

QUALITY OF CHILLED CANINE SEMEN IN TRIS-EGG YOLK EXTENDER SUPPLEMENTED WITH SERICIN

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ABSTRACT

The objective of this research was to evaluate the quality of chilled canine semen in Tris-egg yolk (TEY) extenders containing different concentrations of sericin. Semen were collected from four dogs by massage method. Canine semen was collected using sterile urine pots and evaluated. Sperm-rich fractions were pooled and divided into four equal aliquots, which were then diluted with TEY extenders supplemented with different concentrations of sericin (0%, 0.1%, 0.25%, and 0.5%). The diluted semen aliquots were preserved at 4° C in sterile centrifuge tubes and were then evaluated for spermatozoa motility, viability, plasma membrane integrity, and acrosome integrity every 12 hours up to 72 h. The TEY extenders supplemented with 0.25% and 0.5% sericin resulted in higher spermatozoa motility and viability at 72 h compared to other TEY extenders (P<0.05). The integrity of plasma membrane and acrosome of spermatozoa showed no significant differences among the group's extenders at 72 h. In conclusion, sericin in concentration of 0.25% and 0.5% were able to prevent the motility and viability of canine spermatozoa after storage for 72 h.

Key words: canine, chilled semen, sericin, spermatozoa, sperm-rich fraction

ABSTRAK

Penelitian ini bertujuan mengevaluasi kualitas semen cair anjing pada pengencer Tris kuning telur (TEY) yang disuplementasi sericin dengan konsentrasi berbeda. Semen dikoleksi dari empat anjing menggunakan metode masase. Semen anjing dikoleksi menggunakan urine pots dan dievaluasi. Sperm-rich fractions digabungkan dan dibagi menjadi empat bagian kemudian dimasukkan ke dalam pengencer TEY yang disuplementasi sericin dengan konsentrasi berbeda (0%; 0,1%; 0,25%; dan 0,5%). Semen tersebut kemudian dipreservasi pada suhu 4° C dan kemudian dievaluasi untuk motilitas, viabilitas, integritas membran plasma, dan integritas akrosom setiap 12 jam sampai 72 jam penyimpanan. Pengencer TEY yang disuplementasi sericin 0,25% dan 0,5% menunjukkan hasil tertinggi pada motilitas dan viabilitas pada jam ke-72 jika dibandingkan dengan pengencer lainnya. Integritas membran plasma dan akrosom menunjukkan tidak adanya perbedaan yang nyata pada semua perlakuan pada jam ke-72. Disimpulkan bahwa sericin pada konsentrasi 0,25% dan 0,5% dapat mempertahankan motilitas dan viabilitas spermatozoa anjing sampai penyimpanan 72 jam.

Kata kunci: anjing, semen cair, sericin, spermatozoa, sperm-rich fraction

INTRODUCTION

Artificial insemination is one of the assisted reproductive technologies that is getting more popular among dog breeders and veterinarians. Artificial insemination can be done using fresh semen, chilled semen, and frozen semen. However, chilled semen is commonly used in canine artificial insemination as chilled semen has higher spermatozoa motility and viability compared to frozen semen (Rota *et al.*, 1995). Furthermore, whelping rate in dogs inseminated with chilled semen is higher than that of frozen semen (Linde-Forsberg and Forsberg, 1989). One of the potential benefits of artificial insemination in dogs is that the semen of a proper stud dog can be made worldwide available through such technology (Payan-Carreira *et al.*, 2011). The death of spermatozoa during preservation is not only caused by cold shock but also reactive oxygen species (ROS). Reactive oxygen species can induce lipid peroxidation which leads to loss of membrane, deoxyribonucleic acid (DNA) damage, decreased spermatozoa motility and reduced fertilizing capacity of spermatozoa (Lucio *et al.*, 2016).

Reactive oxygen species are free radicals derived from oxygen which are produced by spermatozoa and leucocytes (Griveau and Le Lannou, 1997). Spermatozoa are very susceptible to oxidative stress and lipid peroxidation due to their high content of polyunsaturated

fatty acid in plasma membrane (Henkel, 2005). Reactive oxygen species is essential for spermatozoa capacitation and acrosome reaction but at low concentration (Saleh and Agarwal, 2002). Spermatozoa has three enzymatic systems that work in synergy to protect them from the damage caused by ROS, which are superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase/reductase system (GPx/GR) (Griveau and Le Lannou, 1997). However, spermatozoa lack of antioxidant enzymes to overcome lipid peroxidation caused by O₂⁻ and H₂O₂ because of cytoplasmic scarcity (Alvarez and Storey, 1989; Storey, 1997). Addition of antioxidant into semen extender is usually done to reduce the effect of oxidative stress during preservation in order to improve chilled semen quality (Michael *et al.*, 2009).

