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Spermatozoa Quality of Kintamani Dogs in Coconut Water-Egg Yolk Diluent with Addition of Moringa Leaves and Carrot Extract

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ABSTRACT

The success of artificial insemination in dogs is significantly influenced by the quality of the semen used. Furthermore, during storage at 5°C, the semen is attacked by free radicals, which adversely affect its quality. Moringa leaves, and carrot extracts are natural ingredients with high antioxidant content, expected to overcome free radical attacks and oxidative stress experienced by sperm during storage. Therefore, this study aims were to examine the effect of adding Moringa leaf extract or carrot juice in coconut water-egg yolk diluent on the sperm quality of Kintamani dogs. The method used a completely random design. Three treatment groups exist for each diluent. The base diluent consists of young coconut water-egg yolk (D1), the base diluent plus moringa leaves extract (D2), and the base diluent plus carrot juice (D3). The life spermatozoa were observed in eight sections, from 0 (control) to 84 hours with 12 hourly observations. Each treatment group was examined for spermatozoa progressive motility, viability, abnormalities, and intact plasma membrane. The results showed that D2 maintained semen quality significantly better ($P<0.05$) than D1 and D3. However, D3 was better than D1 ($P<0.05$). It was concluded that the addition of moringa leaves extract in coconut water-egg yolk diluent maintained the best quality of spermatozoa for 60 hours, with motility, viability, abnormality, and intact plasma membrane of 56.67±19.98, 60.83±18.63, 11.83±2, 64, and 55.83±17.45%, respectively.

Key words: Kintamani Dog, Storage Time of Semen, Moringa Leaves Extract, Carrot Juice.

INTRODUCTION

Kintamani dog is Indonesian germplasm, a breed of local mountain dog group that lives around Sukawana Village, Kintamani District, Bangli Regency, Bali (Sawitri et al. 2021). Puja et al. (2005) stated that it came from a local Balinese species with changes in genetic diversity. This dog has a dashing and beautiful appearance; hence, it is admired worldwide. The breeding is conducted conventionally by naturally mating female dogs with males. This method poses a risk of injury and stress on males and females and is less practical (Sawitri et al. 2021). A solution to this problem is using artificial insemination (AI) technology, which is expected to increase the population and quickly improve genetic quality. The essential aspect of implementing this method is maintaining the quality of the semen, hence, it remains good for a certain period. The use of semen is efficient and produces a high pregnancy rate with large litter size. Therefore, it should be diluted and stored at a temperature of 5°C to maintain optimal quality (Payan-Carreira et al. 2011).

Semen is susceptible to cold shock during storage and is also sensitive to the attack of reactive oxygen species (ROS) (Pintus and Ros-Santaella 2021), which causes lipid peroxidation in cell membranes, leading to membrane integrity loss, increased permeability, enzyme inactivation, damage to DNA structures, and cell death (Dutta et al. 2019). Antioxidants are molecules that can suppress ROS and prevent damage to spermatozoa, hence it is necessary to add them to the semen diluent. Moringa leaves extract (*Moringa oleifera* L.) contains phytochemical compounds such as alkaloids, flavonoids, saponins, triterpenoids/steroids and tannins. It has strong antioxidants, which can protect the body against the bad effects of free radicals (Vergara-Jimenez et al. 2017; Nizioł-Łukaszewska et al. 2020). Antioxidants from moringa leaves can neutralize free radicals, thereby preventing oxidative damage to most biomolecules and providing significant protection (Sreelatha and Padma 2010).

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Sokunbi et al. (2015) stated that this extract could maintain motility, morphology and integrity of bovine semen membranes for more than 72 hours when stored at a cold temperature of 6ºC. Furthermore, Carrots (Daucus carota L.) contain vitamins and minerals, antioxidants, beta carotene, alpha-carotene, phytochemical, glutathione, calcium, potassium, and vitamins A, B1, B2, C and E. This fruit's antioxidant compounds also bind free radicals (Rachmayanti et al. 2021).

