

## Coconut Water Based Extender Effects on Motility, Viability, and DNA Integrity of Chilled Kintamani Dog Semen

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### ABSTRACT

This research was conducted to investigate coconut water based extenders as an alternative extender for chilled Kintamani dog semen. Semen were collected from five dogs using manual stimulation and evaluated microscopically. Sperm were stored at 5°C and divided into two aliquots which were extended with either coconut water based extender and sodium citrate extender in the ratio 1:2, 1:3 and 1:4. Sperm motility, viability and DNA integrity immediately and periodically (0,3 and 6 hour) were evaluated. Result showed that the effect of diluent on sperm motility and integrity of the Kintamani dog exerted a highly significant ( $p < 0.01$ ), whereas it had no effect on the percentage of dead sperm ( $p > 0.05$ ). These results indicate that coconut water extender give good results in maintaining the motility, viability and DNA integrity of Kintamani dog sperm. It is concluded that coconut water suitable for extender of Kintamani dog.

Keywords: kintamani dog, coconut water, viability of spermatozoa, DNA integrity

### INTRODUCTION

Kintamani dog is a native to the village of Sukawana, Kintamani District, Bangli Regency, Bali. This dog has relatively more beautiful appearance compared to other local dogs. Kintamani dogs are described as an intelligent, hardy and gently dog. The good physical and personal characteristics of the Kintamani dogs make them as a popular household pet in Bali (Puja, 2005).

Recently, to increase population, dog breeders and owners tended to mate between dogs from other locations. However, transporting the dogs for mating can cause a stress on the female dogs. To solve this problem, artificial insemination (AI) may overcome the limitation of animal transportation. AI is a way to improve the efficiency of reproduction and production of animals, including dogs. AI has proven to be a very effective reproductive technology that selectively increases genetic gain. The usage of chilled sperm in dogs was successfully reported

in canine AI (Tsutsui *et al.* 2003; Linde-Forsberg, 1995).

A phenomenon, cold shock, may occur during the semen chilling process. The phenomenon can reduce the spermatozoa motility and viability (Enciso *et al.*, 2006). To preserve viability of sperm cells before their being used, semen are stored. For storing the semen, they are generally extended and chilled. Added extender in the stored semen can stabilize sperm cell membranes. As a result, it can improve the preservation of spermatozoa (Ponglowhapan *et al.*, 2004).

Many extenders for chilled dog semen have been evaluated and the most common usage is citrated-egg yolk-glucose extender (Moss *et al.*, 2000). However, chemical extender usage has been reported causing toxic to the spermatozoa. A viable alternative extender is needed to replace the effect of chemicals. It seems that natural extender is essential to be an alternative. Coconut water seems to be suitable as a semen extender in canines due to isotonic, not toxic, cheap,

effective, and simple to be used (Cardoso *et al.*, 2004). In addition, tender coconut water was known very effective for sperm extender on dog (Cardoso *et al.*, 2003). The aim of this study was to evaluate the potential of tender coconut water in different ratio concentration as sperm extender in Kintamani dog semen.

## MATERIALS AND METHODS

### Dog Samples

Four male Kintamani dogs aged 1.5 -2.5 years were used in this study. The dogs were housed in outdoor kennel at Asubali Kennel, Gianyar Bali and fed twice a day with commercial food. Drinking water was given ad libitum.

### Semen Collection and Assessment

Semen was collected using manual manipulation. In this study, second fraction semen was used. Fraction with rich sperm was examined immediately after collection. Semen was evaluated for sperm motility, viability and DNA integrity. Sperm motility and viability were evaluated using light microscope. Sperm viability was evaluated by eosin negrosin stain, while DNA integrity was evaluated using acridine orange stain (Tejada *et al.*, 1984). Only samples with sperm motility  $\geq 80\%$  were used in this study.

### Semen Extenders

Semen sample was divided into two aliquots and each was diluted with different extenders. The first extender was citrate-glucose (Moss *et al.*, 2000), whereas, the second extender was based on coconut water (Cardoso *et al.*, 2003). The semen was diluted in extenders in the ratio 1:2, 1:3 and 1:4. The experimental unit was stored at 5<sup>o</sup> C, and the sperm motility, viability and DNA integrity were evaluated periodically (0, 3, and 6 hour).

### Statistical Analysis

The data of sperm motility, viability and DNA integrity were expressed as means  $\pm$  standard deviation and analyzed using SPSS 17.0 for windows. Analysis of variance was

applied to the data. When a significant difference among treatment was found, a Duncan post hoc test was utilized to compare the significance between treatments (Heath, 2000).

## RESULTS AND DISCUSSION.

The percentage of total spermatozoa motility at 5°C in the control group was range from 64.4 to 74.8%. However, in the tender coconut water extender, motility ranged from 73.6 to 81.0%. In storage times of 0, 3, and 6 hours, sperm motility gradually decreased ( $p < 0.01$ ) respectively in all extenders. At 6 hour storage, the average percentage of sperm motility was less than 70% in the citrat-egg yolk-glucose extenders, while the average percentage of sperm motility in tender coconut water extenders was still more than 70%.

Its recorded that the mean value of progressive motility of the spermatozoa was significantly influenced by the extender and concentration ratio. The extender of tender coconut water with concentration ratio 1:4 showed a high percentage motility up to 6 hours, while the citrat-egg yok-glucose extender showed not as good as the coconut water (Table1).

