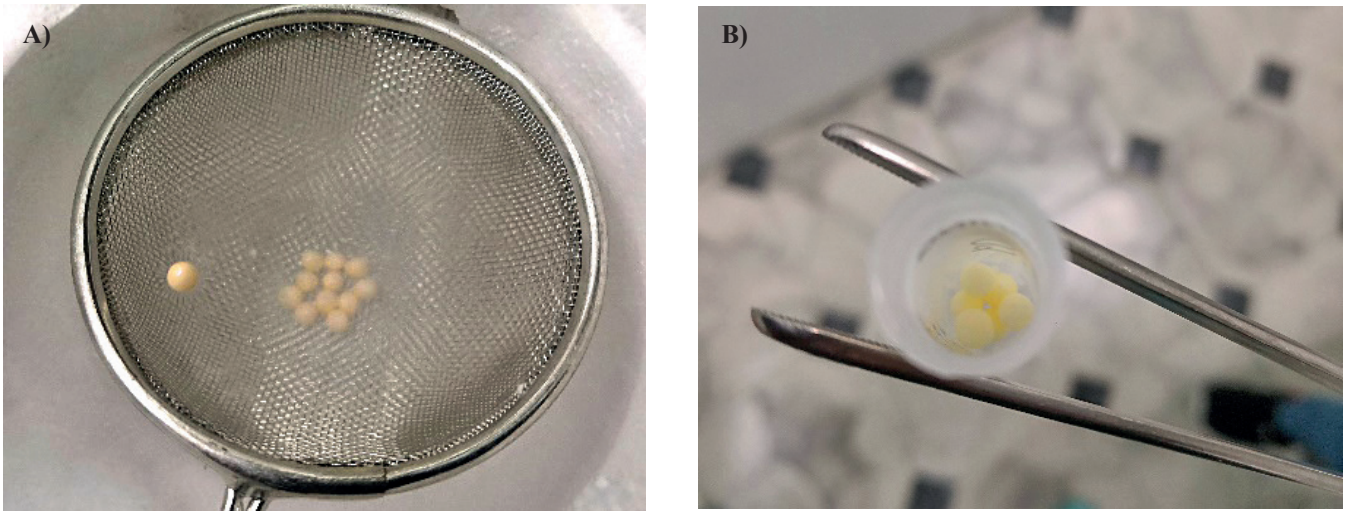

Fig. 1. Solidified canine sperm suspension:
***A* – during the vitrification process into the strainer; *B* – after transfer into cryotube**

first checked for normality. The results were analyzed using ANOVA for repeated measures followed by Tukey's multiple comparisons test and a value for $p < 0.05$ was considered signifcant.

Results

Mean average parameters of the second sperm fraction used in this study were as follows: volume 0.96 ± 0.21 mL, concentration 976 ± 186.02×10⁶ spermatozoa/mL, viability 94.51 ± 1.51, total motility 87.92 ± 1.99%, progressive motility 50.44 ± 3.80% and sperms with normal morphology 83 ± 5.56%. The influence of the cryoprotector, equilibration time and warming regimen on vitrified canine sperm using coconut water extender are presented in Table 1.

Fresh sperm samples showed significantly higher ($p < 0.05$) viability than all of the vitrified samples. The percentage of the viable sperms after devitrification was lowest when vitrification has been performed without previous equilibration. It was significantly improved by presence of equilibration period before vitrification. Sperm viability was significantly lower ($p < 0.05$) when lecithin and sucrose were used as cryoprotectors and devitification was at 37°C. The highest values of 67.22 ± 4.02% viable sperms were detected when equilibration was done for 60 minutes, egg yolk was used as a cryoprotector and devitrification was performed at 42°C (Figure 2).