Sericin is one of the antioxidants that was reported to increase semen quality after supplemented in extender. Sericin constitutes 25 to 30% of the silk protein synthesized inside the silk gland of *Bombyx mori* silk worm (Zhang, 2002). This protein is made of 18 amino acids which most of them possess strong polar side groups such as hydroxyl, carboxyl, and amino group (Wei *et al.*, 2005). The hydroxyl groups in serine and threonine might be responsible for the antioxidant properties of sericin as they chelate trace elements such as copper and iron (Kato *et al.*, 1998). Supplementation of sericin as antioxidant in canine

semen extender has yet to be reported. Therefore, this research aimed to evaluate the quality of chilled canine semen after supplemented with sericin in various concentration.

MATERIALS AND METHODS

Animals

Four healthy and sexually mature Doberman Pinscher were used in this research for semen collection. The dogs aged from two to five years old, weighed between 25 and 45 kg and with proven fertility after natural mating. All dogs were individually housed in kennels with runs attached, good air ventilation, and sheltered from sun and rain. The dogs were fed with a commercial dry canine diet (Hill's® Science Diet® Adult Advanced Fitness Small Bites Dog Food) as much as ± 300 g twice a day and given water ad libitum. Deworming tablets (Drontal Dog Tasty Bone) were also given one tablet per 10 kg of body weight every three months. This research was approved by Animal Care and Use Committee (ACUC) of Bogor Agricultural University (33-2016 IPB).

Extenders

The composition of the extenders used in this research was modified from Bencharif *et al.* (2013), consisted of 3.025 g tris (hydroxymethyl) aminomethane (MERCK, Merck KGaA, Darmstadt, Germany), 1.7 g citric acid monohydrate (MERCK, Merck KGaA, Darmstadt, Germany), 1.25 g D(-) fructose (MERCK, E. Merck, Darmstadt, Germany), 100 mL Milli-Q water ad, 20 mL egg yolk, 0.25 mL penicillin-G (Meiji, PT Meiji Indonesian Pharmaceutical Industries, Jakarta, Indonesia), and 0.25 mL streptomycin sulfate (Meiji, PT Meiji Indonesian Pharmaceutical Industries, Jakarta, Indonesia). The extender was then divided into four equal aliquots added with different concentrations of sericin (Wako, Wako Pure Chemical Industries Ltd., Osaka, Japan), which were 0% (S-0), 0.1% (S-0.1), 0.25% (S-0.25), and 0.5% (S-0.5).

Semen collection and evaluation

Semen collection was done once a week in the morning by massage method with the presence of an estrous bitch as teaser. Sperm-rich fraction was collected from two or three dogs each time and evaluated immediately for volume, color, consistency, pH, spermatozoa motility, spermatozoa concentration, and spermatozoa viability. Only sperm-rich fractions

with spermatozoa motility $>70\%$ and spermatozoa concentration $>100 \times 10^6$ spermatozoa/mL were used in this research.

Semen processing

Sperm-rich fraction from each dog was pooled to obtain a sufficient number of spermatozoa for each experiment and to reduce variability between samples. Pooled sperm-rich fraction was divided into four equal aliquots, which were then diluted with TEY extenders supplemented with different concentrations of sericin (0%, 0.1%, 0.25%, 0.5%) with a final concentration of 100×10^6 spermatozoa/mL. The diluted semen aliquots were preserved at 4° C in sterile centrifuge tubes and were then evaluated for spermatozoa motility, viability, plasma membrane integrity, and acrosome integrity every 12 hours up to 72 hours. The experiment was repeated five times. The motility of spermatozoa was evaluated subjectively under microscope. Eosin-negrosin staining was used to evaluate the viability of spermatozoa. Membran integrity of spermatozoa was evaluated using hypo-osmotic swelling test (HOST). Whereas acrosome integrity was evaluated using coomassie blue staining method as reported by Thiangtum *et al.* (2012)

Statistical Analysis

All data were calculated using Statistical Package for Social Sciences (SPSS) 18.0 for Windows software (SPSS Inc., Chicago, IL, USA). Chilled canine semen data from five treatments were analyzed using two-way analysis of variance (ANOVA) and tested with Duncan multiple range test to define differences among extenders.

RESULTS AND DISCUSSION

Characteristics of Sperm-rich Fractions from Doberman Pinschers

Only sperm-rich fractions were used in this research because according to England and Allen (1992), pre-sperm and prostatic fraction from canine semen contain prostatic fluid that adversely affected the spermatozoa motility and increased the number of morphologically abnormal spermatozoa in chilled canine semen. The characteristics of sperm-rich fractions from Doberman Pinschers are presented in Table 1.

The mean volume of sperm-rich fractions from the Doberman Pinschers in this study was in the normal volume range of canine sperm-rich fraction according

Table 1. Characteristics of sperm-rich fractions from Doberman Pinschersean

Parameter	Mean \pm standard deviation*
Macroscopic	
Volume (mL)	2.07 \pm 084
Colour	Greyish-white
Consistency	Watery
pH	6.07 \pm 0.19
Microscopic	
Motility (%)	86.67 \pm 2.50
Concentration ($\times 10^6$ spermatozoa/mL)	155.83 \pm 65.61
Viability (%)	89.98 \pm 4.89

*Data obtained from 9 samples of sperm-rich fractions originated from 4 dogs

to Feldman and Nelson (2003), which was between 0.5 and 12.0 mL. This result is similar to those obtained by Goericke-Pesch and Failing (2012) on the mean volume of sperm-rich fractions from Doberman Pinschers, which was 2.3 mL. Semen volume does not correlate with canine fertility as it depends on dog's breed, size, testis and prostate gland.

