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Research Article

A Comparative Study on the Effects of Coconut Water Based Extenders on the Quality of Kintamani Dog Semen Preserved at 4°C

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Abstract | The objective of this study was to determined the effects of different extenders on chilled kintamani dog semen quality parameters. In this study, the impact of extenders on motility, viability and DNA integrity of kintamani dog semen were investigated. A total of 12-second fraction of ejaculates were collected from four kintamani dogs using manual stimulation. Sperms were diluted in each of the five extenders at room temperature and then cooled to 4^0 C. Samples were then evaluated every day until five days. Chilled semen samples were assessed for motility, viability and DNA integrity. Results showed that the progressive motility of the sperm cells was significantly higher in extender A and B compared to other extenders. The extender containing 20% egg yolk and 20 % tender coconut water preserved the motility of more than 60% of the spermatozoa up to the day 5 post-sampling. Percentage of live sperm decreased slightly from day 0 to day 5. There was no significant difference in live spermatozoa due to the extender after five day. The DNA was not unaltered by different of extender and storage refrigeration process. It concluded that kintamani dog semen qualities could be maintained for up five days when semen extended with coconut water-based extender with addition fructose and store at 4^0C.

Keywords | Kintamni dog, Chilled semen, Motility, Viability, DNA integrity

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INTRODUCTION

The Kintamani dog is a favorite companion dog in Indonesia. The kintamani dog known as *the gembrong dog* has been bred in a village of Sukawana in the district of Kintamani in Bali for centuries. The Kintamani dog is emerging breed dog from Bali Indonesia. The characteristic temperament of the Kintamani dog is friendly and gentle dog make it a popular pet for the people in Indonesia, and efforts are being made to help it attain recognition by the Federation Cynologique Internationale (Puja et al., 2005). To produce a quality offspring, some breeders still use natural mating between dogs from remote locations. The natural mating often leads to stress dogs because they have to travel a long distance. (Hori et al., 2014). To solve these problems, we are interested in using artificial insemination techniques. As part of breeding management, the use of artificial (AI) is the way to offers excellent potential in accelerating the genetic improvement of kintamani dog.

AI can be performed using fresh chilled semen. Fresh Chilled semen was reported has similar pregnancy rates to fresh semen if applied soon after collection. To keep the survival of sperm after collection is needed a semen extenders. The appropriate extender for the preservation would be to minimize damage and to maintain the viability of spermatozoa. The extenders commonly used for chilled dog semen are based on animal products, such as e-

Table 1: Composition of the extenders used in this study

Ingredients	Extender A	Extender B	Extender C	Extender D	Extender E
Glucose	-	-		0,18	0,18 g
Fructose	-	1,0 g	1,0 g		
Tris	3,025 g	2,4 g	2,4 g	3,025 g	3,025
Egg-yolk	20 ml	-	-	-	-
Citric acid	1,7 g	1,3 g	1,3 g	1,7 g	1,7
Coconut water	-	-	20 ml	20 ml	
Pasteurized coconut water		20 ml			20 ml
Penicilin	0,65mg/ml	0,65mg/ml	0,65mg/ml	0,65mg/ml	0,65mg/ml
Streptomysin	1mg/ml	1mg/ml	1mg/ml	1mg/ml	1mg/ml
Ultra pure water	until 100 ml	To 100	To 100	25%	25%
pH	6,6	6,6	6,6	6,6	6,6

gg yolk. Egg yolk the most common component used in canine semen extenders to protect spermatozoa from cold shock during chilled process (Mason, 2016). However, the presence of an avian influenza outbreak (Abe et al., 2008) and egg yolk affected by microbial contamination (Tarig et al., 2017) causes the desire to replace the egg yolk in extenders. Thus, it is necessary to develop extenders without use egg yolk. As an alternative to replace the egg yolk, coconut water to be especially suitable for chilled semen extender preparation in canine species since pH and ion compositions of coconut water similar to the plasma fluid. Coconut (*Cocos nucifera*) water also contains essential constituents such as sugar, vitamins, minerals, potassium, magnesium, fiber, and proteins (Silva and Bamunuarachchi, 2009) and has been reported to have antioxidant properties (Mantena et al., 2003).

Sugar is one of the main components in semen extenders. The most common sugar for canine semen extenders is fructose and glucose. Fructose and glucose addition to giving an energy substrate to spermatozoa and glucose and fructose are metabolized in separate pathways by freshly ejaculated dog sperm (Ponglowhapan et al., 2004). Spermatozoa metabolize both Glucose and fructose. However, fructose works better to preserve the viability of sperm.

In the interest of developing an efficient and natural extender, it is essential to study the effect of semen extenders containing unpasteurized coconut water, and pasteurized coconut water combined with different sugars on Motility, Viability, And DNA Integrity of Kintamani Dog Semen

MATERIALS AND METHODS

Animal

Four kintamani dogs between two and three years old, clinically healthy were used in this research. The dogs were kept in individual cages in 2x4 m and fed twice daily with commercial dry food and give free access to water.