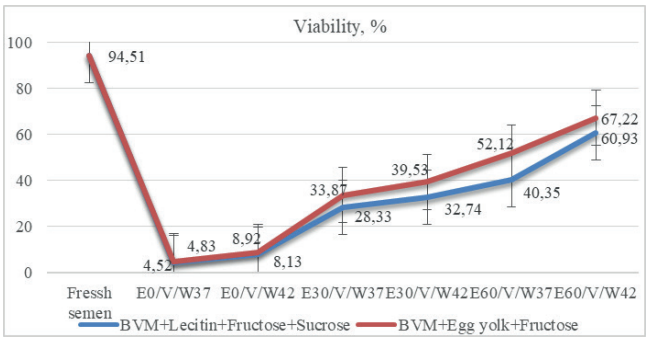

Fig. 2. Viability in canine sperm after devitrification. Values are expressed as mean ± SD

Table 1. Parameters after devitrification of canine semen samples (n = 10) using coconut water extender. Data are expressed as mean ± SD. The values in a row marked with a different superscript differ at P < 0.05. E0 – without equilibration; E30 – 5°C for 30 min; E60 – 5°C for 60 min; V – vitrification; W37 – warming at 37°C; W42 – warming at 42°C; A – BVM+lecithin+fructose+sucrose; B – BVM+egg yolk+fructose

	Fresh semen	Equilibration/ Vitrification/ Warming											
		E0/V/W37		E0/V/W42		E30/V/W37		E30/V/W42		E60/V/W37		E60/V/W42	
		A	B	A	B	A	B	A	B	A	B	A	B
Viability, %	94.51± 1.51a	4.52± 0.85b	4.83± 1.03b	8.13± 2.03c	8.92± 2.18c	28.33± 8.34d	33.87± 6.51d	32.74± 4.71d	39.53± 3.40e	40.35± 4.54e	52.12± 5.74f	60.93± 3.28g	67.22± 4.02h
Total motility, %	87.92± 1.99a	3.20± 1.03b	3.70± 1.16b	5.90± 2.38c	6.30± 2.11c	21.95± 9.59d	28.32± 8.89d	28.62± 5.50d	30.07± 7.79d	37.03± 4.89e	46.89± 7.05f	53.23± 4.56g	58.13± 5.61h
Progressive motility, %	50.44± 3.80a	5.70± 0.67b	6.20± 0.63b	11.10± 0.74c	11.30± 1.06c	15.65± 0.89d	16.43± 1.46d	23.40± 3.59e	29.33± 1.56f	29.62± 1.96f	40.64± 2.07g	45.11± 1.15h	48.98± 1.59a
VCL, µm/s	191.1± 26.6a	138.3± 29.1b	137.1± 23.1b	140.5± 18.9b	139.4± 22.8b	143.3± 21.8b	142.6± 23.1b	137.2± 15.8b	149.6± 12.3b	174.1± 33.7a	173.4± 19.7a	179.3± 26.9a	179.4± 17.3a
VSL, µm/s	129.2± 16.6a	83.1± 23.4b	81.7± 33.6b	84.6± 18.8b	86.7± 21.8b	89.3± 18.7b	88.5± 11.4b	86.1± 12.8b	96.2± 9.9b	117.8± 15.3a	117.5± 13.4a	122.5± 15.6a	123.8± 19.7a
VAP, µm/s	146.3± 15.1a	103.3± 11.3b	103.7± 13.6b	104.8± 14.8b	105.9± 11.7b	108.7± 9.6b	107.6± 11.1b	102.9± 10.5b	119.3± 12.8c	135.2± 19.7a	136.5± 17.4a	143.9± 17.2a	144.3± 13.3a
LIN, %	68.50± 8.36a	58.70± 9.17b	60.53± 7.98b	58.90± 10.56b	60.70± 8.16b	63.11± 7.61ab	62.82± 9.11ab	63.93± 8.33ab	65.35± 6.44ab	64.23± 7.91ab	66.54± 8.97ab	67.68± 6.75a	67.97± 5.54a
STR, %	88.93± 4.31a	78.87± 11.43b	77.87± 8.18b	78.64± 10.86b	79.49± 9.19b	81.42± 5.13b	82.13± 7.41b	81.78± 6.29b	82.87± 7.51b	84.17± 5.89b	84.88± 4.11b	85.38± 4.63b	87.01± 3.57b
ALH, µm	5.11± 0.71a	3.14± 1.90b	3.78± 1.11b	3.42± 1.78b	3.92± 0.99b	4.23± 0.89ab	4.32± 0.88ab	4.27± 1.01ab	4.29± 0.98ab	4.85± 0.73a	4.97± 1.19a	4.89± 0.87a	5.01± 0.89a
BCF, Hz	25.1± 3.6a	12.2± 9.3b	14.7± 5.4b	12.3± 8.7b	16.3± 6.1b	15.4± 5.1b	18.6± 2.9b	17.7± 3.6b	18.6± 3.2b	22.7± 3.2a	23.8± 4.7a	22.5± 4.1a	21.1± 2.8a
Normal morphology, %	83.56± 5.56a	48.67± 6.57b	51.87± 6.21b	51.83± 5.89b	53.56± 4.91b	59.45± 6.78c	63.87± 7.28c	61.28± 5.25c	64.24± 5.87c	69.33± 3.18d	72.24± 4.28d	70.71± 5.12d	73.67± 6.11d