Dilution increases the volume of sperm, making it feasible to inseminate more females in a single ejaculation artificially. A good diluent is cheap, simple, practical, and has a high preservation power (Vasicek et al. 2015). It should also provide nutrients, allow the progressive movement of sperm, be non-toxic, act as a buffer, and protect spermatozoa from cold shock (Bustani and Baiee 2021). The young coconut water diluent with egg yolk contains a nutritional source for spermatozoa metabolism and isotonic properties to semen. It acts as a buffer, protects spermatozoa in the cooling process, and is not toxic (Rochmi and Sofyan 2019; Puja et al. 2018). Furthermore, the optimal ratio of young coconut water and egg yolk is 80:20% (Apriliana et al. 2021; Priharyanthi et al. 2021). Fafo et al. (2016) and Priharyanthi et al. (2021) reported the addition of moringa leaf extract at a concentration of 15% as an antioxidant to semen diluent. This extract can maintain the quality of semen during the cold storage process. According to Ndeta et al. (2015) and Apriliana et al. (2021), adding carrot juice with a concentration of 1% to diluent used as an antioxidant could preserve the quality of sperm during storage.

MATERIALS AND METHODS

Ethical statement

This research was approved by the ethical commission of The Faculty of Veterinary Medicine at Udayana University with No. B/164/UN14.2.9/ PT.01.04/2022.

Semen Collection

Two adult dogs aged 1.5 and 2 years were used in this study. Semen was collected using a manual massage method with gloved hands. The bulbus glandis was massaged until a partial erection was attained and continued to rub the penis; when the dog raised its hind legs, pushing the penis 180° backwards. The semen was ejaculated in 3 fractions, including prostatic fluid, sperm fraction, and prostatic fluid. Furthermore, the outpour was collected in a glass beaker, but only the second fraction was accommodated (Romagnoli 2002). During the ejaculation process in dogs, three fractions of semen are ejaculated. The first fraction was clear, the second fraction which was rich in sperm was whitish in color and the third fraction was clear again. We only accommodate the second faction, the third faction is ignored.

Making Moringa Leaves Extract

Approximately 1kg of young, green moringa leaves was prepared without stems, aerated for 16 hours, and then crushed. It was macerated for 24 hours in a 70% ethanol (1.00893 MERCK) solution until completely submerged. Using Whatman 42 paper, the macerated material was filtered till the filtrate was produced. The filtrate was put into a vacuum rotary evaporator at a temperature of 60ºC and 35rpm for 1 hour to obtain a paste-shaped extract. According to Sokunbi et al. (2015), this extracting process includes weighing 0.5g of moringa leaves paste and adding 50mL of aqua bidest (B3-1704 Intermed) into an Erlenmeyer flask, then homogenized with a magnetic stirrer for 15min at 400rpm. Furthermore, it was poured into a test tube and centrifuged for 2x30min at 1,500rpm. Finally, the sediment was removed, and the supernatant was collected as extract preparation.

Making Carrot Juice

Healthy, fresh, and clean carrots are selected peeled and wash until clean, then they are cut into pieces and process with a juicer. The process results were then filtered twice with Whatman paper measuring 125 mm, and the carrot juice was poured into a measuring cup (Ennouri et al. 2015).

Manufacturing of Diluent and Semen Diluent

The diluent of young coconut water with egg yolks was prepared by adding 80mL of young coconut water to 20mL of chicken egg yolk and homogenized (Apriliana et al. 2021; Priharyanthi et al. 2021), then 5% moringa leaves extract preparations was added (Fafo et al. 2016; Priharyanthi et al. 2021). Furthermore, antibiotic penicillin 1000IU/mL (CAS 69-57-8 Sigma) and streptomycin 1mg/mL (CAS 3810-74-0 Sigma) diluents were mixed and then homogenized.

The diluent was also prepared by adding 80mL of young coconut water to 20mL of chicken egg yolk, then homogenized (Apriliana et al. 2021; Priharyanthi et al. 2021) and 1% of carrot juice preparation (Ndeta et al. 2015; Apriliana et al. 2021). Furthermore, antibiotic penicillin 1000IU/mL (CAS 69-57-8 Sigma) and streptomycin 1mg/mL (CAS 3810-74-0 Sigma) diluents were mixed and then homogenized. The semen was diluted with a final concentration of $100x10^6$cells/mL (Romagnoli 2002; Khye et al. 2021).

Semen Storage

All diluted semen was stored at 4-5ºC in the refrigerator until examination. Evaluation of semen quality was carried out every 12 hours, namely at 0, 12, 24, 36, 48, 60, 72, and 80 hours.

