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EFFECT OF EQUILIBRATION PROCESS ON CANINE SPERMATOZOA AFTER VITRIFICATION USING COCONUT WATER EXTENDER WITH ADDITION OF SOY LECITHIN AND SUCROSE AS NONPERMEABLE CRYOPROTECTANTS

Efecto del proceso de equilibrio en espermatozoides caninos después de la vitrificación con dilutor a base de agua de coco con adición de lecitina de soya y sacarosa como crioprotectores no permeables

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ABSTRACT

The aim of the present study was to evaluate the effect of equilibration process on canine sperms after vitrification using coconut water extender with addition of soy lecithin and sucrose as nonpermeable cryoprotectants. Twelve ejaculates were collected separately by digital manipulation from 12 adult dogs. Only the second fraction of the ejaculate was used in this study, which was evaluated about volume, concetration, viability, total and progressive motility, kinetic parameters and morphology. After evaluation, semen was diluted with a coconut water extender (50% coconut water (v/v), 25% (v/v) distilled water and 25% (v/v) 5% anhydrous monosodium citrate solution) with addition of soy lecithin and fructose at 1% (v/v) and 0.25 M sucrose until final concentration of 100x10^6 spermatozoa/ml. Samples were divided into three aliquots and each of them was processed at different regimens: without equilibration, 5°C for 30 minutes and 5°C for 60 minutes and then vitrified by dropping 30 µl of sperm suspension directly into liquid nitrogen. Sperm pellets were devitrified at least one week later as three of them were dropped into 0.3 mL of CaniPlus AI (Minitüb, Germany), which was previously warmed in a water bath at 37°C for 2 minutes. Sperm concentration and 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 with addition of 1% soy lecithin and 0.25 M sucrose as cryoprotectants were used, presence of equilibration time of 60 minutes returned the best canine sperm quality results.

Keywords: Dog, Semen, Equilibration, Vitrification, Coconut water

RESUMEN

El objetivo del presente estudio fue evaluar el efecto del proceso de equilibrio en los espermatozoides caninos después de la vitrificación utilizando diluyente de agua de coco con adición de lecitina de soja y sacarosa como crioprotectores no permeables. Se recogieron doce eyaculados por separado mediante manipulación digital de 12 perros adultos. En este estudio se utilizó únicamente la segunda fracción del eyaculado, la cual se evaluó en cuanto a volumen, concentración, viabilidad, motilidad total y progresiva, parámetros cinéticos y morfología. Después de la evaluación, el semen se usó diluyente a base de agua de coco (50 % de agua de coco (v/v), 25 % (v/v) de agua destilada y 25 % (v/v) de solución de citrato monosódico anhidro al 5 %) con adición de soya. lecitina y fructosa al 1% (v/v) y sacarosa 0,25 M hasta concentración final de 100x10^6 espermatozoides/ml. Las muestras se dividieron en tres alícuotas y cada una de ellas se procesó a diferentes regímenes: sin equilibrar, a 5°C durante 30 minutos y a 5°C durante 60 minutos y luego se vitrificó dejando caer 30 µl de suspensión de esperma directamente en nitrógeno líquido. Los pellets de semen se desvitrificaron una semana después, colocando 03 pelets vitrificados en 0,3 ml de CaniPlus AI (Minitüb, Alemania), que se calentó previamente en un baño de agua a 37 °C durante 2 minutos. Los parámetros de motilidad y concentración de esperma se analizaron utilizando un sistema de análisis de esperma asistido por computadora (CASA), la viabilidad mediante la técnica de tinción supravital y los parámetros de morfología se evaluaron

en muestras de semen teñidas con Haemacolor®. En conclusión, nuestros resultados demuestran que cuando se usaron vitrificación y diluyente de agua de coco con la adición de lecitina de soya al 1 % y sacarosa 0,25 M como crioprotectores, la presencia de un tiempo de equilibrio de 60 minutos arrojó los mejores resultados de calidad del esperma canino.