Similar tendency was observed in total and progressive motility. The longer equilibration process, using egg yolk as a cryoprotector and devitrification at 42°C resulted in the greatest significant values of 58.13 ± 5.61% for total canine sperm motility ($p < 0.05$) (Figure 3). Progressive motility was also improved by these factors and the highest levels of 48.98 ± 1.59% were even not significantly different with the fresh semen samples before vitrification (Figure 4). Vitrification also caused significant changes ($p < 0.05$) in the sperm kinematic parameters and the highest values were obtained when using 60 min equilibration, egg yolk based coconut extender and warming at 42°C (Table 1).

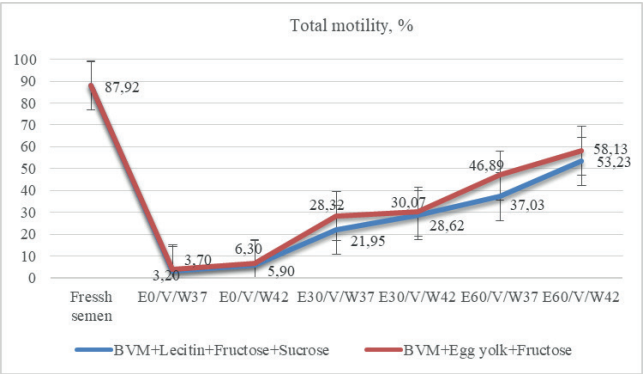

Fig. 3. Total motility in canine sperm after devitrification. Values are expressed as mean ± SD

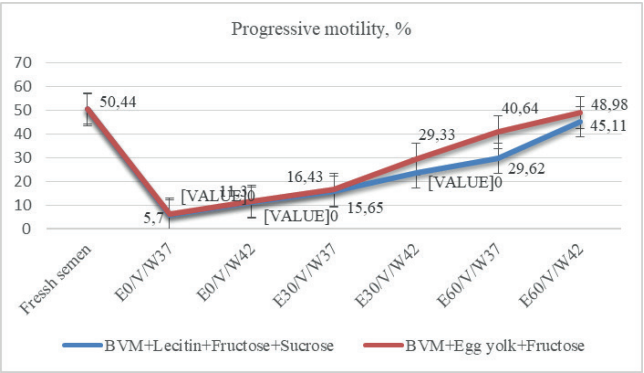

Fig. 4. Progressive motility in canine sperm after devitrification. Values are expressed as mean ± SD

Comparing the different type of the cryoprotector, protocols for equilibration and warming regimen it was found that highest percentage of sperms with normal morphology were found in fresh samples ($p < 0.05$). Vitrification of spermatozoa in a coconut water extender after equilibration for 60 minutes resulted in a significantly higher ($p < 0.05$) percentage of spermatozoa with normal morphology compared to other vitrified samples, but without statistical difference ($p > 0.05$) when containing egg yolk or lecithin and sucrose as cryoprotector or comparing devitrification temperature (Figure 5).

