

SHORT COMMUNICATION

Preservation of semen from Kintamani Bali dogs by freezing method

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ABSTRACT

Objective: To explore the effect of glycerol at different concentrations using different extenders on DNA fragmentation and motility of frozen-thawed Kintamani Bali dog spermatozoa.

Materials and Methods: Sample was collected from four mature Kintamani Bali dogs. Each ejaculate was prepared for cryopreservation with two different semen extenders; egg yolk Tris extender and coconut water-based extender. For each extender, three different glycerol concentrations were used; 4%, 6%, and 8%. Each of the six aliquots was loaded into 0.5 ml cryotube, placed on a styrofoam box 5 cm over liquid nitrogen for 10 min, and immersed in liquid nitrogen up to 8 min. Then, the frozen cryotubes were transferred into liquid nitrogen container. The cryotubes were thawed in a water bath at 38.5°C for 120 sec. After equilibration and thawing, each sample was assessed for motility parameters and for DNA fragmentation.

Results: The addition of 6% glycerol to extenders revealed the most effective addition of glycerol on motility and sperm DNA fragmentation after equilibrium and post-thawing.

Conclusion: It is concluded that both extenders with the addition of 6% glycerol are safe to be used as an extender in Kintamani Bali dog semen preservation, and DNA fragmentation of Kintamani Bali dog spermatozoa was not influenced by the freezing procedure.

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Introduction

The Kintamani Bali dog known as the gembrong dog is an emerging breed dog from Indonesia and is precisely a native of Sukawana, Kintamani, Bali, Indonesia. Characterized physically by the coat thick, wavy bristle, and long at withers, tail, and thigh, as well as friendly and gentle, the Kintamani Bali dog is very popular among the people in Indonesia, and efforts are being made to help it attain recognition by the Federation Cynologique Internationale [1].

To increase population, some breeders still use natural mating between dogs. Some owners choose natural mating with the same breeds from remote locations. However, transport of dogs in far location markedly causes stress on female dogs. To solve these problems, it is necessary to use artificial insemination technology. This objective can be achieved only through the availability of the frozen semen

with the quality standards. The benefits of using frozen semen to the breeding program are the semen can be stored for long-term and can be shipped to long distances [2]. The frozen semen can widespread dissemination of the superior stud dog minimizes the costs of keeping or transporting live animals [3].

Semen cryopreservation has been used as a tool in semen banks. Although cryopreservation has been developed and optimized over the past decades, frozen sperm storage for a long time brings many inconveniences [4] and affects sperm quality [5]. The process still decreases the biological properties of canine spermatozoa [6]. During cryopreservation, the decreasing temperature during cooling and the formation or intra- and extra-cellular ice during freezing can affect sperm cell and causing the death

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of some sperm and reduce the longevity of sperm in the female reproductive tract and has a significant impact on fertility [7]. Therefore, the pregnancy rate of current artificial insemination pregnancy with frozen-thawed dog semen is not completely satisfactory [8].

The freezing process that begins with dilution with the extender, chilling, freezing, and addition of cryoprotective agent, and thawing could decrease the fertilizing capability of sperm [9,10]. Among these processes, the addition of cryoprotective agent plays a crucial role in cryopreservation. To protect cryodamage against the damaging effects of freezing, most semen freezing protocols incorporate a permeating cryoprotectant, such as glycerol. Glycerol can penetrate the sperm cell and has positive intracellular and extracellular effects. In order to protect sperm from freeze/thaw damage, it is necessary to have at least 4% glycerol in the semen extender; thus, all semen extenders have at least 4% glycerol in their extenders.

In domestic dogs, sperm motility is only one of the many important attributes of a fertile spermatozoon. It is also a metric for the viability of semen samples [11]. However, other sperm quality parameters such as DNA fragmentation should be evaluated with equal importance. The reason for this evaluation is spermatozoa with damaged DNA can significantly impair embryo development and causes pregnancy loss. In order to make sperm banks with highly-fertile sperms, this study aimed to analyze whether different semen dilutions with different glycerol concentrations affect the motility and DNA fragmentation of frozen-thawed Kintamani Bali dog semen.

Materials and Methods

Ethical approval

The current study was done following the principles and guidelines of the Animal Ethics Committee, Faculty of Veterinary Medicine, Udayana University.

Semen collection

Semen was collected from four mature Kintamani Dogs by manual stimulation method [12] at Asubali Kennel, Gianyar, Bali. The semen was transferred to the laboratory within few minutes. Semen samples were examined for sperm cell concentration, the percentage of motility, and spermatozoa life. Semen samples with a volume >0.6 ml, concentration > 200 × 10⁶ spermatozoa/ml, and sperm motility >80% were used in the study. The semen was given a holding time for 10 min in a water bath at 38°C before dilution.

