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Comparative Analysis of One-step and Two-step Dilution on Quality of Frozen Semen in Kintamani Dogs

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ABSTRACT

Preservation of sperm by freezing allows breeding dogs that are separated over long distances. To increase the fertility of frozen and then thawed spermatozoa, they must be able to survive the process. The current study aimed to evaluate the sperm motility and DNA integrity of Kintamani dogs extended in extenders with one-step and two-step dilution techniques. Ejaculates collected from four dogs were used in the current study. The semen was divided into two equal parts and diluted with extenders using two different dilution techniques, namely One-step dilution in Tris egg yolk containing 7% glycerol, and a two-step dilution technique diluted in an initial 2:1 with an extender, containing 20% egg yolk without glycerol. The same volume of the second extender was added, including 14% glycerol. The sample was loaded into 0.25 ml straws, cooled to 4°C for 4 hours, equilibrated, and then plunged into the liquid nitrogen. The sperm motility was evaluated using Computer-Assisted Sperm Analysis (CASA), and DNA integrity was assessed using Acridine Orange (AO) stained. Results showed that the sperm motility of Kintamani dogs in extenders using two-step dilution was significantly higher compared to the one-step dilution technique. In addition, the obtained results indicated that two types of dilution steps in Kintamani dog semen were not detrimental to the sperm DNA integrity during the freezing process. In conclusion, extenders with two types of dilution techniques could maintain sperm motility above 30%, and no difference between one and two steps dilution was detected.

Keywords: Dilution techniques, DNA integrity, Egg yolk, Kintamani dog, Motility

INTRODUCTION

The Kintamani dog is a purebred dog native to Bali. The Kintamani dogs were designated as a world breed by the FCI (Federation Cynologique Internationale) with a provisional category on February 20, 2019. According to the FCI rules, the Kintamani dog will be designated as the definitive breed if the quality is maintained and the population increases in the next 10 years. If there is no development within that period, the Kintamani dog will be removed from the world list (FCI, 2022). Therefore, in the next decade, there must be an effort to increase the population and genetic quality of Kintamani dogs.

Artificial insemination (AI) is the reproductive technology that can be applied to increase the population and improve genetic quality. In artificial insemination, semen is collected from male dogs manually and inserted into the female dog's reproductive tract using a catheter and place so that the process of fusion of gametes can occur without natural mating (Dutta and Dutta, 2020). Artificial insemination is one of the assisted reproductive techniques mainly done on animals, but its application to dogs is not as advanced as in cattle. In recent decades, advances in the field of reproductive physiology in female dogs and advances in canine semen processing techniques have made this service available worldwide (Mason, 2018), which allows the union of male gamete cells with female gamete cells (Jain et al., 2015; Patel et al., 2017). Until now, there have been many successful reports of the occurrence of conceptus by artificial insemination. The occurrence of pregnancy in artificial insemination is an indication of the quality of the semen used (da Cunha et al., 2017). The basic technique and methods of artificial insemination are relatively easy and have promising prospects (Jain et al., 2015). However, frozen semen for Kintamani dogs is not available yet. In all animals, cryopreservation of semen can be used to retain and preserve fertility for genetic improvement, and to increase the breeding efficiency of important breeds (Patti et al., 2021). Therefore, it is necessary to make efforts to provide Kintamani dog frozen semen.

Kintamani dog semen has been successfully preserved using a diluent based on fresh young coconut water and Tris egg yolk diluent (Puja et al., 2018). Sperm freezing in Kintamani dog has been attempted previously by either diluting semen 1:3 in coconut water and Tris egg yolk containing 7% glycerol, the process of adding extenders to the semen vary.

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Some add extenders used one-step dilution, and others used a two-step solution. Although one-step dilution is considered optimal, no studies have evaluated the effects of the two-dilution step protocol. The advantages of one-step and two-step semen dilution techniques are still debated (Arif et al., 2020).

The one-step dilution method is more practical because the extender is mixed with semen at room temperature, packaged, equilibrated, and frozen. The two-step semen dilution method includes using the former extension without glycerol, followed by the addition of the second extender containing glycerol (García-Alvarez et al., 2010). In cattle, two-step dilution showed no significant difference compared to one-step dilution (Arif et al., 2020). However, one step dilution technique significantly improved the quality of post-thaw African wild dog semen (Van den Berghe et al., 2018). The different dilution techniques used are not yet scientifically compared. Therefore, it is necessary to evaluate the most appropriate and practical dilution techniques in an effort to improve the quality of frozen semen for Kintamani dogs. Therefore, this study was conducted to test the hypothesis that two-step dilution methods maintain the quality of the spermatozoa of Kintamani dogs after the thawing.