The results for sperm extender made of tender coconut water were better than those of the egg yolk – citrate – glucose. The tender coconut water extender enables good preservation of chilled semen. It has been reported that fresh coconut water is an effective extender for canine semen (Cardoso *et al.*, 2003). Additionally, coconut water extender is adequate for preservation of canine semen at 37°C for 180 minutes (Uchoa *et al.*, 2002). Tender coconut water is one known to be a source of electrolytes and other good ingredients such as: sugar, vitamins, magnesium, potassium, fibre, proteins, minerals, and antioxidants (Silva and Bamunarachchi, 2009). In this research, coconut water is considered to be an important buffer consisting of essential inorganic compounds that mantaining life of the spermatozoa. The coconut water extender

might stabilize physical chemical conditions during storage and thereby prolong the life of spermatozoa in storage. The longer time of the sperm storage can affect the sperm motility. The motility will decrease because of the lack of energy stuff availability used by spermatozoa. The storage time up to 6 hours showed the motility of spermatozoa in tender coconut water extender is still relatively high. This can potentially use for artificial

insemination diluent as it performs sperm motility > 60% (Johnston, 1991).

Average values of viability differ between groups ( $p < 0.01$ ). The tender coconut water extender provided significantly better sperm viability compared to citrat-egg yolk glucose extender. Viability of spermatozoa decreased along storage time in all treatments (Table 2). After 6 hours, sperm viability on tender coconut water extender was still higher than the other extender.

Tabel 1. Sperm Motility Percentage in Extenders with Different Concentration Ratio in 5° C

Extenders	Concentration Ratio (sperm extender)	Storage Time (hours)		
		0	3	6
Citrate-Egg Yolk – Glucose	1:2	74.8 ± 0.44	72.8 ± 0.44	68.2 ± 1.09
	1:3	71.6 ± 1.14	68.8 ± 1.09	67.0 ± 1.87
	1:4	71.2 ± 0.83	67.6 ± 1.67	64.4 ± 1.87
Tender Coconut Water	1:2	79.2 ± 0.83	76.8 ± 1.09	74.6 ± 1.14
	1:3	79.4 ± 2.19	76.6 ± 0.89	73.6 ± 0.89
	1:4	81.0 ± 3.16	78.2 ± 2.58	74.6 ± 2.70

Tabel 2. Viability percentage of spermatozoa in extender with difference concentration in 5° C

Extender	Concentration Ratio (sperm extender)	Storage Time (hours)		
		0	3	6
Citrate-Egg Yolk - Glucose	1:2	91.8 ± 0.83	88.4 ± 2.19	85.8 ± 1.30
	1:3	91.4 ± 1.14	86.8 ± 3.03	84.6 ± 0.89
	1:4	89.8 ± 4.14	87.2 ± 4.96	83.0 ± 3.39
Tender Coconut Water	1:2	92.4 ± 1.14	88.4 ± 2.07	84.4 ± 2.07
	1:3	92.8 ± 1.48	91.0 ± 1.41	87.4 ± 3.28
	1:4	91.4 ± 2.19	89.0 ± 2.82	86.0 ± 2.73

Table 3. DNA Integrity Percentage in Extender with Difference Concentration ratio in 5° C

Extender	Concentration Ratio (sperm extender)	Storage Time (hours)		
		0	3	6
Citrate-Egg Yolk - Glucose	1:2	92.8 ± 0.44	90.6 ± 1.34	88.4 ± 0.54
	1:3	91.6 ± 0.89	89.6 ± 1.51	85.6 ± 1.51
	1:4	91.4 ± 0.89	89.4 ± 0.89	85.2 ± 1.30
Tender Coconut Water	1:2	95.6 ± 3.20	94.0 ± 3.67	92.0 ± 4.30
	1:3	97.8 ± 1.30	95.6 ± 1.81	93.0 ± 2.12
	1:4	95.6 ± 1.94	93.4 ± 1.51	91.6 ± 2.19

The present research clearly demonstrate that coconut water based extender affords a medium to sustain the viability of Kintamani dog sperm storage at 5°C, because it could maintain the sperm viability until 6 hours storage and better than other extender. The

coconut water is considered having energy sources to support life of spermatozoa. Tender coconut water extender contains fructose and glucose that used by the spermatozoa for energy source. The reduction in viability observed in the present experiment during

storage at 5°C may due to some factors, one of them is reduced extracellular pH which can cause a gradual decline in cell metabolic activity.

Based on evaluation in this research, the DNA integrity of spermatozoa decreased along the storage time in all extenders. In citrate egg yolk glucose extender, the sperm DNA integrity at 0 hour storage found higher than 90%, while in 3 to 6 hours storage it was observed under 90% in average in all ratio. However, DNA integrity in tender coconut water gave better result in maintaining the Kintamani dog sperm DNA integrity as it demonstrates the integrity higher than 90% from 0 to 6 hours of the storage in all ratio (Table 3).

The staining technique differentiates sperm cells with intact DNA (green stain) and sperm cells with denaturated DNA (orange). Integrity of sperm DNA in tender coconut water extender showed higher percentage ( $92,2 \pm 2,88\%$ ) than egg yolk – citrate – glucose extender ( $86,4 \pm 1,76\%$ ). The DNA was altered by the type and concentration of extenders, ratio extenders, and time of storage (86.4% to 92.2% of intact DNA). This is not similar with previous researches on DNA integrity in other species, such as in horse around 4-14% (Morrell *et al.*, 2008) and in human around 6-37% (Erenpreiss *et al.*, 2004). In this research, total denaturated DNA wasn't clearly showed. In fluorescence microscope observation, the denaturated DNA sperm is generally indicated by red or orange color (Tejada *et al.*, 1984). In this research, the early denaturated spermatozoa was indicated by greenish yellow color in fluorescence microscope observation.

### CONCLUSION

Tender coconut water is potential to be an extender for Kintamani dog semen.

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