Semen processing

Tris-citric acid-egg yolks (TCY) contained 3.025 gm of Tris, 1.7 gm of citric acid, and 20 ml of egg yolk and coconut

water-based extender (CW) contained 2.4 gm Tris, 1.0 gm fructose, 1.3 gm citric acid, and 20 ml coconut water dissolved in 100 ml of distilled water were used as an extender. All extenders contained 1,000 IU penicillin and 1 mg streptomycin per milliliter and the pH 6.8. The semen samples were split into six parts. Three parts were prepared for cryopreservation with TCY and the other parts was prepared with coconut water-based extender. The semen samples were diluted into 1:2 ratio (one part of semen: two part of extender) at room temperature with glycerol at concentrations of 4%, 6%, and 8% separately and equilibrated at 4°C for 3 h. The evaluation was done for the effects of three different glycerol concentrations in TCY extender and CW-based extender on the motility and sperm DNA fragmentation on immediately after equilibration and day 1 after preservation

Freezing and thawing techniques

For the freezing process, the cryotube after equilibration was placed vertically on the plastic rack above the surface of liquid nitrogen vapor (-120°C) at 5 cm in styrofoam box 35 × 17 × 20 cm for 15 min. Immediately, the frozen straws were immersed in liquid nitrogen at -196°C up to 8 min. Then, the frozen cryotubes were transferred into liquid nitrogen container. Thawing of frozen cryotubes was done in a water bath at 38.5°C for 120 sec.

Statistical analyses

The mean and standard deviation (SD) of spermatozoa concentration, motility and alive sperm were measured by using descriptive statistics. Data regarding the motility of spermatozoa and DNA fragmentation were analyzed by analysis of variance and *t*-test using SPSS ver.17. The difference between values was considered significant when the *p* value was less than 0.05 [13].

Result

The results of the examination of the characteristics of fresh semen of the Kintamani dog was cloudy or milky in color. The average concentration, percentage of motility, and percentage of spermatozoa life were shown in Table 1. No significant differences were detected among them and the dog showed a homogenous population.

The results for after equilibration and post-thaw percent total motility (%) and sperm DNA fragmentation are presented in Table 2. The addition of 6% glycerol to extenders revealed the most effective addition of glycerol on motility and DNA fragmentation. After equilibration, the total motility was not different for the two extenders (*p* > 0.05). However, for post-thaw, using the TCY extender significantly resulted in greater sperm motility compared to using the CW extender. After thawing, a reduction in

motility was observed in both extenders, which showed significant differences between TCY extender and CW extender for motility ($p < 0.05$). The motility varied from 34.66% to 55.66% after freezing and thawing. The motility was significantly better in TCY than in CW groups. No difference was observed in the DNA damage in sperm using two extenders after equilibration and post-thawing. The DNA damage following staining with AO showed 1.50–1.88 (Fig. 1). This study demonstrated that both TCE and CW-based extenders produced a similar effect on sperm DNA fragmentation.

Discussion

Based on the characteristic of the sperm-rich fraction of the fresh semen, all ejaculates were within the physiologic range for dogs [14]. Sperm extenders used in this research are adequate medium to sustain the motility and integrity of sperm, contributing to the preservation of sperm energy, protect sperm from thermal shock during refrigeration, and prevent losses of membrane phospholipids during cryopreservation process. Egg yolk contained in TEY extender provides a function to protect from cold [15] and prevents losses of membrane phospholipids. Coconut water extenders contained essential constituents such as sugars, vitamins, minerals, and amino acids [16]. Sugar as a source of energy and essential amino acids play an important role in cell membrane fragmentation [17]. All contents in coconut water provide the function for strong antioxidant activities that protect sperm against oxidative damage [18].

Table 1. Means and standard deviations of the characteristics of fresh semen obtained from dogs ($n = 4$).

Semen Characteristics	Mean \pm SD	Range
Spermatozoa concentration ($\times 10^6$)	690 \pm 72.57	590–760
Motility (%)	90.75 \pm 2.21	89–94
Alive sperms (%)	94.25 \pm 1.50	90–95

Sperm motility is an important parameter in assessing the quality of spermatozoa, as it will affect the success of fertilization [19,20]. In this study, the results showed that after freezing, the sperm motility decreases significantly. It is similar to the report by Gunawan et al. [21], which reported that the chilling factor for sperm preservation decreases the motility of Kintamani Dog spermatozoa. Post-thaw sperm motility in TEY and CW extenders was above the acceptable range of progressive motility for artificial insemination. However, the post-thaw sperm motility revealed in TEY extender with 6% glycerol addition was the best. These values were acceptable for the use of frozen semen in TEY and CW extenders in artificial insemination. According to Concannon and Battista [22], for successful artificial insemination, the needed motility was approximately 30% and the post-thawing motility needed for pregnancy was 20%–30% [23].