The color and consistency of the sperm-rich fractions obtained in this research were in agreement with Greer (2015) and Payan-Carreira *et al.* (2011) who state that normal canine sperm-rich fractions are greyish-white in color with watery consistency. The intensity of the color depends on the spermatozoa concentration; thus, semen color is one of the indicators of canine semen quality. However, cloudy or milky semen samples should always be checked microscopically for conformation as dogs with azoospermia occasionally shed excessive fat droplets into semen, giving the appearance of normal semen (Robert *et al.*, 2016). The mean pH of the sperm-rich fractions is similar to the results of Payan-Carreira *et al.* (2011) and Goericke-Pesch and Failing (2012), which were 6.10 and 6.20 respectively.

The results from microscopic evaluation showed that the mean percentage of motile spermatozoa in the sperm-rich fractions was in the normal range as stated by England and von Heimendahl (2010), which was between 75 and 90%. The mean spermatozoa concentration of the sperm-rich fractions was in accordance with Johnston *et al.* (2001) that stated the normal range of spermatozoa concentration of canine sperm-rich fraction is 4 to 400 x 10⁶ spermatozoa/mL. Spermatozoa concentration of canine sperm-rich fraction varied between breeds and affected by age, size of testis, dog body weight (Payan-Carreira *et al.*, 2011), and frequency of semen collection (Johnson, 2008). Similarly, the mean percentage of live spermatozoa of the sperm-rich fractions was also in the normal range as reported by Johnston *et al.* (2001) that the percentage of live spermatozoa in normal canine semen is above 80%.

Quality of Chilled Semen in Extenders Supplemented with Sericin

The mean percentage of motile spermatozoa in S-0, S-0.1, and S-0.25 extender groups decreased significantly from 6 h until 72 h of storage, whereas spermatozoa in extender S-0.5 showed significant decrease in spermatozoa motility after 24 h ($P < 0.05$; Table 2). This result is consistent with the findings of Iguer-Ouada and Verstegen (2001) and Tsutsui *et al.*

(2003) who reported that number of motile spermatozoa in chilled canine semen decreased gradually during storage at 4° C. This decrease in spermatozoa motility is due to initial increase of spermatozoa metabolism that increased uptake of oxygen and energy, and hence resulted in increased lactic acid concentration and decreased pH (Verstegen *et al.*, 2005). Apart from that, osmotic pressure increases upon storage due to the accumulation of metabolic end products (Salisbury and Lodge, 1962). Songsasen *et al.* (2002) reported that canine spermatozoa progressive motility was very sensitive to osmotic stress. Although storage of chilled semen at 4° C may reduce spermatozoa metabolism, spermatozoa are still able to carry out metabolic activities at lower limit which in turn produce metabolites and free radicals that will affect the osmolality of the extender and spermatozoa motility (Setiadi *et al.*, 2007).

The motility of spermatozoa was similar among the groups until 36 h of storage ($P > 0.05$). At 48 and 60 h of storage, the motility of spermatozoa was higher in all of sericin groups compared to spermatozoa preserved in extender without sericin ($P < 0.05$). After then at 72 h, the spermatozoa in extender with 0.25% and 0.5 % have motility higher than those of S-0, and S -0.1 groups ($P < 0.05$). The decrease of spermatozoa motility may also occur because ROS alter glyceraldehyde 6-phosphate dehydrogenase (G6PDH) that functions in producing ATP through synthesis and oxidation of nicotinamide adenine dinucleotide phosphate (NADPH) in the spermatozoa metabolism. Glyceraldehyde 6-phosphate dehydrogenase (G6PDH) is highly vulnerable to oxidative stress, thus spermatozoa motility will decrease due to low level of ATP when G6PDH is damaged by ROS. The end product of lipid peroxidation such as malondialdehyde (MDA) inhibits cellular enzyme like ATPase that may affect spermatozoa motility (Saraswat *et al.*, 2014). After 40 hours of storage, it was speculated that sericin played a role in protecting the spermatozoa from the ROS generated in this research, and hence extenders added with sericin had higher spermatozoa motility if compared to the extender that was not added with sericin. This finding is similar to that of Kumar *et al.* (2015) who reported supplementation of 0.25 to 0.5% sericin in semen extender improved spermatozoa motility in frozen buffalo semen. Kato *et al.* (1998) hypothesized that sericin's antioxidant properties is due to its high content in serine and threonine, where the hydroxyl groups from those two amino acids chelate trace elements such as copper and iron. Iron (Fe^{2+}) and

Table 2. Mean percentage (\pm standard deviation) of motile spermatozoa in extenders added with different concentrations of sericin during 72 hours of preservation at 4° C