Palabras clave: Perro; Semen; Equilibrio; vitrificación; Agua de coco

INTRODUCTION

The main method for assisted reproductive biotechnology in male organisms and increasing their reproductive capacity is cryopreservation of spermatozoa, which is useful for extending their lifespan and viability (Gharajelar et al., 2016). Until now, two types of sperm cryopreservation are developed-conventional and 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 high percentage of 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 (LN2) without ice crystal formation in fast and inexpensive manner (Isachenko, 2004; Amirat-Briand et al., 2010; Magnotti et al., 2018). It is widely used for embryo, oocyte or tissue storage (Isachenko et al., 2004; Rosato and Iaffaldano, 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 and 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 (Gharajelar et al., 2016).
Cell survival after the freezing procedure is provided by mandatory addition of cryoprotectors to semen extenders. Two groups of cryoprotectors are available. First are permeable cryoprotectants, e. g. glycerol, which prevents intracellular ice crystals formation, 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, lecithin or egg yolk) (England, 2000; Isachenko et al., 2004). They prevent water precipitation and formation of intracellular or extracellular ice crystals by greatly increasing viscosity of the suspension (Isachenko et al., 2011).

During the conventional cryopreservation process, canine semen is first diluted with a suitable extender, equilibrated with a cryoprotectant by cooling at 4-5°C for a relatively long time (1-3 hours) and stored frozen into liquid nitrogen (Okano et al., 2004). Equilibration process provides permitting sperm membrane changes or ionic flux which increases their resistance during the freezing 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.

In order to protect spermatozoa from harmful factors different semen extenders were discovered and developed (Bustani and Baiee, 2021). 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 and 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 and Bamunuarachchi et al., 2009) with high antioxidant properties (Mantena et al., 2019). It is suitable as a part of canine semen extender due to isotonic, not toxic, cheap, effective, and simple to be used (Cardoso et al., 2003). There are no data about the possible potential of using coconut water extender for dog sperm vitrification in the literature until now.

Therefore, our study demonstrated the effect of equilibration time on canine sperm quality after vitrification with coconut water extender and nonpermeable cryoprotectants.

MATERIALS AND METHODS

1. Experimental animals and management

Twelve ejaculates were collected by digital manipulation from 12 adult private-owned dogs (English buldog-4, French bulldog-2, English cocker spaniel-2, French Mastiff-2, English pointer-1, Cane corso-1), aged 3-7 years and weighted 13-63 kg, which were presented at the University Veterinary Hospital, Trakia university, Stara Zagora, Bulgaria. The dogs were previously found cryotolerant by conventional semen freezing method. 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.

2. Semen collection and evaluation

The collection was performed separately for the three fractions in sterile plastic tubes by the same operator. In order to provide stimulation semen collection was done in a presence of a teaser bitch. Immediately after the manipulation, 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, concentration, viability, motility parameters and morphology.

The volume was measured by a graduated pipette. 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 concentration (x10^6/mL) and 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).

3. Preparation of extenders and semen processing

After the initial evaluation, the sperm rich fraction was diluted with coconut water extender, which consisted of 50% (v/v) water from green coconut, 25% (v/v) distilled water and 25% (v/v) 5% anhydrous monosodium citrate solution with addition of soy lecithin and fructose at 1% (v/v) and 0.25 M sucrose until final concentration of 100x10^6 spermatozoa/ml. The extended samples were divided into three aliquots and each of them was processed at different regimens: without equilibration (E0), 5°C for 30 minutes (E30) and 5°C for 60 minutes (E60).

4. Vitrification and warming

Vitrification was based on the methodology previously described by Shah et al. (2019) for human sperm. Aliquots of 30 µl of the sperm suspension were directly dropped with a micropipette into styrofoam box filled with liquid nitrogen (LN2) and contained a stainless-steel strainer from a height of 10 cm. After solidification process the droplets settled down into the strainer (Figure 1), transferred into pre-cooled cryotubes and stored in LN2 for a week until devitrification for evaluation.

Figure 1. Solidified canine sperm suspension during the vitrification process into the strainer.

The devitrification process was performed as three of the sperm pellets were dropped into 0.3 mL of CaniPlus AI (Minitüb, Germany), which was previously warmed in a water bath at 37°C for 2 minutes. Sperm viability, motility parameters and morphology were evaluated as previously described.

5. 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 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 0.95±0.23 mL, concentration 968±179.05×10^6 spermatozoa/mL, viability 93.59±1.63, total motility 87.74±1.97%, progressive motility 52.11±2.89% and sperms with normal morphology 83.09± 5.59%. The influence of equilibration time on vitrified canine sperm using coconut water extender are presented in Table 1.

Table 1. Parameters after devitrification of canine semen samples (n = 12) 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 minutes; E60-5°C for 60 minutes V-vitrification; W-warming at 37°C.