It is well known that cryopreservation causes a decrease in sperm motility. The decrease in motility is associated

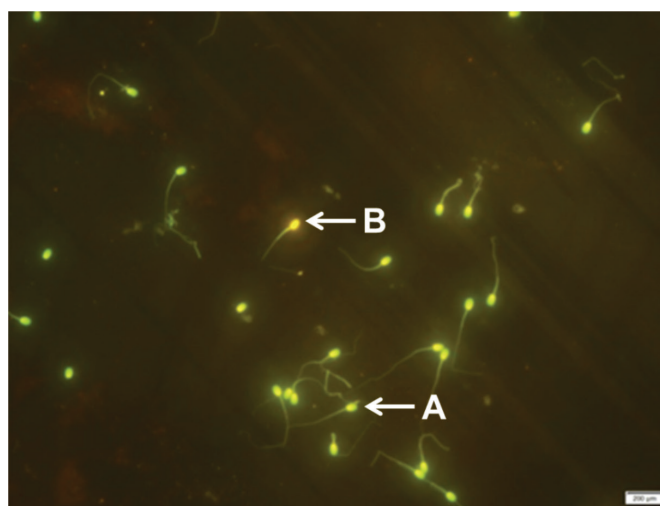


Figure 1. Photomicrographs of fluorescently stained Kintamani Dog sperm: the living sperm stained bright green (A) and the DNA fragmentation stained red (B).

Table 2. Mean of motile spermatozoa and DNA Fragmentation after equilibration and post-thaw.

Extenders	Glycerol concentration	Motility		DNA fragmentation	
		After equilibration	Post thaw	After equilibration	Post thaw
TCE	0.4	87.66 \pm 0.81	38.83 \pm 3.54	1.50 \pm 0.54	1.66 \pm 0.51
	0.6	88.50 \pm 1.22	55.66 \pm 2.58	1.50 \pm 0.54	1.66 \pm 0.81
	0.8	87.83 \pm 1.16	40.00 \pm 4.38	1.50 \pm 0.54	1.50 \pm 0.54
CW	0.4	87.16 \pm 1.47	36.00 \pm 2.28	1.50 \pm 0.54	1.50 \pm 0.54
	0.6	88.16 \pm 1.47	34.66 \pm 4.71	1.50 \pm 0.54	1.50 \pm 0.54
	0.8	85.50 \pm 1.37	34.66 \pm 1.75	1.66 \pm 0.81	1.83 \pm 1.16

with the overproduction of reactive oxygen species and reduction in the antioxidant capacity as manifested by a decrease in intracellular GSH content which induces damage in the spermatozoal membrane. Cryodamage induced by freezing and thawing can be decreased by adding the suitable cryoprotectant in the semen extender. Cryoprotectants reduce the physical and chemical stress exerted by the freezing process on spermatozoa [24]. Glycerol is the most used cryoprotectant agent in cryopreservation of semen [25].

The optimal concentration of glycerol in extender represents a balance between its toxic and protecting effects because high and low concentrations can also affect the spermatozoa [26]. Studies on semen cryopreservation using TEY and coconut water-based extenders in different glycerol concentration in different species have yielded a promising result [17,18]. In the dog, tris-yolk extender addition with 6% of glycerol was optimal for canine semen cryopreservation [27,28] and addition with 4% glycerol was the ideal concentration in tris yolk extenders [29]. According to Farstad [30], the concentration of glycerol has been varied between 2% and 10%. In this study, the best level to produce the highest motility was 6% glycerol in the extender.

Cryopreservation induces lipid peroxidation and increases the levels of metabolites called reactive oxygen species (ROS), which play a key role on sperm oxidative stress [2] and disrupt the balance between free radicals and antioxidant system of frozen-thawed spermatozoa [31]. Prolonged exposure of spermatozoa to ROS compromises plasma membrane fragmentation, causes a rapid loss of intracellular ATP and axonemal damage, decreases the sperm motility, and leads to the loss of intracellular enzymes and DNA fragmentation. DNA integrity assessment has a pivotal diagnostic and prognostic role in infertility [32]. DNA damage can be assessed indirectly by means of sperm chromatin integrity assay by acridine orange. This method distinguishes single-stranded DNA from double-stranded DNA based on the fluorescence emission of sperm. AO fluoresces green when it binds to normal DNA (double stranded) and red when it binds to denatured DNA (single-stranded) as an aggregate. This method has been widely used to assess DNA integrity of spermatozoa since its first description in 1980 [11]. In this research, the sperm DNA fragmentation of frozen-thawed semen was not affected by extenders and freezing procedure. The factors that affected the sperm DNA damage during cryopreservation are not known and various hypotheses have been suggested; this characteristic seems to be species-specific [33]. According to Lee and Cho [34], the DNA status after thawing depends on the presence of protamine 1 (P1) and 2 (P2). The sperm deficient in P2 resisted damage during freezing and thawing. The lack of P2 in Kintamani Dog

spermatozoa might explain the resilience of its DNA to the freezing/thawing procedure.

Conclusion

It is concluded that both extenders with the addition of 6% glycerol are safe to be used as an extender in Kintamani semen preservation. DNA fragmentation of Kintamani Dog spermatozoa was not affected by the freezing procedure.

Conflict of Interest

The authors declare that there is no conflict of interest with regards to the publication of this manuscript.

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Authors' contribution

I. Ketut Puja designed the study, analyzed and interpreted the data, and drafted the manuscript. Ni Made Sawitri and Nisa Maharani collected the data. Luh Gde Sri Surya Heryani and Anak Agung Gde Oka Dharmayudha also contributed to manuscript preparation. I. Wayan Nico Fajar Gunawan took part in preparing and critical checking of this manuscript.

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