Spermatozoa Quality Evaluation

The quality of Kintamani dog semen was evaluated macroscopically and microscopically. For the fresh semen, macroscopic examination includes volume, pH, consistency/thickness, and odor. Meanwhile, the microscopic examinations observed are concentration, motility, viability, abnormalities, and intact plasma membrane (Bustani and Baiee 2021). While, for the diluted semen, the evaluation only used microscopic examination included motility, viability, abnormalities, and intact plasma membrane. Furthermore, the proportion of motile spermatozoa is the percentage that moves progressively (move forward). The motility was evaluated subjectively in eight fields of view with a 400x magnification light microscope. Finally, the number ranged between 0 and 100% with a 5% scale.

According to Kondracki et al. (2017), spermatozoa viability and abnormalities were examined using eosin-nigrosine staining. This was prepared by mixing 6.7g/L Eosin Y (1.15935.0025 MERCK) and 9g/L Nigrosine (1.15924.0025 MERCK) in 9g/L sodium chloride (1.06404.0500 MERCK). Afterwards, 50µL of semen with 5µL of eosin-nigrosine was mixed and then homogenized. The smear preparations were made after 30 seconds and dried by aerating, after which approximately 200 spermatozoa were observed using a microscope with 400x magnification. The dead spermatozoa appear red, while those alive are not stained/transparent. To measuring the spermatozoa abnormalities, a microscope with 400x magnification was used to observe up to 200 spermatozoa. Those with abnormal shapes in the head, body and tail were calculated as the percentage showing abnormalities.

The percentage of the intact plasma membrane was evaluated by the hypoosmotic swelling (HOS) test method (Ramu and Jeyendran 2013). The solution consists of 0.73g sodium citrate (1.06448.0500 MERCK) and 1.35g fructose (1.05323 MERCK) dissolved in distilled water to a volume of 100mL. A total of 200mL of hypoosmotic solution was added and mixed with 20mL of semen until homogeneous, then incubated at 37°C for 45 minutes. Thin smear preparations were made on a glass object evaluated using a light microscope with a magnification of 400 times. Spermatozoa with intact plasma membranes are characterized by coiled or bubbled tails, while those that are damaged are represented by straight tails (Zampini et al. 2020).

Data Analysis

Data on motility, viability, abnormalities and intact plasma membrane of spermatozoa were analyzed using analysis of variance (ANOVA). This is continued with Duncan's test with a 95% confidence interval when there is a significant difference between treatments. Furthermore, the analysis was conducted using the SPSS for Windows version 25 program.

RESULTS AND DISCUSSION

Characteristics of Kintamani Dog Fresh Semen

Table 1 shows that the semen collected from the two Kintamani dogs were combined, homogenized, and examined macroscopically and microscopically.

The volume of collected semen for each dog was 1.8mL. A total of 1.2mL of ejaculate was accommodated only in the second practice, which was rich in spermatozoa. Romagnoli (2002) obtained dog semen volume in the second practice from the range of 0.1-3mL (average 1.17mL) depending on the size of the testes; Karger et al. 2016 got 1.20mL (range 0.8-1.5mL). Furthermore, this is identical to that of the Kintamani's dog semen. The spermatozoa concentration examination in this study was 380x10^{6}cells/mL. This is almost the same as in previous studies, where it ranges from 417.3±170.4x10^{6}cells/mL (Martínez-Barbitta and Salinas 2022) but higher than what Domosławska et al. (2013) found was 205.32±132.16x10^{6}cells/mL. The progressive motility examination of Kintamani dog semen showed 87%. Previous studies by Rijsselaere et al. (2011), Domosławska et al. (2013), Jarosz et al. (2016), and Karger et al. (2016) showed 80, 85.31±8.8, 70.6±10.2, and 83%, respectively. Meanwhile, from the viability of the Kintamani dog's semen with eosin-nigrosine staining, the live sperm were transparent, and the dead were stained red, as shown in Fig 1. The results indicate 92%, while other studies reported 90% (Rijsselaere et al. 2011). The abnormal semen examination of Kintamani dogs yielded 7% abnormal forms, as shown in Fig. 2, 3, and 4, while other studies reported 10% (Rijsselaere et al. 2011) and 9-18% (Karger et al. 2016). Furthermore, the analysis of the semen on intact plasma membrane was 89%, while other studies had 91% (87-96) (Karger et al. 2016). While, the sperm with a good intact plasma membrane is shown in Fig. 5.