MATERIAL AND METHODS

The chemicals used were obtained from Sigma Chemical Company (St. Louis, MO, USA). Semen was collected from four adult male Kintamani dogs of proven fertility, aging from 2 to 3 years. The dogs used were clinically healthy. Dogs are kept separately in cages of the same size (2×4 m), fed a mixture of meat and commercial feed, and given drinking water *ad libitum*. All experimental animals were managed according to the guidelines of the Institutional Committee for the Care and Use of Experimental Animals at the Faculty of Veterinary Medicine, Udayana University, Bali, Indonesia.

Sample collection

To obtain sufficient semen, the ejaculates were pooled from four dogs. A total of four pooled samples were collected. Semen was collected from all dogs twice a week using the manual stimulation method (Puja et al., 2019). Only samples with a minimum of 70 motility, the normal morphology of spermatozoa of ≥ 70%, and the total sperm concentration ≥ 600 × 106 were considered for this study (Hermansson et al., 2021).

Extenders

The extender used in this research were prepared fresh. The compositions used are shown in Table 1. A Tris egg yolk extender was prepared and modified according to the procedure described by Hermansson et al. (2021).

Table 1. The diluent composition of frozen semen of Kintamani dogs

Material	Amount
Tris (g)	3.025
Fructose (g)	1.25
The citric acid (g)	1.7
Egg yolk (ml)	20
Streptomycin sulfate (mg/ml)	1
Penicillin (IU/ml)	1000
Glycerol (ml)	7
Distilled water (ml)	100
pH	6.5-6.7

Sample processing

After all the semen was collected, the ejaculate was centrifuged for 6 minutes at 700 g, and the supernatant was removed. The ejaculate sample was divided into two aliquots, and each aliquot was frozen using different dilution techniques (Korochkina et al.,2014).

One-step dilution

Semen was diluted 1:1 (one volume of semen diluted in one volume of extenders) with extenders containing 7% glycerol at room temperature. Then, the diluted semen was packed in 0.25 ml straws. Furthermore, it was equilibrated at a temperature of 4°C in a refrigerator for four hours, then the freezing process was carried out (Van den Berghe et al., 2017).

Two-step dilution

Semen was diluted within an initial 2:1 (two volumes of semen diluted in one volume of extenders containing lower glycerol concentration (3% v/v glycerol) dilution at room temperature. The dilute semen was then added (3:1

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dilution; 1 volume of the pre-cooled second extender containing 11% v/v glycerol so that the final concentration of glycerol in the diluent was 7%. Then, the diluted semen is packed in 0.25 ml straws, followed by equilibration at 4°C in a refrigerator for four hours, then the freezing process is carried out (Van den Berghe et al., 2017).

Freezing techniques

In the sperm freeze procedure, the straw was placed upper tray at 6 cm height above the liquid nitrogen surface in a 50 × 33 × 35 cm Styrofoam box for 15 minutes. After that, the frozen straws were immediately immersed in liquid nitrogen at -196°C and left in LN2 for 10 minutes. The frozen straw was transferred to a liquid nitrogen container for further storage (Puja et al., 2019).

Thawing procedure

The thawing process was carried out by placing the straws into a water bath at 38.5°C for 30 seconds. The content was immediately put into a pre-warmed Leja counting chamber (Domosławska et al., 2013).

Evaluation of frozen-thawed sperm

Sperm motility was assessed using the Hamilton-Thorne Sperm Analyzer IVOS II (IMV-Technologies, Aigle, France). After thawing, an aliquot of the sperm is loaded onto a prewarm Leja slide (Leja, Nieuw-Vennep, The Netherlands). This was placed on the stage of the HTM-IVOS, the temperature of which was stabilized at 37°C. Analysis was based on capturing 30 frames at 50 Hz and counting a minimum of 200 cells. DNA integrity was evaluated using the Acridine Orange (AO) stained (Mohammed et al., 2015). For each sample, 100 cells were counted and classified with intact and denatured DNA. Cells with intact DNA integrity show green, orange, or red color if denaturation.

Statistical analysis

The percentage of sperm motility and DNA integrity of the fresh semen were analyzed using descriptive analysis. To compare differences, data regarding the percentage of sperm motility and DNA integrity of frozen semen were analyzed with Paired sample t-tests using SPSS version 25. The difference between values was considered significant when the P value was less than 0.05 (Heat, 2000). All data are presented as mean values ± standard deviation.

RESULTS

The results of macroscopic observations showed that the color of ejaculate was cloudy or milky. In this study, the characteristics of semen produced from all male dogs were good. Table 2 shows the mean total sperm number, percentage of motility, percentage of spermatozoa life, and normal sperm morphology, which are generally normal so that all semen is suitable for processing. The results also indicated that individual variation led to no significant differences in semen individual characteristics.

