



CANINE EPIDIDYMAL SPERM VITRIFICATION WITH EGG YOLK AND COCONUT WATER EXTENDER

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Summary

Antonov, A., B. Ivanova, R. Vasileva & V. Nedev, 2025. Canine epididymal sperm vitrification with egg yolk and coconut water extender. *Bulg. J. Vet. Med.* (online first).

The aim of this study was to assess the efficiency of coconut water extender with addition of 20% egg yolk (CWE+EY) for canine epididymal sperm vitrification. Twenty epididymides were removed from ten adult dogs. Sperm samples were retrieved in Petri dishes by the float-up method on a heating stage (37.0 °C). The epididymides and proximal ductus deferentes were incised and suspended in 6 mL coconut water extender with addition of 1% fructose. After 45 min, the samples were collected and sperm quality parameters (viability, motility, morphology) were assayed using a computer-aided sperm analysis (CASA) system. After centrifugation, the supernatant of each sample was removed and the semen pellets were resuspended in the same extender with added egg yolk. Equilibration was performed at 5 °C for 60 minutes, semen was vitrified by direct dropping into liquid nitrogen in spheres with a volume of 30 µL. After a week of storage in cryotubes, spheres were devitrified as three of them were dropped into 0.5 mL of CaniPlus AI (Minitüb, Germany) at 37 °C for 2 minutes and the same parameters were reevaluated. Vitrification resulted in significantly ($P < 0.05$) lower percentage of viable sperms, total and progressive motilities (81.38 ± 19.33 vs 45.47 ± 23.19 ; 63.86 ± 13.45 vs 35.19 ± 12.58 and 38.24 ± 8.11 vs 21.48 ± 8.57 , respectively), but abnormal sperms (51.18 ± 17.53 vs 59.27 ± 11.57) and kinetic variables did not differ ($P > 0.05$) in relation to pre-freezing samples. Our results demonstrated that vitrification with CWE+EY extender as cryoprotectant had excellent potential for canine male gamete preservation.

Key words: dog, coconut water, egg yolk, epididymis, sperm, vitrification

INTRODUCTION

The most popular method for assisted male reproductive biotechnology is semen cryopreservation (Gharajelar *et al.*, 2016). Recently, dog semen cryopreservation gained popularity and breeders use sperm banks for short- and long-term storage of

frozen spermatozoa, mainly before international exportation (Domain, 2023). Different semen freezing methods were investigated and two groups were developed: slow-gradual and ultra-rapid. The first (conventional) showed variable cryo-

survival results due to moderately poor semen quality after thawing. The second (known as vitrification) consists of liquid solidification into glassy state by direct immersion into liquid nitrogen (LN₂) thus avoiding formation of ice crystals (Isachenko *et al.*, 2004; Amirat-Briand *et al.*, 2010; Magnotti *et al.*, 2018). The method was firstly developed for embryo, oocyte and tissue storage (Isachenko *et al.*, 2004; Rosato & Iaffaldano, 2013), but was successfully performed for sperm preservation in humans (Isachenko *et al.*, 2011), and different animal species: fish (Merino *et al.*, 2011), salmon (Figuerola *et al.*, 2015), mouflons (Pradiee *et al.*, 2015; 2017), felids (Swanson *et al.*, 2017), donkeys (Diaz-Jimenez *et al.*, 2018) and stallions (Hidalgo *et al.*, 2018). In dogs, conventional sperm cryopreservation is still preferred, but the ultra-rapid freezing has become a useful alternative option during the last decade (Sánchez *et al.*, 2011; Kim *et al.*, 2012; Gharajelar, 2016; Caturla-Sánchez *et al.*, 2018, Pipan *et al.*, 2020; Galarza *et al.*, 2021; Antonov & Ivanova, 2022; 2023; Ivanova & Antonov, 2022; 2023).

Different methods allow for long-term storage of sperms, yet most of them were performed with ejaculated semen and only few used epididymal collection of spermatozoa. Cryopreservation of dog epididymal semen could be a suitable technique to conserve genetic material from domestic canines, which either died under unexpected situations or underwent orchiectomy for medical reasons, as well as in endangered non-domestic canine species (Varesi *et al.*, 2013; Galarza *et al.*, 2021).