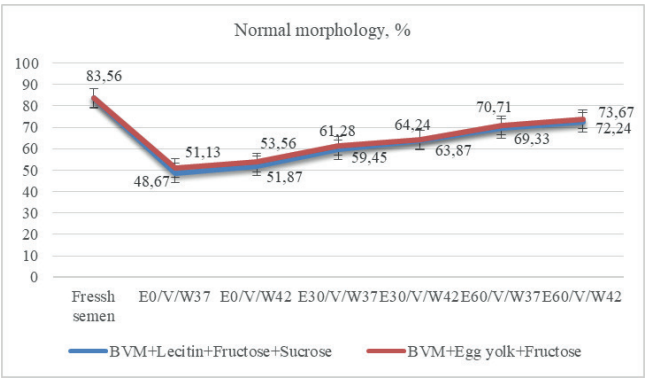

Fig. 5. Persentage of normal morphology canine sperms after devitrification. Values are expressed as mean ± SD

Discussion

Conventional slow freezing methods with permeable cryoprotectants are usually employed in canine semen preservation for last decades (Sánchez et al., 2011). Vitrification also requires using cryoprotectants in order to prevent sperm damage during freezing process and as it was mentioned, it was developed in dogs, but further studies are needed to improve protocol for this ultra-rapid semen freezing. Thus, our experiment was conducted to compare different cryoprotectants, possible effect of presence and duration of equilibration time and warming regimen on the quality of dog spermatozoa. Moreover, we used a very cheap extender, based on natural buffer solution such as coconut water and the quality of preserved canine semen after devitrification was even better than any previously reported results (Sánchez et al., 2011; Kim et al., 2012; Gharajelar et al., 2016; Caturla-Sánchez et al., 2018; Pipan et al., 2020; Galarza et al., 2021).

Cryoprotectant is an obligatory part of the semen extender in order to prevent cold shock during the freezing process and vitrification requires presence of nonpermeable ones such as carbohydrates or proteins. Caturla-Sánchez et al. (2018) investigated vitrification media containing different carbohydrates for preservation of dog semen and found that the addition of 0.25M sucrose provided the best sperm quality results, but without acceptable motility. Sanchez et al. (2011) investigated the ability of combination of sucrose and bovine serum albumin to protect spermatozoa and found it could effectively preserve important physiological param-

eters during ultra-rapid cryopreservation in canine sperm. Kim et al. (2012) vitrified canine semen samples with egg yolk based extenders without acceptable motility and viability results. Gharajelar et al. (2016) used cryopreservation mediums on the basis of glycerol, milk and egg yolk and found that milk had better effects on the cryopreservation of semen than glycerol and egg yolk. Pipan et al. (2020) replaced successfully the foreign animal protein with a combination of soy lecitin and sucrose. In the present study, we have successfully used both egg yolk or a combination of soy lecitin and sucrose in addition to coconut water extender and compared their cryoprotective activity.

Our results are in agreement with most of previous studies, according to which a protein source is necessary to be added to carbohydrates in order to provide sperm survival during and after the cryopreservation process. Egg yolk is believed to act at the level of the cell membrane (Sánchez et al., 2011) and soy lecithin has antioxidant activity and protects semen from oxidative stress resulting in high viability and motility after cryopreservation (Dalmazzo et al., 2017). In the scientific literature the best canine sperm viability and total motility reported after vitrification were 59% and 50% respectively with TRIS based extender containing 1% soy lecitin and 0.25M sucrose concentration (Pipan et al., 2020). In the present study, we observed even better sperm viability and total motility using coconut water extender and the same lecitin and sucrose concentration. Furthermore, samples vitrified in egg yolk based extender resulted in a significantly higher ($p<0.05$) percentage of the examined sperm parameters compared to lecitin and sucrose. It has been concluded that both egg yolk or the combination of lecitin and sucrose can effectively preserve important physiological parameters of canine sperm during ultra-rapid cryopreservation.