The Kintamani dog semen quality check concluded that the sample is good quality and is suitable for further processing. According to Silva et al. (2005), good quality dog semen has a volume of 0.6mL, concentration of >200x10^{6}cells/mL, and progressive motility of 80%.

Table 1: Quality of Kintamani dog semen

Semen Characteristics	Total
Volume (mL)	3mL (two dogs 1.8 and 1.2)
Concentration (10^6/mL)	380
Progressive Motile (%)	87
Viability (%)	92
Abnormality (%)	7
Intact Plasma Membrane (%)	89

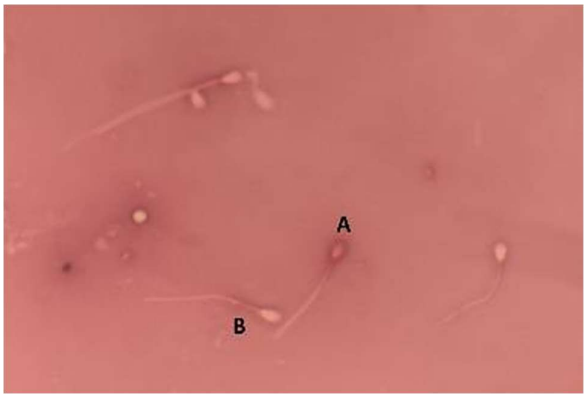

Fig. 1: Spermatozoa viability (400x), died sperm (A/colored red), live sperm (B/transparent).

Fig. 2: Abnormal sperm. (400x). Double tail (A). Young spermatozoa (B).

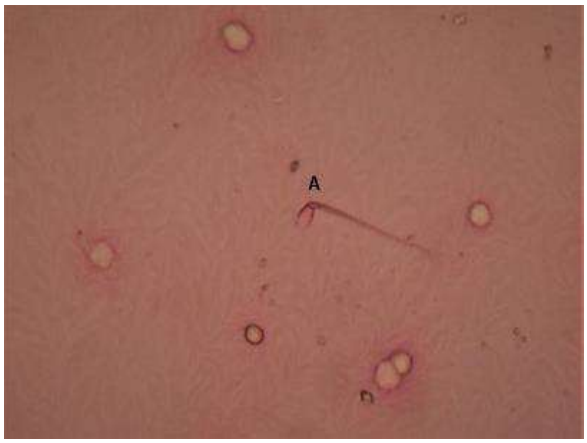

Fig. 3: Abnormal sperm. (400x). Crooked neck (A).

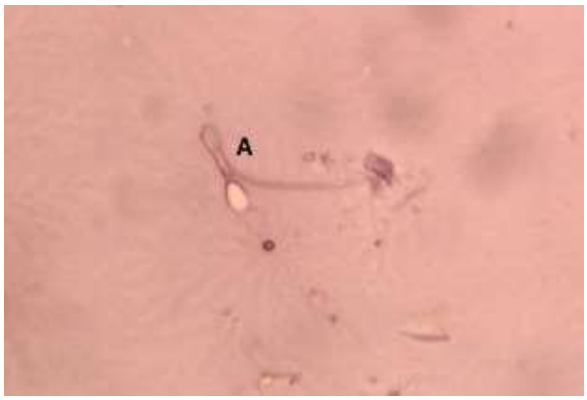

Fig. 4: Sperm abnormal. (400x). Tail folded (A).

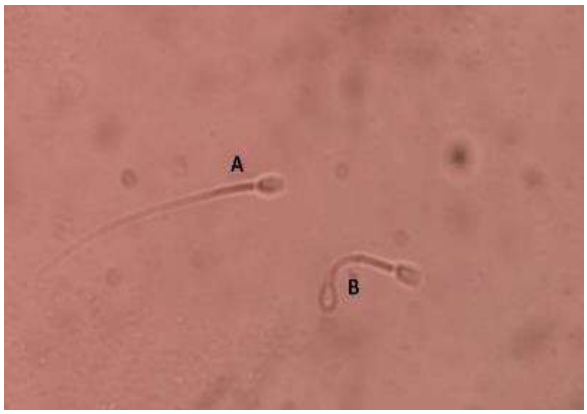

Fig. 5: Magnification (400x). Incomplete plasma membrane (A). Intact plasma membrane (B).