The selection of a proper semen extender is an important element of semen processing, as its constituents must protect sperm cells from harmful factors during

cryopreservation (Peterson *et al.*, 2007; Ogbu *et al.*, 2014). Numerous commercial extenders for canine semen cryopreservation combined with different cryoprotectants are described in the scientific literature. Many researchers have explored different natural extracts and infusions. One of the most popular bioingredients of semen extenders is the coconut water (Cardoso *et al.*, 2003; 2005; 2006; Gunawan *et al.*, 2016; Puja *et al.*, 2018), which is isotonic, effective, non-toxic, non-expensive, and easy to use (Cardoso *et al.*, 2003). Additionally, coconut water has strong antioxidant properties (Cardoso *et al.*, 2003; 2006; Mantena *et al.*, 2003; Silva & Bamunuarachchi, 2009), due to high content of phytohormones (Nunes & Sales, 1993), sugars, vitamins, electrolytes and amino acids (Yong *et al.*, 2009). Most of the studies using coconut water extender have used conventional canine semen cryopreservation and only few have used vitrification (Antonov & Ivanova, 2022; 2023; Ivanova & Antonov, 2022; 2023). In order to reduce spermatozoa damage during the cryopreservation process, addition of a cryoprotectant is mandatory. One of the most widely used cryoprotectors, especially in conventional semen freezing, is egg yolk. It protects spermatozoa from initial cold shock (Layek, 2016; Abdel-Aziz Swelum, 2019), inhibits sperm capacitation (Mahiddine & Kim, 2021) and increases the viscosity of the extender, which prevents the formation of intracellular and extracellular ice crystals (Isachenko, 2011). Limited data are available about its preservation potential during ultra-rapid canine sperm cryopreservation and for epididymal semen in particular.

All of these investigations were performed with ejaculated semen. To our best knowledge there are no data about the

potential of using coconut water as extender for canine epididymal sperm vitrification. Therefore, the current study was aimed to evaluate the potential of using coconut water extender supplemented with egg yolk to preserve canine epididymal sperm.

MATERIALS AND METHODS

Ethics statement

The epididymal samples used in this research were collected after orchietomy. All dog owners have signed a written informed consent before the castration that the research will be performed and results published.

Epididymal sample and sperm collection

Twenty testes and epididymides were obtained from ten healthy dogs, scheduled for elective spaying at the University Veterinary Hospital of the Faculty of Veterinary Medicine, Trakia University, Stara Zagora, Bulgaria (Fig. 1). The dogs were from different breeds and aged from 1 to 5 years. The testes together with the epididymides were placed in 50 mL plastic

tubes containing saline solution preheated at 37 °C and transported to the laboratory within an hour after orchietomy.

Cauda epididymis and ductus deferens of each testis was separated with a scalpel blade and placed in a dry Petri dish (Fig. 2). Sperm samples were collected using a float-up method (Simons et al., 2019) on a heating stage at 37 °C. Incisions on the epididymis and *ductus deferens* sparing the blood vessels were performed and immediate spontaneous leakage of semen was observed. Semen collected from both epididymides was combined into a single sample. Six mL coconut water extender (2 parts coconut water, 1 part distilled water and 1 part 5% anhydrous monosodium citrate solution) was immersed into the Petri dish with epididymal samples and was covered with a metal plate to protect sperm cells from light. After 45 min the medium with extracted spermatozoa was pipetted in a test tube and sperm parameters were evaluated.

Sperm evaluation

Sperm concentration and velocity parameters were assessed with the Motility and



Fig. 1. Testis with the epididymis after orchietomy.



Fig. 2. Spermatozoa retrieval by float up method.

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

Sperm morphology was evaluated with the analytical software module Morphology of Sperm Class Analyser (SCA) (Microptic, S.L., Barcelona, Spain). The samples were stained with SpermBlue (Microptic, Spain) for 2 minutes and analysed by SCA of a minimum of 50 optic fields.

Eosin-nigrosin staining was used for sperm viability assessment. At least 500 sperm cells were counted by light microscopy under oil immersion at 400 \times . Non-coloured (white) spermatozoa were con-

sidered vital and coloured ones (pink or red) were identified as non-vital.