According to Caturla-Sanchez et al. (2018), a period of equilibration with vitrification solution, at 5°C for 30 min, may contribute to negative effects on sperm motility and may be harmful for dog spermatozoa. Controversial, our results showed better viability, total and progressive motility, velocity parameters and highest percentage of normal morphology spermatozoa when equilibration was performed at 5°C during 1 h, compared to lack of equilibration or 5°C for 30 min, which is in agreement with Hidalgo et al. (2018) that equilibration temperature had shown to be essential for sperm vitrification. According to Domoslawska et al. (2013), the most useful method for discrimination between semen of fertile and infertile dogs is the evaluation of velocity parameters (VAP, VSL, VCL) and BCF, which are important for the progression of sperms into cervical mucus and penetration of zona pellucida of oocytes (Verstegen et al., 2018). Our results showed no significant differences ($p > 0.05$) in velocity parameters between fresh semen samples and when equilibration was at 5°C for 1 h, so presence of equilibration time is necessary for sperms in order to adapt for a certain period of time before vitrification, as it is in conventional cryopreservation. This may surve as an evidence that during vitrification canine sperms also need a period of adaptation, during, which they develop higher resistance to the effects of freezing and the lack of equilibration may be harmful for dog spermatozoa, increasing morphological defects and decreasing their fertilizing capacity.

In order to provide best sperm survival, the freezing rate must be added by a suitable thawing temperature regimen, which could be even more critical than the cooling rate in vitrification (Mazur et al., 2011). In previous investigations of dog sperm vitrification, warming protocol has largely been ignored or unintentionally missed, but as it is already known, both are highly correlated. During thawing, the osmotic balance is reversed, rehydration occurs and the lipid protein configuration of the membrane is restored similarly as the events are induced during freezing (Simons, 2018). Previous results suggest that slow warming (37°C) helps prevent damage to vitrified dog spermatozoa compared to rapid warming process (65°C) (Caturla-Sánchez et al., 2018). In present study, we also used slow warming and higher recovery rates were registered when temperature regimen of 42°C was used compared to 37°C. Our results are in agreement with Fizer et al. (1993) that warming also has a very critical role in sperm survival as cooling does, because sperm survival and damage depends on the intermediate zone of temperature between -10 to -60°C and they have to traverse through it twice during a cryopreservation protocol.

Oxidative stress is one of the major problems in stored semen (Dalmazzo et al., 2017) and our superior results could be due to lowering it by high antioxidant properties of coconut water. Present study demonstrated that canine spermatozoa vitrification in a coconut water extender containing egg yolk or the combination of lecitin and sucrose could be successfully performed as alternative to conventional cryopreservation due to it is much faster, simpler, cheaper and it could provide a high recovery of fertile spermatozoa after warming. Our results confirm, that coconut water could successfully replace some of the expensive chemical ingredients of semen extenders. Another advantage is that as a component of plant origin and in combination with other animal free ingredients of extenders, coconut water could not surve as a reason for restrictions in worldwide semen transport. Therefore, further research on fertility studies should be conducted and investigated to detect true measure of successful dog sperm vitrification with coconut water extender.

Conclusions

Our results demonstrate that when vitrification and coconut water extender were used, egg yolk as a cryoprotectant, presence of equilibration time of 60 min and warming at 42°C for 2 min provided the best canine sperm quality results.

Acknowledgments

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Conflict of interest statement

The authors declare there is no conflict of interest.