	Fresh semen	E0/V/W	E30/V/W	E60/V/W
Viability, %	93.59±1.63a	4.54±0.86b	28.35±8.32c	40.35±4.54d
Total motility, %	87.74±1.97a	3.22±1.12b	22.11±9.56c	37.03±4.89d
Progressive motility, %	52.11±2.89a	6.74±0.73b	15.63±1.72c	39.68±2.18d
VCL, µm/s	190.2±26.4a	139.3±29.2b	143.2±21.7b	174.3±32.8a
VSL, µm/s	129.3±16.3a	83.4±23.2b	89.1±18.6b	117.9±15.2a
VAP, µm/s	146.1±15.2a	103.7±11.4b	108.6±9.3b	135.3±19.6a
LIN, %	68.53±8.35a	58.72±9.15b	63.12±7.64ab	64.24±7.90ab
STR, %	88.91±4.32a	78.87±11.43b	81.42±5.13b	84.17±5.89b
ALH, µm	5.12±0.74a	3.14±1.90b	4.23±0.89ab	4.85±0.73a
BCF, Hz	25.1±3.5a	12.2±9.3b	15.4±5.1b	22.7±3.2a
Normal morphology, %	83.09±5.59a	48.67±6.57b	59.45±6.78c	69.33±3.18d

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 and highest values of 40.35±4.54% viable sperms were detected when equilibration was done for 60 minutes.

Similar tendency was observed in total and progressive motility. The longer equilibration process resulted in the greatest significant values of 37.03±4.89% for total canine sperm motility ($p<0.05$). Progressive motility was also improved by the presence of equilibration and the highest levels of 39.68±2.18% were detected when it was for 60 minutes. Vitrification also caused changes in the sperm kinematic parameters, but the highest values obtained when using 60 minutes equilibration were even not significantly different ($p>0.05$) with the fresh semen samples before vitrification (Table 1).

Comparing the different protocols for equilibration, it was found that vitrification of spermatozoa in a coconut water extender with nonpermeable cryoprotectants resulted in a significantly lower ($p<0.05$) percentage of spermatozoa with normal morphology compared to fresh samples and highest result was found when equilibration was performed for 60 minutes.

DISCUSSION

Conventional slow freezing methods are usually employed in canine semen preservation for last decades (Sánchez et al., 2011). In last few years, vitrification was also developed in dogs, but further studies are needed to improve protocol for this ultra-rapid semen freezing. Thus, the aim of our experiment was to evaluate the effect of presence and duration of equilibration on the quality of vitrified dog spermatozoa. Moreover, we used a very cheap extender, based on natural buffer solution such as coconut water with addition of soy lecithin and sucrose as nonpermeable cryoprotectors and the quality of preserved canine semen after devitrification was similar to previously reported results (Sánchez et al., 2011, Kim et al., 2012, Gharajelar et al., 2016, Caturla-Sánchez et al., 2018, Pipan et al., 2020 and Galarza et al., 2021).

It was previously found that presence of equilibration at 5°C for 30 minutes with vitrification solution may result in negative effects on dog sperm motility and may be harmful for spermatozoa (Caturla-Sanchez et al. (2018). 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 for 1 hour, compared to lack of equilibration or 5°C for 30 minutes. Our results are in agreement with Hidalgo et al. (2018) that presence of equilibration had shown to be essential for sperm vitrification. According to Domoslawska et al. (2013), the most useful method for comparing semen from 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., 2002). 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 hour. In our opinion, presence of equilibration time is also 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 evidence that during vitrification canine sperms also need a period of adaptation in order to enhance their resistance to the effects of cryopreservation and the lack of equilibration may be harmful for dog spermatozoa, increasing morphological defects and decreasing their fertilizing capacity.

Our results demonstrated that canine spermatozoa vitrification in a coconut water extender containing combination of lecithin and sucrose could be successfully used 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. On the other side they 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 with addition of soy lecithin and sucrose were used, presence of equilibration time of 60 minutes provided the best canine sperm quality results after warming.

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AUTHOR CONTIBUTIONS
Conceptualization, A.A. and B.I.; Methodology, A.A.; Software, B.I.; Validation, B.I. and A.A.; Formal Analysis, A.A.; Investigation, B.I.; Resources, B.I.; Data Curation, B.I.; Writing – Original Draft Preparation, B.I.; Writing – Review&Editing, A.A.; Visualization, B.I.; Supervision, A.A.

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