Semen dilution, vitrification and warming procedures

After pre-freezing semen quality evaluation, each test tube containing previously described semen samples were centrifuged at 37 $^{\circ}\text{C}$ (2000 rpm, 10 min). The supernatant was discarded and the spermatozoa were resuspended in the coconut water extender with addition of 20% (v/v) egg yolk (CWE-EY) and 1% fructose until final sperm concentration of 100×10^6 spermatozoa/mL. After equilibration at 5 $^{\circ}\text{C}$ for 60 min, the samples were vitrified using micropipette by direct dropping of 30 μL sperm suspension from a 10-cm height on a stainless steel strainer, placed into liquid nitrogen (LN_2) (Fig. 3A). The solidified pellets were transferred into cryotubes (Fig. 3B) and stored in LN_2 for 7 days. Sperm pellets were devitrified by placing three of them in 0.5 mL of Cani-Plus AI (Minitüb, Germany) and warmed at 37 $^{\circ}\text{C}$ for 2 min, followed by immediate evaluation of the abovementioned parameters.

Statistical analysis

The results were processed by statistical program. The data are presented as mean \pm standard deviation (SD) and were analysed by repeated measures ANOVA and the Tukey's test Statistica v. 7.0 (Stat-Soft., Tulsa, USA). A value of $P < 0.05$ was considered significant.

RESULTS

The values of evaluated parameters and the effect of vitrification on canine epididymal semen using CWE+EY as cryoprotectant are presented in Table 1. The vitrification affected the quality of spermato-

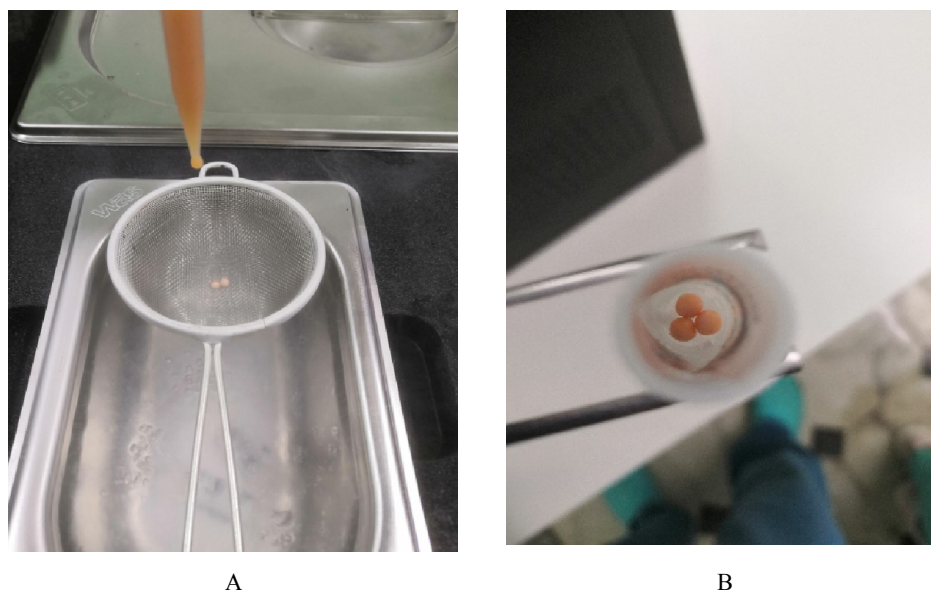


Fig. 3. Solidified canine epididymal sperm suspension: **A.** during the vitrification process into the strainer, placed into liquid nitrogen; **B.** sperm pellets after transfer into cryotube.

Table 1. Parameters of fresh and canine epididymal semen samples devitrified using coconut water extender. Data are expressed as mean \pm SD (n=10)

Parameter	Fresh semen	Vitrified semen
Viability, %	81.38 \pm 19.33	45.47 \pm 23.19 a
Total motility, %	63.86 \pm 13.45	35.19 \pm 12.58 a
Progressive motility, %	38.24 \pm 8.11	21.48 \pm 8.57 a
VCL, μ m/s	187.2 \pm 23.40	178.4 \pm 22.19
VSL, μ m/s	128.1 \pm 16.60	124.5 \pm 16.3
VAP, μ m/s	145.1 \pm 16.23	143.2 \pm 15.9
LIN, %	68.54 \pm 9.63	67.67 \pm 7.24
STR, %	89.83 \pm 4.39	86.27 \pm 4.68
ALH, μ m	5.19 \pm 0.88	4.91 \pm 0.89
BCF, Hz	24.9 \pm 3.69	23.1 \pm 3.87
Abnormal morphology, %	51.18 \pm 17.53	59.27 \pm 11.57

Devitrified semen parameters marked with a superscript differ from those of fresh semen at $P < 0.05$.

zoa. Fresh semen showed significantly superior ($P < 0.05$) viability, total and progressive motility than vitrified semen samples.