References

Amirat-Briand, L., Bencharif, D., Vera-Munoz, O., Pineau, S., Thorin, C., Destrumelle, S,... & Tainturier, D. (2010). *In vivo* fertility of bull semen following cryopreservation with an LDL (low density lipoprotein) extender: Preliminary results of artificial inseminations. *Animal Reproduction Science*, *122*(3-4), 282-287.

Bustani, G. S. & Baiee, F. H. (2021). Semen extenders: An evaluative overview of preservative mechanisms of semen and semen extenders. *Veterinary World, 14*(5), 1220-1233.

Cardoso, R. C. S., Silva, A. R., Uchoa, D. C. & Silva, L. D. M. (2003). Cryopreservation of canine semen using a coconut water extender with egg yolk and three different glycerol concentrations. *Theriogenology, 59,* 743-751.

Cardoso, R. C. S., Silva, A. R. & Silva, L. D. M. (2005). Use of the powdered coconut water (ACP-106®) for cryopreservation of canine spermatozoa. *Anim. Reprod., 2*(4), 257-262.

Cardoso, R. C. S., Silva, A. R. & Silva, L. D. M. (2006). Comparison of two dilution rates on canine semen quality after cryopreservation in a coconut water extender. *Animal Reproduction Science, 92*(3-4), 384-391.

Caturla-Sánchez, E., Sánchez-Calabuig**, M., Gutiérrez, J. F. P., Cerdeira, J., Castaño, C. & Santiago-Moreno, J.** (2018). Vitrification of dog spermatozoa: Effects of two cryoprotectants (sucrose or trehalose) and two warming procedures. *Cryobiology, 80,* 126–129.

Curry, M. R. (2000). Cryopreservation of semen from domestic livestock. *Rev. Reprod., 5*(1), 46-52.

Dalmazzo, A., Losano, J., Rocha, C. C., Tsunoda, R. H., Angrimani, D. D. S. R., Mendes, C. M., Assumpção, M. E. O. D., Nichi, M. & Barnabe, V. H. (2017). Effects of Soy Lecithin Extender on Dog Sperm Cryopreservation. *Anim. Biotechnol., 29,* 174–182.

Diaz-Jimenez, M., Dorado, J., Pereira, B., Ortiz, I., Consuegra, C., Bottrel, M., Ortiz, I. & Hidalgo, M. (2018). Vitrification in straws conserves motility features better than spheres in donkey sperm. *Reprod. Domest. Anim., 53,* 1–3.

Domosławska, A., Zduńczyk, S., Niżański, W. & Janowski, T. (2013). Assessment of semen quality in infertile dogs using computer-assisted sperm analysis by the Hamilton-Thorne Semen Analyser. *Bull. Vet. Inst. Pulawy, 57,* 429-432.

England, G. C. W. (2000). Semen evaluation, artificial insemenation. In: Etinger JS, Feldman EC (Eds). *Veterinary Internal Medicine.* Philadelphia, USA: WB Saunders Company, 1571-1585.

Falah, B., Bassam, A. A.-W., Asaad, A., Lee, A., Yu, L., Wan-Nor, F., Abd, W. H. & Ghadeer, S. B. (2020). Effect of vitrification on spermatozoa quality in bull semen. *Eurasia J. Biosci., 14,* 3897-3904.

Figueroa, E., Merino, O., Risopatron, J., Isachenko, V., Sanchez, R., Effer, B., Isachenko, E., Farias, J. G. & Valdebenito, I. (2015). Effect of seminal plasma on Atlantic salmon (*Salmo salar*) sperm vitrification. *Theriogenology, 83,* 238–245.

Fiser, P. S., Fairfull, R. W., Hansen, C., Panich, P. L., Shrestha, J. N. B. & Underhill, L. (1993). The effect of warming velocity on motility and acrosomal integrity of boar sperm as influenced by the rate of freezing and glycerol level. *Mol. Reprod. Dev., 34,* 190-195.