Extender	Spermatozoa motility (mean percentage \pm standard deviation)						
	6 h	12 h	24 h	36 h	48 h	60 h	72 h
S-0	83.0 \pm 2.7 ^A	76.0 \pm 2.2 ^B	66.0 \pm 4.2 ^C	54.0 \pm 5.5 ^D	43.0 \pm 4.5 ^{aE}	30.0 \pm 6.1 ^{aF}	8.0 \pm 2.7 ^{aG}
S-0.1	83.0 \pm 2.7 ^A	75.0 \pm 3.5 ^B	69.0 \pm 2.2 ^C	61.0 \pm 6.5 ^D	51.0 \pm 5.5 ^{bE}	40.0 \pm 3.5 ^{bF}	21.0 \pm 4.2 ^{bG}
S-0.25	83.0 \pm 2.7 ^A	77.0 \pm 2.7 ^B	70.0 \pm 5.0 ^C	62.0 \pm 5.7 ^D	55.0 \pm 5.0 ^{bE}	42.0 \pm 2.7 ^{bF}	31.0 \pm 4.2 ^{cG}
S-0.5	83.0 \pm 2.7 ^A	77.0 \pm 2.7 ^{AB}	71.00 \pm 4.18 ^B	62.0 \pm 7.6 ^C	56.0 \pm 7.4 ^{bC}	44.0 \pm 2.2 ^{bD}	34.0 \pm 2.2 ^{cE}

^{a, b}Different superscripts in the same column indicate significant differences among extenders ($P < 0.05$); ^{A, AB, B, C, D, E, F, G}Different superscripts in the same row indicate significant differences among time ($P < 0.05$). The extender was added sericin at concentration of which 0% (S-0), 0.1% (S-0.1), 0.25% (S-0.25), and 0.5% (S-0.5)

copper (Cu²⁺) are the catalysts in the formation of hydroxyl radical (OH·) from hydrogen peroxide (H₂O₂) through Fenton and Haber-Weiss reaction (Ayala *et al.*, 2014).

The mean percentage of live spermatozoa in all extenders decreased significantly from 60 h of storage (P<0.05; Table 3). Significant differences in the mean percentage of live spermatozoa between all extenders were only found at 36 h, 60 h and 72 h (P<0.05). Extender S-0.25 and S-0.5 had higher mean of live spermatozoa if compared to other extenders at 36 h (P<0.05). At 72 h, extender S-0.5 had the highest mean of live spermatozoa among all extenders (P<0.05). However, it was not significantly different from extender S-0.1 and S-0.25 at 72 h (P>0.05). In contrast, extender S-0 showed the lowest mean of live spermatozoa at 72 h (P<0.05). This result is similar to the findings of Versteegen *et al.* (2005) who reported that number of live spermatozoa in chilled canine semen decreased continuously during storage at 4° C. Reactive oxygen species are also produced by dead spermatozoa through reaction catalyzed by aromatic amino acid oxidase (AAAO) (Upreti *et al.*, 1998). Therefore, it can be indicated that the concentration of ROS increased with time during storage as the number of dead spermatozoa also increased throughout the research. High concentration of ROS damage spermatozoa membrane through lipid peroxidation and affect mitochondria permeability and spermatozoa

respiration, leading to ATP depletion as a result of insufficient axonemal phosphorylation (Bansal and Bilaspuri, 2010). Protein as one of the major components in the organelles of spermatozoa can also be damaged by ROS through protein fragmentation, amino acid modification and cross linking. Furthermore, ROS also damage spermatozoa DNA through oxidation of DNA bases such as guanine (Saraswat *et al.*, 2014). The damage caused by ROS on the lipid, protein and DNA will lead to loss of motility and viability of spermatozoa.

This finding also showed that extenders supplemented with sericin were able to maintain spermatozoa viability better than extender that was not added with sericin. Dash *et al.* (2008) identified sericin as a potential antioxidant as it inhibited cell apoptosis by scavenging ROS. Sonseeda *et al.* (2015) demonstrated that chilled chicken semen supplemented with 0.25% sericin resulted in higher spermatozoa viability as sericin protected the spermatozoa from oxidative stress. It was observed that the mean percentage of live spermatozoa was higher than the mean percentage of motile spermatozoa in all extenders at 72 h. This is because live spermatozoa may be motile or non-motile, thus spermatozoa viability will be always higher than spermatozoa motility (Mundt and Shanahan, 2010).