Effect of Diluent on Spermatozoa Quality

Table 2 shows the effect of diluent on the quality of semen stored at 5ºC. The results showed that the diluent has a significant impact (P<0.05) on the quality of spermatozoa. Adding moringa leaves extract to it maintained progressive motility, viability, and intact plasma membrane significantly longer (P<0.05). Furthermore, spermatozoa abnormalities were significantly less (P<0.05) when compared to the addition of carrot juice and controls. Carrot juice was able to maintain progressive motility, viability, and intact plasma membrane, which was significantly longer (P<0.05) and considerably less abnormality (P<0.05) than the control.

Table 2: Effect of diluent on spermatozoa quality of Kintamani dogs

Parameters	Diluent		
	AKK	AKK+EK1%	AKK+SW15%
Motility	50.50±30.93a	70.81±21.93b	68.19±24.17c
Viability	54.25±29.24a	75.69±21.99b	69.62±25.39c
Abnormality	12.56±4.98a	9.44±1.50b	10.38±2.24c
Intact Plasma Membrane	49.25±29.33a	70.44±21.32b	65.87±24.61c

Alphabets that are different in a row indicate significant differences (P<0.05). AKK=egg yolk-coconut water thinner; AKK+EK15%=egg yolk coconut water thinner + moringa leaves extract 15% and AKK+SW1%=egg yolk coconut water + carrot juice 1%.

During the storage process of spermatozoa at a temperature of 5ºC, the metabolic activity remains active, which physiologically produces reactive oxygen compounds. Physiologically oxygen plays an essential role in the metabolic process by producing energy in the mitochondria through redox reactions, which oxidize molecules to produce Adenosine Tri Phosphate (ATP) as an energy source. During this process, oxygen has several derivatives of free radicals or reactive oxygen species (ROS) (Juan et al. 2021; Martemucci et al. 2022). Free radicals are very dangerous for the survival of spermatozoa because they have very reactive properties to gain electrons. They will attack and collect electrons from unsaturated fatty acids that make up the phospholipids of cell plasma membranes. An autocatalytic reaction (chain reaction) can ultimately damage all phospholipids of the spermatozoa plasma membrane when it is not prevented (Prihantoko et al. 2020). During semen collecting, dilution, and storage, lipid peroxidation occurs in the plasma membrane, which produces free radicals (Medica et al. 2021; Rizkallah et al. 2022).

During the sperm preservation process, there will be a process of free radical formation. The process, among others, can go through two processes. First, free radicals are secondary products of metabolic activity in mitochondria in an effort to produce energy through the NADPH oxidase 5 (NOX5) pathway (Pandey and Fulton 2011). Second, immature and abnormal spermatozoa. The formation of free radicals is mediated by a cytosolic enzyme called glucose-6-phosphate dehydrogenase (G6PD). Its formation through two pathways, namely the nicotinamide adenine dinucleotide phosphate (NADPH) pathway which is located in the sperm plasma membrane; and the NADPH-dependent oxidase-reductase pathway that occurs in mitochondria via the respiratory mechanism (Frederiks and Vreeling-Sindelárová 2001; Sanocka and Kurpisz 2004). The presence of leukocytes in semen plasma includes polymorphonuclear leukocytes (PMN). Semen contains 50-60% leukocytes and 40-50% macrophages which can produce free radicals in the inflammatory process (Sabeti et al. 2016). Free radical formation in spermatozoa with abnormal morphology was positively correlated with high number of PMN (Sabeti et al. 2016) and ROS (Kobayashi and Suda 2012).

Moringa leaves and carrot are classified as natural antioxidants. The ethanol extract of moringa leaves

contains alkaloids, flavonoids, saponins, triterpenoids/ steroids and tannins (Putra et al. 2016). While carrot juice contains beta carotene, alpha-carotene, phytochemical, glutathione, calcium, potassium. Both also contain vitamins A, B1, B2, C and E. According to Gan et al. (2010) the content of phenolic compounds and other antioxidants found in Moringa leaves and carrots have –OH groups and double bonds (>C=C<) which are able to bind to free radicals.