The kinematic parameters of fresh and devitrified canine spermatozoa did not

differ significantly ($P > 0.05$). The percentage of abnormal sperm cells after vitrification tended to increase, but a significant difference between fresh and devitrified semen was not determined.

DISCUSSION

Conventional canine semen freezing is largely used in increasing the reproductive capacity of stud dogs (Sanchez *et al.*, 2011), but unfortunately, sometimes results in large percent of sperm mortality and morphological abnormalities (Baiee *et al.*, 2020; Falah, 2020). Thus, many scientists working in the field of canine assisted reproductive biotechnologies focused their research on discovering new semen cryopreservation techniques. In order to increase the potential of dog semen preservation, studies have been also conducted to preserve epididymal sperm by conventional or ultra-rapid freezing (Mota Filho *et al.*, 2014; Simons *et al.*, 2019; Galarza *et al.*, 2021).

In the present study, the float-up method for epididymal sperm recovery was used. It was preferred due to its simplicity and previously reported satisfactory results in terms of viability, motility and morphology parameters despite the presence of some cellular debris and blood content (Simons *et al.*, 2019; Ali Hasan *et al.*, 2023). Sperm retrieval from the epididymis was successfully performed using coconut water extender and even outperformed previously reported results (Mota Filho *et al.*, 2014; Galarza *et al.*, 2021).

In recent years, vitrification by direct plunging of spermatozoa was successfully developed in dogs (Sánchez *et al.*, 2011; Kim *et al.*, 2012; Gharajelar, 2016; Caturla-Sánchez *et al.*, 2018, Pipan *et al.*, 2020; Galarza *et al.*, 2021; Antonov & Ivanova, 2022; 2023, Ivanova & Antonov, 2022; 2023). Being a novel method, further studies are required to improve the protocol (Ivanova & Antonov, 2022). Therefore, the current experiment was focused on evaluation of the effect of vitrification on the quality of canine epididymal spermatozoa. The choice of coconut water

extender was based on its availability, ease of use, natural, cheap ingredient, with high antioxidant properties. The results confirmed that it could be successfully used as a component of semen extender in ultra-rapid freezing, even for epididymal sperm cryopreservation. An evidence for this statement is that semen characteristics after devitrification in the present investigation were of even better quality than those from previously reported results (Galarza *et al.*, 2021).

Motility of the spermatozoa is one of the most important criteria for their fertile capacity (Partyka *et al.*, 2012) with rates of 30–50% in frozen semen considered as acceptable (Concanon & Battista, 1989). In our study demonstrated significant difference of the motility parameters between fresh and vitrified samples, but the average sperm motility percentage after devitrification was above the mentioned levels and thus can be successfully used for artificial insemination.

Sperm velocity parameters evaluation was also accepted in the scientific literature as useful for differentiation between fertile and infertile dogs (Domasławska *et al.*, 2013). Galarza *et al.* (2021) found reduction of kinetic characteristics of cryopreserved dog epididymal sperm, but our study detected no significant differences ($P>0.05$) between the velocity parameters of fresh and vitrified samples. This is in agreement with previously reported results of our team (Ivanova & Antonov, 2022; 2023; Antonov & Ivanova, 2022; 2023) and confirms the convenience of vitrification as an alternative to conventional semen freezing.

CONCLUSION

Coconut water extender supplemented with egg yolk as cryoprotectant could be

used in ultra-rapid freezing of canine epididymal semen for preservation of canine male gametes.

ACKNOWLEDGEMENTS

We would like to thank the owners of dogs, included in this study for their support.

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Paper received 09.12.2024; accepted for publication 12.05.2025

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