The mean percentage of spermatozoa with intact plasma membrane decreased significantly from 6 h until 72 h in all group extenders (P<0.05; Table 3). All

Table 3. Mean percentage (± standard deviation) of live spermatozoa in extenders added with different concentrations of sericin during 72 hours of preservation at 4° C

Extender	Spermatozoa viability (mean percentage±standard deviation)						
	6 h	12 h	24 h	36 h	48 h	60 h	72 h
S-0	87.4±3.6 ^A	86.3±3.6 ^A	85.3±2.80 ^A	81.9±4.1 ^{aAB}	81.3±7.4 ^{AB}	77.1±5.1 ^{aB}	76.2±5.6 ^{aB}
S-0.1	89.9±2.6 ^A	88.3±2.6 ^A	86.7±3.17 ^{AB}	85.3±2.6 ^{abAB}	84.2±6.1 ^{ABC}	81.6±3.7 ^{abBC}	80.3±3.8 ^{abC}
S-0.25	90.4±3.4 ^A	88.9±3.4 ^{AB}	87.84±3.23 ^{AB}	86.4±2.8 ^{bABC}	85.5±2.9 ^{BC}	82.4±3.7 ^{abC}	82.3±3.18 ^{bc}
S-0.5	91.5±3.7 ^A	90.4±3.7 ^{AB}	88.72±3.02 ^{ABC}	86.7±2.6 ^{bBCD}	85.1±2.0 ^{CD}	83.1±2.3 ^{bd}	83.0±2.3 ^{bd}

^{a, ab, b}Different superscripts in the same column indicate significant differences among extenders (P < 0.05); ^{A, AB, ABC, BC, BCD, C, CD, D}Different superscripts in the same row indicate significant differences among time (P<0.05). The extender was added sericin at concentration of which 0% (S-0), 0.1% (S-0.1), 0.25% (S-0.25), and 0.5% (S-0.5)

Table 4. Mean percentage (± standard deviation) of hypo-osmotic swelling test-positive spermatozoa in extenders added with different concentrations of sericin during 72 hours of preservation at 4° C

Extender	Hypo-osmotic swelling test-positive spermatozoa (mean percentage±standard deviation)						
	6 h	12 h	24 h	36 h	48 h	60 h	72 h
S-0	90.4±4.6 ^A	88.8±5.76 ^A	85.7±5.2 ^{AB}	80.4±5.78 ^{aBC}	78.5±6.7 ^{aBC}	76.7±5.1 ^{aC}	76.6±5.4 ^C
S-0.1	93.2±4.3 ^A	91.1±3.6 ^{AB}	88.9±3.7 ^{AB}	86.8±3.9 ^{bBC}	82.7±5.1 ^{abCD}	80.8±5.3 ^{abCD}	79.4±5.3 ^D
S-0.25	93.3±3.7 ^A	91.7±3.4 ^{AB}	88.8±2.8 ^{AB}	87.2±3.4 ^{bBC}	83.9±4.1 ^{abCD}	82.1±3.4 ^{abD}	81.1±3.2 ^D
S-0.5	92.9±2.6 ^A	91.2±3.3 ^{AB}	88.9±3.5 ^{AB}	86.8±3.2 ^{bBC}	86.6±4.9 ^{bBC}	83.1±2.9 ^{bCD}	81.6±2.6 ^D

^{a, ab, b}Different superscripts (a-b) in the same column indicate significant differences among extenders (P<0.05); ^{A, AB, BC, C, CD, D}Different superscripts in the same row indicate significant differences among time (P<0.05). The extender was added sericin at concentration of which 0% (S-0), 0.1% (S-0.1), 0.25% (S-0.25), and 0.5% (S-0.5)

Table 5. Mean percentage (± standard deviation) of spermatozoa with intact acrosomes in extenders added with different concentrations of sericin during 72 hours of preservation at 4° C

Extender	Spermatozoa with intact acrosomes (mean percentage±standard deviation)						
	6 h	12 h	24 h	36 h	48 h	60 h	72 h
S-0	97.6±0.7 ^{aA}	96.3±1.5 ^{AB}	95.0±1.5 ^{ABC}	94.02±2.06 ^{BCD}	93.2±2.2 ^{CD}	92.9±2.4 ^{CD}	92.1±2.5 ^D
S-0.1	98.4±0.4 ^{ba}	97.4±1.6 ^{AB}	96.3±1.7 ^{AB}	95.40±2.79 ^{AB}	95.0±3.0 ^{AB}	94.4±3.4 ^B	93.8±3.4 ^B
S-0.25	98.9±0.7 ^{ba}	97.2±1.6 ^{AB}	96.7±2.4 ^{AB}	96.20±2.41 ^{AB}	95.4±2.5 ^B	95.0±2.8 ^B	94.7±3.1 ^B
S-0.5	98.8±0.3 ^{ba}	97.4±2.5 ^{AB}	96.4±2.2 ^{AB}	96.30±2.46 ^{AB}	94.6±2.8 ^B	95.0±3.2 ^B	94.7±3.2 ^B

^{a, b}Different superscripts (a-b) in the same column indicate significant differences among extenders (P<0.05); ^{A, AB, ABC, B, BCD, C, CD, D}Different superscripts (A-D) in the same row indicate significant differences among time (P<0.05). The extender was added sericin at concentration of which 0% (S-0), 0.1% (S-0.1), 0.25% (S-0.25), and 0.5% (S-0.5)

extenders only showed significant differences in the mean percentage of hypo-osmotic swelling test-positive (Figure 1) spermatozoa from 36 h until 60 h ($P < 0.05$). Extender S-0.5 had the highest mean percentage of hypo-osmotic swelling test-positive spermatozoa at 72 h. However, it was not significantly different from other extenders ($P > 0.05$). This decrease in the mean percentage of hypo-osmotic swelling test-positive spermatozoa may be due to the damage on the spermatozoa plasma membrane caused by ROS. Reaction of spermatozoa towards hypo-osmotic solution will not be perfect when their plasma membranes are damaged, considering the plasma membranes of the spermatozoa are no longer able to maintain osmotic equilibrium between intracellular and extracellular (Sankai *et al.*, 2002). Dobranic *et al.* (2005) reported a strong positive correlation between hypo-osmotic swelling test and spermatozoa progressive motility. This finding may be due to spermatozoa motility partially depends on the functional integrity of the membrane and spermatozoa metabolism (Jeyendran *et al.*, 1984).