Moringa leaves extract was added to the semen diluent in several studies. Priharyanthi (2021) added moringa leaves extract to egg yolk-coconut water diluent to dilute pork semen and Rizal et al. (2021) added to the egg yolk lactose diluent for goat semen. Adding this extract with an optimum concentration in the diluent maintained the quality of spermatozoa during storage than the control. This is due to the contain high antioxidant level of moringa leaves extract, which can protect the body against free radicals (Vergara-Jimenez et al. 2017; Nizioł-Łukaszewska et al. 2020). Plavonoids combat free radicals by scavenging against superoxide radicals (Chun et al. 2003), peroxyl radicals (Nakao et al. 1998; Akhlaghi and Bandy 2009), and peroxynitrite radicals (Pollard et al. 2006). Alkaloids are effective in tackling free radicals by performing scavengers against hydroxyl radicals (Gurudeeban et al. 2015). Saponins are effective against free radicals by scavenging the radicals, -diphenyl-β-picrylhydrazyl (DPPH), and 2,20 -azino-bis(3-ethylbenzothiazoline-6-sulfonic acid (ABTS) and also H2O2 (Khan et al. 2022) Triterpenes, -amyrin, oleanolic acid and ursolic acid combat free radicals by scavenging against nitric oxide (NO•), superoxide (•O2-) and lipid peroxides (LOO•) radicals (Santiago et al. 2014). These can neutralize free radicals, thereby preventing oxidative damage to most biomolecules and providing significant protection (Sreelatha and Padma 2010).

According to Thomassen et al. (2006), the feasibility of semen used for artificial insemination in dogs should have progressive motility of 50% with abnormal morphology < 20%. Furthermore, adding moringa leaves extract to egg yolk-coconut water diluent stored at 5ºC is suitable for artificial insemination with the average progressive motility, viability, abnormality, and intact plasma membrane of 70.81±21.93, 75.69±21.99, 9.44±1.50, and 70.44±21.32%, respectively.

The addition of carrot extract into semen diluent was previously conducted. Hardiyanti and Kurniawan (2020) added carrot extract to egg yolk coconut water diluent to dilute free-range chicken semen, Apriliana et al. (2021) added carrot extract to duck egg yolk coconut water to dilute pork semen, and Barek et al. (2020) added carrot juice to egg yolk citrate diluent to dilute Bligon goat semen. The addition of this extract to diluent at the right concentration maintained the quality of the semen during the storage process compared to the control. Carrot juice contains essential substances needed by cells, including carbohydrates used by spermatozoa as energy substrates, vitamin C and β-carotene as antioxidant compounds, as well as various minerals to maintain semen quality during storage (Parera et al. 2009). In this study, the addition of this extract to the purifier of egg yolk coconut water resulted in high semen quality, including motility, viability, abnormality, and intact plasma membrane of 61.19±24.08%, 69.62±25.31, 10.38±2.25%, and 65.88±24.62 respectively. This is suitable for artificial insemination in dogs because, according to Thomassen et al. (2006), the feasibility of semen used for AI should have progressive motility of 50% with abnormal morphology <20%.

Effect of Storage Time on Spermatozoa Quality

Table 3 and Fig. 6, 7, 8, and 9 show the advantages of adding moringa and carrot extracts in the semen dilution in slowing down spermatozoa damage.

The results showed that adding moringa and carrot extract had a significant effect (P<0.05) on the quality of spermatozoa during the storage time. The addition of these compounds can reduce the damage to spermatozoa. The results showed that the diluted semen was suitable for artificial insemination for 60 hours. Furthermore, the quality obtained include motility, viability, abnormalities, and intact plasma membranes of 56.67±19.98, 60.83±18.63, 11.83±2.64, and 55.83±17.45%, respectively. According to Thomassen et al. (2006), the feasibility of semen used for artificial insemination in dogs has progressive motility of 50% with abnormal morphology <20%.

According to the storage time, there is a decrease in the semen quality of the storage process due to the aging process of spermatozoa during the storage process (Beirão et al. 2019). The food supply in the diluent will be depleted and eventually exhaust the food sources (Rochmi and Sofyan 2019; Tvrdá et al. 2020). The ability of antioxidants in the diluent to bind free radicals is decreasing. Therefore, ROS can damage cell plasma membranes, resulting in reduced motility, viability, intact plasma membranes and increased abnormalities (Bebas et al. 2016). Furthermore, there is a decline in the pH of the diluent, which can reduce the quality of spermatozoa (Zhou et al. 2015; Mishra et al. 2018).