Collection of Ejaculated Semen

The ejaculates were obtained by manual stimulation into sterile tubes according to methods described by Puja (Puja, 2011). The first and third fractions of the ejaculate were discarded. Only the second fraction of the ejaculates was collected for the experiments.

Evaluation of Fresh Semen

The semen quality of was assessed immediately after collection. For the dogs to be included in the research before use is checked for motility. Only semen has >80% motility used for this study. Semen is brought to the laboratory with a 4^0 C icebox. Sperm motility was evaluated using a light microscope (100x) according to the procedures of Johnston et al. (2001). The percentage of live spermatozoa were measured by eosin-nigrosin stain and evaluated microscopically (1000x) from 200 cells per slide. The DNA integrity assays for the quantification of DNA damage by AO test (Shamsi et al., 2011).

Sperm Dilution

All extenders were prepared in the laboratory using reagent chemicals purchased in Sigma. After evaluation, the sample was extended in unpasteurized coconut water (CW) and pasteurized coconut water (PCW) based extender. Coconut water was pasteurized by boiling for 15 minutes at 65°C. The coconut extender was combined with Two different of sugar. An extender containing Tris Egg yolk citric acid and no sugar supplementation served as a control. The composition of Five extenders was tested in this research reported in Table 1. Sperms were diluted in each of the five extenders at room temperature and then cooled to 4^0 C in a refrigerator. 100 µl of each extender studied were placed in separate test tubes maintained at a room, then 200 µl of semen was added to each of the tubes and then cooled to 4^0 C in a refrigerator.

Table 2: Mean of motile spermatozoa in chilled canine semen from day 0 to day 5 in five extenders.

Extender	Motilities (%)					
	0 day	1 day	2 days	3 days	4 days	5 days
A	87,83±2,13	85,00± 2,68	81,83± 1,94	74,06± 1,78	68,66±3,26	65,10± 2,40
B	87,50± 2,16	84,66± 2,16	81,50 ±1,87	73,50± 1,37	68,50±1,04	62,83±0,75
C	87,16± 1,94	83,00± 1,78	78,66± 2,42	67,50±1,37	63,33±1,50	57,83±1,16
D	87,00± 1,78	84,16± 1,94	78,83± 1,47	64,33±1,63	60,50±1,41	54,16±1,47
E	86,83± 1,47	84,00± 1,78	79,50± 1,37	63,66±0,81	61,50±1,37	57,00±2,09

Table 3: Percentage of live spermatozoa preserved at +4˚C in five the extender.

Extender	LIVE spermatozoa (%)					
	0 day	1 day	2 days	3 days	4 days	5 days
A	92,16± 2,75	90,50± 3,08	88,66±3,61	87,16±3,60	85,50±4,03	84,16±3,76
B	91,83±2,63	90,83±2,31	89,16±3,31	87,00±3,34	85,16±4,07	83,5±3,93
C	92,00±2,75	90,00±2,96	87,66±3,55	86,00±2,75	84,00±3,34	82,16±4,52
D	92,16±2,99	89,83±2,78	87,83±3,12	86,16±3,06	84,16±3,60	82,33±3,38
E	92,16±2,99	90,50±2,33	88,16±2,78	86,16±3,06	84,83±3,12	83,33±3,14

Table 4: Percentage of DNA integrity preserved at +4˚C in five extender.

Extender	DNA Integrity (%)					
	0 day	1 day	2 days	3 days	4 days	5 days
A	96.83± 1.16	96,83±1.16	96.66±1.21	96.50±1.04	96.00±0.89	95.00±1.22
B	97.16±0.98	96.50±1.22	96.33±1.36	96.14±1.16	96.00±1.26	95.83±1.17
C	97.16±0.75	96.66±0.81	95.83±1.16	95.83±1.16	95.66±1.21	95.50±1.22
D	97.16±0.75	96.66±0.81	96.16±0.98	95.83±1.16	95.83±1.16	95.66±1.12
E	97.00±0.63	96.33±1.03	96.16±0.98	95.83±0.75	95.83±0.75	95.50±0.83

Statistical Analysis

The effect of extenders on motility, viability and DNA integrity were performed by ANOVA (Heath, 2000). Data gathered in this experiment were statistically analyzed using SPSS version 23.0.

RESULT

Evaluation of Fresh Ejaculate Semen

In fresh ejaculate semen, the average volume of the second (sperm-rich) fraction in a kintamni dog was 1.2 ± 0.18 ml with a sperm concentration of 643.33 x 10^6/ml. The average percentage of life was 93.25%, and the DNA integrity was 98.00. Spermatozoa motility was 91.00.

Evaluation of Sperm Motility after Chilled

In this research, the percentage of motility, live and DNA integrity were compared in five different extenders on the day of the sampling and daily until the five days after sampling. Mean Sperm motility after treatment is shown in Table 2. The progressive motility of the sperm cells was significantly higher than the in extender A and B compared to other extenders ($p<0.01$). Semen quality based on motility after five days seen the same result in extender A and B ($p>0.05$). The extender containing 20% egg yolk and 20 % pasteurized coconut water and an addition of 1.0 g fructose preserved the motility of more than 60% of the spermatozoa up to the 5- day post-sampling. The type of coconut water and sugar affected motility. The percentage of motility decreased significantly from day 1 until day 5 in all treatments.