The mean percentage of spermatozoa with intact acrosomes in all extenders decreased significantly from 6 h until 72 h ($P < 0.05$; Table 5). Significant differences in the mean percentage of spermatozoa with intact acrosomes in all extenders were only found at 6 h ($P < 0.05$). Extender S-0.1, S-0.25, and S-0.5 showed higher mean percentage of spermatozoa with intact acrosomes than extender S-0 at 6 h ($P < 0.05$). However, the mean percentage of spermatozoa with intact acrosomes of extender S-0.1, S-0.25 and S-0.5 were not significantly different among those three extenders ($P > 0.05$).

This result is consistent with the findings of Iguer-Ouada and Verstegen (2001) who also reported that the decrease in the mean percentage of spermatozoa with intact acrosomes was lower than other measured parameters. In addition, Yu (2006) stated that spermatozoa acrosome integrity was the most resistant characteristic to changes. According to Jeyendran *et al.* (1984), plasma membranes of the head and the tail function separately, and hence the integrity of spermatozoa membrane will not always indicate that the spermatozoa head membrane will function normally during capacitation and acrosome reaction. Kumi-Diaki and Badtram (1994) demonstrated that there was no correlation between spermatozoa acrosome defect and other functional parameters, thus spermatozoa motility and plasma membrane integrity might be independent of the integrity of spermatozoa acrosome. The mean percentage of spermatozoa with intact acrosomes in all extenders remained relatively high at 72 h, which was above 90% in average. This finding may be related to the composition of the chilled semen used in this research that did not contain glucose. Mahi and Yanagimachi (1978) stated that the number of acrosome-reacted spermatozoa were higher in chilled canine semen extender that was added with glucose than extender that was not added with glucose. Apart from that, the high mean percentage of spermatozoa with intact acrosomes can be due to the presence of egg

yolk in all extenders used in this research. This can be supported by the findings of Iguer-Ouada and Verstegen (2001) who showed a higher number of spermatozoa with loss acrosomes in EDTA extender than extender added with egg yolk.

Extender S-0 showed the lowest mean percentage of spermatozoa with intact acrosomes at 6 h ($P < 0.05$). The findings of Strezek *et al.* (2009) showed that total antioxidant status (TAS) of canine pre-sperm and prostatic fraction were $36.02 \pm 9.28\%$ and $30.01 \pm 9.87\%$ respectively, whereas the TAS of sperm-rich fraction was only $16.23 \pm 6.85\%$. The pre-sperm and prostatic fraction were not included in this research and this might be the reason the spermatozoa in extender S-0 lacked of endogenous and exogenous antioxidants that were responsible to reduce the oxidative stress caused by ROS. The overall quality of chilled semen deteriorated gradually during storage at 4°C . This finding is similar to that of Michael *et al.* (2008) and Michael *et al.* (2009) who showed that canine spermatozoa motility, viability, plasma membrane integrity and acrosome integrity decreased throughout the storage at 4°C . The deterioration of semen quality might be due to the increase of ROS concentration (Michael *et al.*, 2009). Therefore, supplementation of antioxidant into semen extender should be done in order to counteract ROS. The findings of this research revealed that sericin is a potential antioxidant as it was able to protect spermatozoa from free radicals and lipid peroxidation. Moreover, the findings of Isobe *et al.* (2012) showed that addition of 0.5% sericin into in vitro maturation medium was able to enhance the development of bovine embryo by preventing oxidative stress. Hajarian *et al.* (2016) also demonstrated that supplementation of 0.1 and 0.5% sericin into ovine in vitro maturation medium and in vitro embryo culture promoted the cleavage rate and blastocyst yield by chelating trace elements that were involved in lipid peroxidation.

CONCLUSION

In conclusion, the TEY extenders supplemented with 0.25% and 0.5% sericin resulted in higher spermatozoa motility and viability at 72 h if compared to other TEY extenders. The integrity of plasma membrane and acrosome of spermatozoa showed no significant difference between all the TEY extenders at 72 h.

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REFERENCES

- Alvarez, J.G. and B.T Storey. 1989. Role of glutathione peroxidase in protecting mammalian spermatozoa from loss of motility caused by spontaneous lipid peroxidation. *Gamete Res.* 23:77-90.
- Ayala, A., M. F. Munoz, and S. Arguelles. 2014. Lipid peroxidation: Production, metabolism and signaling mechanism of malondialdehyde and 4-hydroxy-2-nonenal. *Oxid. Med. Cell Longev.* Doi:10.1155/2014/360438.