The motility percentage reduced significantly after the equilibration process ($p < 0.05$) in both dilution techniques. However, both types of dilution techniques maintained motility above 60% after the equilibration process (Table 3). In this study, a significant effect of the equilibration process was seen on motility immediately after equilibration ($p < 0.05$). Immediately after equilibration, the equilibration process significantly affects the motility of spermatozoa ($p < 0.05$). The motility of fresh semen was 91.5%, and 72.4% in one-step dilution after equilibrium and 75% in two-step dilution. However, after equilibration, there was no significant difference in the effect between one-step dilution and two-step dilution on motility ($p > 0.05$). The percentage of spermatozoa having intact DNA integrity after the equilibration process was still above 95% (Table 3). The analysis showed that the dilution technique did not significantly affect the percentage of DNA integrity after the equilibration process ($p > 0.05$).

The result of motility and DNA integrity post-thawing are shown in Table 4. The percentage of motility in post-thawed was 40.40 in Two-step dilution and 37.60 in one-step dilution, respectively. The mean motility significantly differed in two-step dilution compared to one-step dilution ($p < 0.05$). The mean motility of spermatozoa was significantly different between two-step dilution to one-step dilution ($p < 0.05$). After post-thawing, the average percentage of spermatozoa DNA was still above 95% (Table 4). This indicates that the dilution steps are suitable for Kintamani dog semen. The results of the analysis showed that the type of dilution technique did not significantly affect the percentage of DNA integrity ($p > 0.05$).

Table 2. Fresh semen quality of Kintamani dogs

Parameter	Value	Range
The mean total sperm number ($x10^6$)	653.33	510-750
Motility (%)	91.50	89 -94
Live sperm (%)	94.45	90-95
Normal sperm morphology (%)	96.33	96-97

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Table 3. Motility and DNA integrity percentage of spermatozoa after equilibration using two different dilution techniques in studied dogs

Dilution techniques	Motility (Percentage)	DNA Integrity (Percentage)
One-step dilution	72.40	99.00
Two-step dilution	75.00	99.00

Table 4. Motility and DNA integrity percentage of spermatozoa after thawing using two different dilution techniques in Kintamani dogs

Dilution techniques	Motility (Percentage)	DNA integrity (Percentage)
One step dilution	37.60	98.80
Two step dilution	40.40	98.60

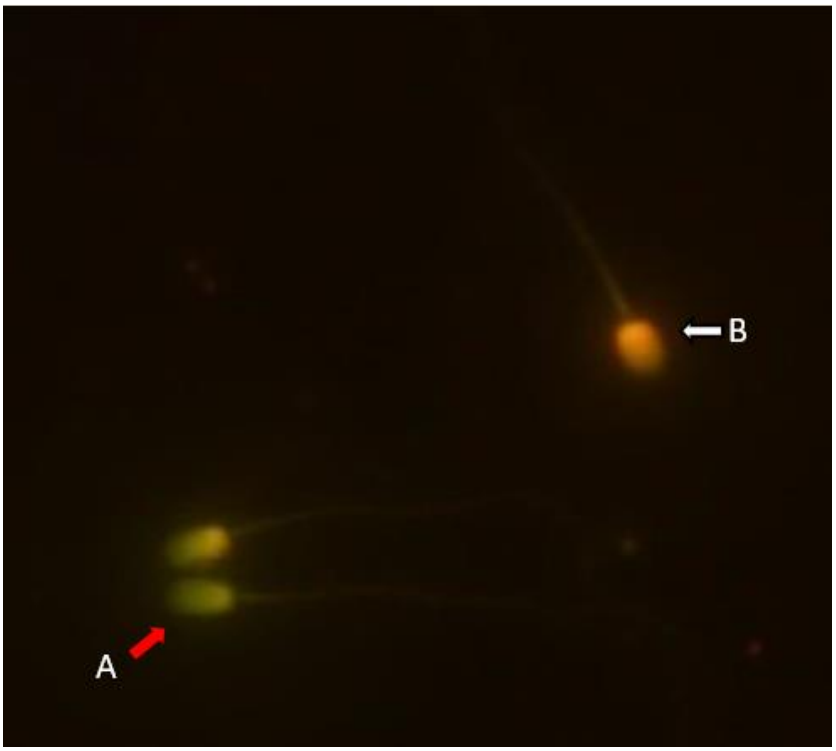

Figure 1. Sperm DNA denaturation as assessed by acridine orange test in Kintamani dog. Spermatozoa with intact DNA integrity were green (**A**: Red arrow) and denaturized were orange (**B**: white arrow)

DISCUSSION

In this study, the results indicated Kintamani dog semen could be successfully frozen with one-step or two steps dilution in Tris egg yolk extenders. Sperm motility decreased in both dilution techniques. Moreover, the DNA integrity of spermatozoa does not appear to be damaged by the freezing process. However, the extenders with one-step or two steps dilution provide sufficient post-thaw spermatozoa of Kintamani dogs for artificial insemination. This study showed that post-thawing spermatozoa motility in both dilution steps was still in the range of progressive motility that could still be used and accepted for artificial insemination in dogs. The minimal post-thawing sperm motility required for artificial insemination is around 30%. The motility between 30-65% was classified as normal spermatozoa (Hollinshead et al., 2017).