Galarza, D. A., Landi, G., Mejía, E., Samaniego, J. X., Mendez, S., Soria, M. E., Taboada, J., Sanchez-Calabuig, M. J., Castano, C. & Santiago-Moreno, J. (2021). Cryopreservation of dog epididymal spermatozoa by conventional freezing or ultra-rapid freezing with nonpermeable cryoprotectant. *Cryobiology, 103,* 15-21.

Gharajelar, S. N., Sadrkhanloo, R. A., Onsori, M. & Saberivand, A. (2016). A comparative study on the effects of different cryoprotectants on the quality of canine sperm during vitrification process. *Vet. Res. Forum. Int. Q. J., 7,* 235–239.

Gunawan, I. W. N. F., Kardena, I. M., Suatha, I. K. & Puja, I. K. (2016). Coconut Water Based Extender Effects on Motility, Viability, and DNA Integrity of Chilled Kintamani Dog Semen. *Veterinary Science and Medicine Journal, 4,* 17-21.

Hidalgo, M., Consuegra, C., Dorado, J., Diaz-Jimenez, M., Ortiz, I., Pereira, B., Sanchez, R. & Crespo, F. (2018). Concentrations of non-permeable cryoprotectants and equilibration temperatures are key factors for stallion sperm vitrification success. *Anim. Reprod. Sci., 196,* 91–98.

Holt, W. V. (2000). Fundamental aspects of sperm cryobiology: The importance of species and individual differences. *Theriogenology, 53*(1), 47-58.

Igna, V., Hanganu, M., Tulcan, C., Mircu, C., Otava, G., Knop, R., Cernescu, H. & Ardelean, V. (2008). The influence of equilibration time on dog semen cryopreservation. *Scientific Works-Lucrari Stiintifice, C series, LIII,* 1222-5304.

Isachenko, E., Isachenko, V., Sanchez, R., Katkov, I. I. & Kreienberg, R. (2011). Cryopreservation of spermatozoa: Old routine and new perspectives. *In Principles and Practice of Fertility Preservation. Cambridge: University Press,* 176–198.

Isachenko, E. (2004). DNA integrity and motility of human spermatozoa after standard slow freezing versus cryoprotectant-free vitrification. *Hum. Reprod., 19,* 932–939.

Isachenko, V., Isachenko, E., Katkov, I. I., Montag, M., Dessole, S., Nawroth, F. & Van Der Ven, H. (2004). Cryoprotectant-free cryopreservation of human spermatozoa by vitrification and freezing in vapor: Effect on motility, DNA integrity,

and fertilization ability. *Biol. Reprod., 71,* 1167–1173.

Kim, S., Lee, Y., Yang, H. & Kim, Y.-J. (2012). Rapid freezing without cooling equilibration in canine sperm. *Anim. Reprod. Sci., 130*, 111–118.

Magnotti, C., Cerqueira, V., Lee-Estevez, M., Farias, J. G., Valdebenito, I. & Figueroa, E. (2018). Cryopreservation and vitrification of fish semen: a review with special emphasis on marine species. *Reviews in Aquaculture, 10*(1), 15-25.

Mantena, S. K., Badduri, S. R., Siripurapu, K. B. & Unnikrishnan, M. K. (2003). *In vitro* evaluation of antioxidant properties of Cocos nucifera Linn. water. *Nahrung, 47,* 126-131.

Mazur, P. & Seki, S. (2011). Survival of mouse oocytes after being cooled in a vitrification solution to -196°C at 95° to 70,000°C/min and warmed at 610° to 118,000°C/min: a new paradigm for cryopreservation by vitrification. *Cryobiology, 62,* 1-7.

Merino, O., Risopatrón, J., Sánchez, R., Isachenko, E., Figueroa, E., Valdebenito, I. & Isachenko, V. (2011). Fish (Oncorhynchus mykiss) spermatozoa cryoprotectant-free vitrification: Stability of mitochondrion as criterion of effectiveness. *Anim. Reprod. Sci., 124,* 125–131.