- Bansal, A.K. and G.S. Bilaspuri. 2010. Impacts of oxidative stress and antioxidants on semen functions. **Vet. Med. Int.** Doi:10.4061/2011/686137.
- Bencharif, B., L. Amirat-Briand, J. Le Guillou, Vialtelle, M. Anton, E. Schmitt, S. Desherces, P. Barriere, and D.Tainturier. 2013. Refrigeration of canine sperm at +4° C: Comparative study of four different extenders for the refrigeration of canine sperm at +4° C: LDL, Tris egg yolk, Equex® and INRA96®. **Revue. Méd. Vét.** 164(5):252-262.
- Dash, R., C. Acharya, P.C. Bindu, and S.C. Kundu. 2008. Antioxidant potential of silk protein sericin against hydrogen peroxide-induced oxidative stress in skin fibroblasts. **BMB Rep.** 41(3):236-241.
- Dobranic, T., Samardzija, M. Cergolj, and N. Prvanovic. 2005. Determination of membrane integrity of canine spermatozoa. **Vet. Arhiv.** 75(1):23-30.
- England, G.C.W. and W.E. Allen. 1992. Factors affecting the viability of canine spermatozoa. II. Effects of seminal plasma and blood. **Theriogenology.** 37:373-381.
- England, G.C.W. and A. von Heimendahl. 2010. **BSAVA Manual of Canine and Feline Reproduction and Neonatology.** 2nd ed. BSAVA, Gloucester.
- Feldman, E. and C. Nelson. 2003. **Canine and Feline Encrinology and Reproduction.** 3rd ed. Saunders, Philadelphia.
- Goericke-Pesch, S. and K. Failing. 2012. Retrospective analysis of canine semen evaluations with special emphasis on the use of the hypoosmotic swelling (HOS) test and acrosomal evaluation using Spermac®. **Reprod. Dom. Anim.** 48(2):213-217.
- Greer, M.L. 2015. **Canine Reproduction and Neonatology.** Teton NewMedia, Wyoming.
- Griveau, J.F. and D. Le Lannou. 1997. Reactive oxygen species and human spermatozoa: Physiology and pathology. **Int. J. Androl.** 20:61-69.
- Hajarian, H., H.K. Shabankareh, and F. Aghaz. 2016. Enhanced in vitro developmental competence of sheep embryos following sericin supplementation of the in vitro maturation and in vitro culture media. **Small Ruminant. Res.** 136:257-273.
- Henkel, R. 2005. The impact of oxidants on sperm function. **Andrologia.** 37:205-206.
- Iguer-Ouada, M. and J.P. Versteegen. 2001. Long-term preservation of chilled canine semen: Effect of commercial and laboratory prepared extenders. **Theriogenology.** 55:671-184.
- Isobe, T., Y. Ikebata, T. Onitsuka, M. Wittayarat, Y. Sato, M. Taniguchi, and T. Otoi. 2012. Effect of sericin on preimplantation development of bovine embryos cultured individually. **Theriogenology.** 257(78):747-752.
- Jeyendran, R.S., H.H. Van Der Ven, M. Perez-Pelaez, B.G. Crabo, and L.J.D. Zaneveld. 1984. Development of an assay to assess the functional integrity of the human sperm membrane and its relationship to other semen characteristics. **J. Reprod. Fert.** 70:219-228.
- Johnson, C. 2008. Semen Quality and Artificial Insemination. <http://veterinarycalendar.dvm360.com/semen-quality-and-ai-proceedings>.
- Johnston, S.D., M.V. Root Kustritz, and P.N.S. Olson. 2001. **Canine and Feline Theriogenology.** Saunders, Philadelphia.
- Kato, N., S. Sato, A. Yamanaka, H. Yamada, N. Fuwa, and M. Nomura. 1998. Silk protein, sericin, inhibits lipid peroxidation and tyrosinase activity. **Biosci. Biotechnol. Biochem.** 62:145-147.
- Kumar, P., D. Kumar, P. Sikka, and P. Singh. 2015. Sericin supplementation improves semen freezability of buffalo bulls by minimizing oxidative stress during cryopreservation. **Anim. Repro. Sci.** 152:26-31.
- Kumi-Diaki, J. and G. Badtram. 1994. Effect of storage on sperm membrane integrity and other functional characteristics of canine spermatozoa: in vitro bioassay for canine semen. **Theriogenology.** 41:1355-1366.
- Linde-Forsberg, C. and M. Forsberg. 1989. Fertility in dogs in relation to semen quality and the time and site of insemination with fresh and frozen semen. **J. Reprod. Fert. Suppl.** 39:299-310.
- Lucio, C.F., F.M. Regazzi, L.C. Silva, D.S. Angrimani, M. Nichi, and C.I. Vannucchi. 2016. Oxidative stress at different stages of two-step semen cryopreservation procedures in dogs. **Theriogenology.** 85(9):1568-1575.