Evaluation of Live Sperm after Chilled

The mean lives sperm percentage in five different extenders on the day of the sampling and daily until the five day after sampling recorded was above 80% with the range from 82,16 to 84,16%. Mean of live Sperm after treatment is shown in Table 3. Percentage of live sperm decreased slightly from day 0 to day 5. However, The mean of live sperm in did not decrease significantly from Day 0 to Day 1 (($p<0,01$). In the analysis of the percentage of live sperm showed that the semen samples in five different did not differ ($P > 0.05$).

Assesment of DNA Integrity of Sperm after Chilled

This is used to assess damage to the cellular DNA. Sperm DNA integrity after treatment could be seen in Table 4.

The DNA integrity was unaltered by the chilled process. The percentages of sperm with DNA integrity did not differ ($P > 0.05$) among the extender. The result of five different extenders was preserved the DNA integrity of more than 95% However the DNA integrity decreased slightly with the storage time.

DISCUSSION

The characteristics of the fresh semen it is shown that the total number of spermatozoa, the percentage of motility and DNA integrity were within the physiologic range for dogs (Johnston et al., 2001).

In this study, the extenders used in this study were provided an adequate medium to sustain the kintamani dog sperm quality. The pasteurized coconut water extenders addition with fructose were efficient conserving up to the 5-day post-sampling. The mean motility of sperm in pasteurized coconut water was significantly different than unpasteurized. The obtained results indicate that that pasteurized the biochemical characteristic unchanged during boiled and eradicate the microbiological load (Adubofuor et al., 2016) This extender has preserved the motility of more than 60%. In this result demonstrated that coconut water-based extender with addition fructose seen to the ability to maintain the sperm motility, viability and DNA integrity same than the egg yolk citrate extenders (control).

It was observed that chilling was the most suitable method of semen storage and chilling still survive approximately 4-5 days of collection (England and Ponzio, 1996). This result indicated that coconut water-based extenders good effect for the preservation of kintamani dog semen for a short-time (5 days).The survival of sperm observed in the present study could be linked to essential constituents such as sugar, minerals and amino acids, and ion in coconut water (Vigliar et al., 2006) and antioxidant activities (Silva and Bamunuarachchi, 2009).

The supplementation of different type of sugar in coconut water-based extender improves quality of motility. In a recent study, coconut water-based extender with addition fructose has same effectiveness in preserving the motility sperm at 4°C as an egg-yolk citrate extender. The type of sugar significantly affected motility during storage (Yildiz et al., 2000). The role of fructose as in extenders is known to increase the osmotic potential of cells and protect the membrane from chilling-induced injury and fructose in coconut water extender could be used by spermatozoa for energy resources. (Ponglowhapan et al., 2004).

Analysis percentage of live spermatozoa showed no significant differences between extenders and control extender. The mean of live sperm is higher than the mean of motile spermatozoa (Bearden and Fuquay, 1997) because the sperm that lives is not necessarily motile, but some spermatozoa are not motile sometimes still alive (Campbell et al., 2003). The presence of antioxidants, sugar, vitamins, electrolytes and amino acids and essential inorganic compounds in the coconut extenders (Yong et al., 2009) then the effects of cold shock can be minimized so that the spermatozoa deaths can be prevented. The Extender used in this study is considered to be an important buffer and nontoxic that determine the success of sperm storage (Cardoso et al., 2005).

Besides motility and viability of spermatozoa, the DNA integrity is required to the assessment of the quality of spermatozoa (Kim et al., 2010). DNA integrity as one of the parameters determining male fertility (Tejada et al., 1984; Agarwal and Said, 2003; Chohan et al., 2006). Tests for DNA integrity are most promising to asses the sperm reproductive potential (Shamsi et al., 2011). The Acridine Orange (AO) test is used to assess the DNA integrity. This staining technique differentiates between cells with intact DNA; AO fluoresced green whereas AO associated with denatured DNA fluoresced orange or red (Hasegan et al., 2012). The DNA was not unaltered by different of extender and storage refrigeration process. This conclusion is supported by the study of Prinosilova et al. (2011) who suggested that chilled and cryopreservation did not cause any significant changes in DNA integrity. According to the presented results during refrigeration, very little denaturation is seen (Bencharif et al., 2013). Different results were found by Urbano et al. (2017) evaluating the DNA integrity in cooled dog sperm using the SCD. It seems that the extenders and storage temperature of 4 °C can protect sperm DNA integrity of semen.

CONCLUSION

The addition of fructose in coconut water-based extender able to maintain motility, viability, and spermatozoa DNA integrity of kintamani dog during refrigeration process. This result indicated that coconut water-based extenders good effect for the preservation of kintamani dog semen for a short-time (5 days).

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CONFLICT OF INTERESTS

The authors declare that they have no conflict of interests.

AUTHORS CONTRIBUTION

All of the authors have read and approved the manuscript.

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