Table 3: Effect of long storage time on spermatozoa quality of Kintamani dogs

Long storage time at 5ºC (hours)	Observations			
	Progressive Motility (%)	Viability (%)	Abnormalities (%)	Plasma Membrane Intact (%)
0	86.50 ±0.55a	92.33±0.82a	7.67±0.52a	88.00±0.63a
12	83.67±2.50b	90.17±0.75b	8.33±0.52b	86.17±0.75b
24	81.00±4.29c	82.50±5.89c	8.83±0.41c	78.83±6.62c
36	75.83±8.84d	75.17±12.38d	9.50±0.55d	68.33±12.45d
48	67.67±13.34e	67.67±17.85e	10.12±0.75de	62.00±16.75e
60	56.67±19.98f	60.83±18.63f	11.83±2.64ef	55.83±17.45f
72	43.50±21.86g	48.33±18.75g	14.17±3.87ef	43.67±19.21g
84	10.50±8.67h	15.17±6.62h	15.83±3.46g	12.00±7.51h

Different alphabets in a column indicate significant difference (P<0.05).

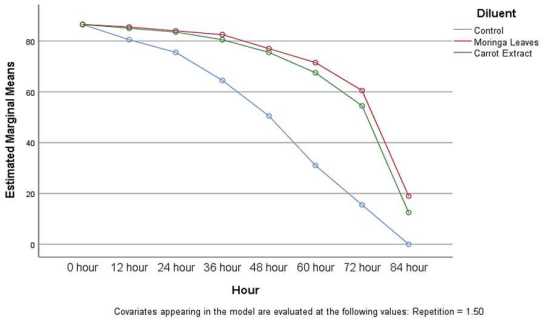

Fig. 6: Graph of effect of diluent and long storage time on spermatozoa motility (%).

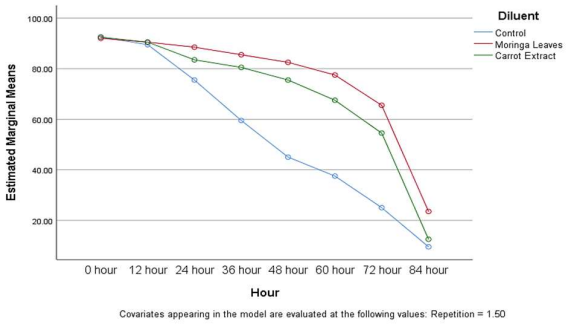

Fig. 7: Graph of effect of diluent and storage time on spermatozoa viability (%).

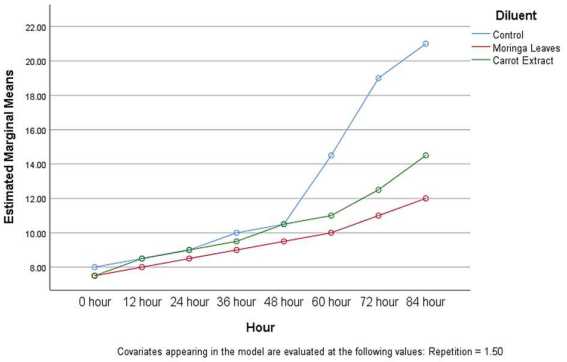

Fig. 8: Graph of effect of diluent and long storage time on spermatozoa abnormalities.

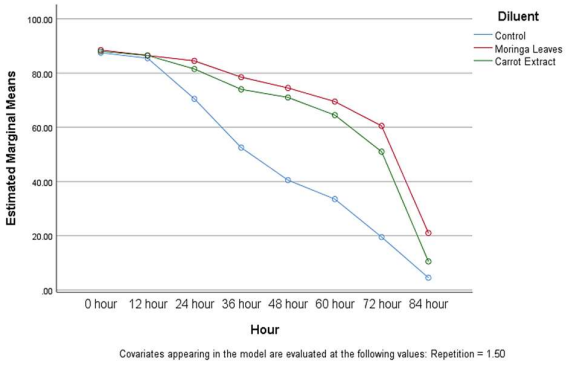

Fig. 9: Graph of effect of diluent and storage time on intact plasma membranes (%) of spermatozoa.

Conclusion

It was concluded that adding moringa leaves extract and carrot juice in coconut water-egg yolk diluent maintained the sperm quality of Kintamani dogs stored at 5^{o}C for 60 hours. However, adding moringa leaves extract was the best for maintain the quality of Kintamani dog sperm.

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Author's contribution

WB: Designed research project, collected samples, performed laboratory work and wrote the manuscript. IWG: collected samples and performed the laboratory work. KKA: performed the laboratory work, analyzed the data and wrote the manuscript. All authors approved final version of the manuscript.

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