Most studies establish the ideal extenders for cryopreservation dog semen in canine reproduction. The extender was used in cattle and has been adapted for use in a dog (Martinez-Rodriguez et al., 2020). This procedure, commonly used for the production of beef bull semen straws, includes ingredients such as Tris, 7% glycerol, and 20% egg yolk (Khalil et al., 2018). Egg yolk is routinely used as the primary diluent for sperm cryopreservation in the domestic animal. Egg yolk contains lipoproteins and lecithin, which play a role in the protection and helps sperm cells in resisting against cold shock. Egg yolk provides excellent protection for semen, and can minimize the effect of cold shock that occurs during freezing and thawing (Corcini et al., 2016).

Sperm motility is one of the most important parameters in semen examination and has been the most widely used indicator for fertility in domestic dogs (Dorado et al., 2011). However, other sperm assessment, such as DNA integrity also is important because DNA integrity is associated with fertility potential (Park et al., 2018). DNA plays an important role in the function of spermatozoa that do not undergo a selection process in the female reproductive tract (Kumaresan et al., 2020). In addition, spermatozoa with broken DNA do not always show a decrease in other sperm quality parameters, but such breaks of DNA can interfere with embryonic development (Cho and Agrawal, 2018; Park et al.,

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2018). In this study, the motility of post-thawing spermatozoa was significantly different between one-step dilution and two dilution steps. The two-step dilution technique preserves the motility higher than the one-step dilution technique.

In this study, equilibration with cryoprotectants for 4 hours reduced sperm motility. According to Belala et al. (2016), the ideal equilibration time for cryopreservation of canine semen is 6 hours. Using SM extenders, the equilibration time was 3 hours (Abe et al., 2018), but based on this study, equilibration time is 4 hours in two-step dilution with a final glycerol concentration of 7%. In addition to motility, the percentage of DNA integrity is needed to determine the quality of spermatozoa. According to Agarwal and Said (2003), fertilization success is influenced by DNA integrity which is one of the parameters that must be assessed in determining the quality of spermatozoa. The AO test determines changes in DNA structure, such as DNA denaturation, which occurs in the cell nucleus. This staining technique was used to differentiate between cells with intact DNA (green staining) and cells with damaged DNA (orange). In the current study, DNA was not modified by different extenders and cryopreservation processes. DNA integrity was not significantly different in both post-thawing. Egg yolk with one-step or two-step dilutions could maintain the sperm DNA integrity of the Kintamani dog, which indicates that the type of dilution step used in this study is good for Kintamani dog semen. The results showed that the percentage of DNA integrity was not affected by the type of dilution technique used. Semen extenders can demonstrate the ability to minimize DNA fragmentation. In this study, completely denatured spermatozoa were found, which were indicated by the orange color of the sperm (Figure 1) under a fluorescence microscope as reported by Tejada et al. (1984). Considering the DNA integrity after thawing evaluated by the AO Stain and the results obtained in this study are a high percentage of sperm intact to those from Khalil et al. (2018), that observed 81% of sperm intact in frozen bull semen.

CONCLUSION

In conclusion, sperm motility was influenced by the type of dilution step technique. The motility was quite high in the two-step dilution technique. The extenders with this dilution technique could maintain motility above 30%. From the results of observations of DNA motility and integrity in both types of the dilution step technique with a final glycerol concentration of 7%, they were able to maintain motility and DNA integrity acceptable for artificial insemination during equilibration and freezing. It is, therefore, recommended to use two-step-dilution techniques for the production of frozen Kintamani dogs in Indonesia

DECLARATION

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Authors' contribution

I Nyoman Sulabda and I Ketut Puja designed the research, executed it, and wrote the manuscript. Anak Agung Gde Oka Dharmayudha assisted in collecting data and analysis. All authors read and approved the final version of the manuscript for publishing in the present journal.

Conflicts of interests

Authors have declared that no competing interests

Ethical consideration

All authors have checked the ethical issue such as plagiarism, consent to publish, misconduct, data fabrication and falsification, and redundancy.

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