Ogbu, C. C., Ugwu, S. O. & Ezebili, C. V. (2014). Use of co-conut milk as an extender for cock semen. *Res. Opin. Anim. Vet. Sci., 4*(10), 571–577.

Okano, T., Murase, T., Asano, M. & Tsubota, T. (2004). Effects of Final Dilution Rate, Sperm Concentration and Times for Cooling and Glycerol Equilibration on Post-Thaw Characteristics of Canine Spermatozoa. *Journal of Veterinary Medical Science, 66*(11), 1359-1364.

Peterson, K., Kappen, M. A., Ursem, P. J., Nothling, J. O., Colenbrander, B. & Gadella, B. M. (2007). Microscopic and flow cytometric semen assessment of Dutch AI-bucks: effect of semen processing proce-dures and their correlation to fertility. *Theriogenology, 67*(4), 863–871.

Pipan, M., Casal, M. L., Šterbenc, N., Virant Klun, I. & Mrkun, J. (2020). Vitrification Using Soy Lecithin and Sucrose: A New Way to Store the Sperm for the Preservation of Canine Reproductive Function. *Animals, 10,* 653.

Pradiee, J., Esteso, M. C., Castaño, C., Toledano-Díaz, A., Lopez-Sebastián, A., Guerra, R. & Santiago-Moreno, J. (2017). Conventional slow freezing cryopreserves mouflon spermatozoa better than vitrification. *Andrologia, 49,* 4.

Pradiee, J., Esteso, M. C., Castaño, C., Toledano-Díaz, A., Lopez-Sebastián, A., Guerra, R. & Santiago-Moreno, J. (2017). Conventional slow freezing cryopreserves mouflon spermatozoa better than vitrification. *Andrologia, 49,* e12629.

Pradiee, J., Esteso, M. C., Lopez-Sebastián, A., Toledano-Díaz, A., Castaño, C., Carrizosa, J. A., Urrutia, B. & Santiago-Moreno, J. (2015). Successful ultrarapid cryopreservation of wild Iberian ibex (*Capra pyrenaica*) spermatozoa. *Theriogenology, 84,* 1513–1522.

Puja, I. K., Sawitri, N. M., Maharani, N., Gunawan, I. W. N. F. & Heryani, L. G. S. S. (2018). A comparative study on the effects of coconut water based extenders on the quality of kintamani dog semen preserved at 4^{O}C. *Adv. Anim. Vet. Sci., 6*(5), 192-196.

Rosato, M. P. & Iaffaldano, N. (2013). Cryopreservation of rabbit semen: Comparing the effects of different cryoprotectants, cryoprotectant-free vitrification, and the use of albumin plus osmoprotectants on sperm survival and fertility after standard vapor freezing and vitrification. *Theriogenology, 79,* 508–516.

Sánchez, R., Risopatrón, J., Schulz, M., Villegas, J., Isachenko, V., Kreinberg, R. & Isachenko, E. (2011). Canine sperm vitrification with sucrose: Effect on sperm function. *Andrologia, 43,* 233–241.

Shah, D., Rasappana, S. & Karthik, G. (2019). A simple method of human sperm vitrification. *MethodsX, 6,* 2198-2204.

Silva, P. & Bamunuarachchi, A. (2009). Manufacture of carbonated tender coconut water and development of a process for the utilization of coconut flesh. *Asian Journal of Food and Agro-Industry, 2,* 210-213.

Simons, N. (2018). Collection and cryopreservation of epididymal spermatozoa in dogs. Dissertation, Ghent University.

Swanson, W. F., Bateman, H. L. & Vansandt, L. M. (2017). Urethral catheterization and sperm vitrification for simplified semen banking in felids. *Reprod. Domest. Anim., 52,* 255–260.

Verstegen, J., Iguer–Ouada, M. & Onclin, M. (2002). Computer assisted semen analyzers in andrology research and veterinary practice. *Theriogenology, 57,* 149–179.

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