- Mahi, C.A. and R. Yanagimachi. 1978. Capacitation, acrosome reaction, and egg penetration by canine spermatozoa in a simple defined medium. **Gamete Res.** 1:101-109.
- Michael, A.J., C. Alexopoulos, E.A. Pontiki, D.J. Hadjipavlou-Litina, P. Saratsis, H.N. Ververidis, and C.M. Boscós. 2008. Quality and reactive oxygen species of extended canine semen after vitamin C supplementation. **Theriogenology.** 70:827-735.
- Michael, A.J., C. Alexopoulos, E.A. Pontiki, D.J. Hadjipavlou-Litina, P. Saratsis, H.N. Ververidis, and C.M. Boscós. 2009. Effect of antioxidant supplementation in semen extenders on semen quality and reactive oxygen species of chilled canine spermatozoa. **Anim. Reprod. Sci.** 112:119-135.
- Mundt, L.A. and K. Shanahan. 2010. **Graff's Textbook of Urinalysis and Body Fluids.** Lippincott Williams & Wilkins, Pennsylvania, USA.
- Payan-Carreira, R., S. Miranda, and W. Nizanski. 2011. Artificial Insemination in Dogs. In **Artificial Insemination in Farm Animals.** M. Manafi (ed.). Intech, Rijeka.
- Robert, M.A., G. Jayaprakash, M. Pawshe, T. Tamilmani, and M. Sathiyabarathi. 2016. Collection and evaluation of canine semen-a review. **Int. J. Environ. Sci. Technol.** 5(3):1586-1595.
- Rota, A., B. Strom, and C. Linde-Forsberg. 1995. Effects of seminal plasma and three extenders on canine semen stored at 4° C. **Theriogenology.** 44:885-900.
- Saleh, R.A. and A. Agarwal. 2002. Oxidative stress and male infertility: From research bench to clinical practice. **J. Androl.** 23(6):737-752.
- Salisbury, G.W. and J.R. Lodge. 1962. Metabolism of Spermatozoa. In **Advances in Enzymology and Related Areas of Molecular Biology.** F.F. Nord (ed.). John Wiley & Sons, Inc, New Jersey.
- Sankai, T., H. Tsuchiya, and N. Ogonuki. 2002. Short-term nonfrozen storage of mouse epididymal spermatozoa. **Theriogenology.** 55(8):1759-1768.
- Saraswat, S., S.D. Kharache, and S.K. Jindal. 2014. Impact of reactive oxygen species on spermatozoa: a balancing act between beneficial and detrimental effects. **Iranian J. Appl. Anim. Sci.** 4(2):247-255.
- Setiadi, M.A., Yulnawati, and A. Suprayogi. 2007. Kualitas spermatozoa epididymis anjing selama penyimpanan pada suhu 4° C. **J. Ilmu Ternak Vet.** 12(2):134-138.
- Songsasen, N., I. Yu, S. Murton, D.L. Paccamonti, B.E. Eilts, R.A. Godke, and S.P. Leibo. 2002. Osmotic sensitivity of canine spermatozoa. **Cryobiology.** 44:79-90.
- Sonseeda, P., S. Somtao, M. Duanginda, and T. Vongpralub. 2015. Effects of sericin supplementation on the sperm survival of cooled stored chicken semen. **Khon. Kaen. Agr. J.** 43(2):103-106.
- Storey, B.T. 1997. Biochemistry of the induction and prevention of lipoperoxidative damage in human spermatozoa. **Mol. Hum. Reprod.** 3:203-314.
- Strzezek, R., M. Kozirowska-Gilun, M. Kowalówka, and J. Strzezek. 2009. Characteristics of antioxidant system in dog semen. **Pol. J. Vet. Sci.** 12:55-60.
- Thiangtum K., T. Hori, and E. Kawakami. 2012. Effect of catalase and superoxide dismutase on motility, viability and acrosomal integrity of canine spermatozoa during storage at 5° C. **Thai J. Vet. Med.** 42(4):447-453.
- Tsutsui, T., T. Tezuka, Y. Mikasa, H. Sugisawa, N. Kirihara, T. Hori, and E. Kawakami. 2003. Artificial insemination with canine semen stored at a low temperature. **J. Vet. Med. Sci.** 65:307-312.
- Upreti, G.C., K. Jensen, R. Munday, D.M. Duganzich, R. Vishwanath, and J.F. Smith. 1998. Studies on aromatic amino acid oxidase activity in ram spermatozoa: Role of pyruvate as an antioxidant. **Anim. Reprod. Sci.** 51:275-287.
- Versteegen, J.P., K. Onclin, and M. Iguer-Ouada. 2005. Long-term motility and fertility conservation of chilled canine semen using egg yolk added Tris-glucose extender: In vitro and in vivo studies. **Theriogenology.** 64:720-733.
- Wei, T., M.Z. Li, and R.J. Xie. 2005. Preparation and structure of porous silk sericin materials. **Macromol. Mater. Eng.** 290:188-194.
- Yu, L.J. 2006. Evaluation of glucose-BSA as extender of chilled canine spermatozoa for long-term storage. **Korean J. Emb. Trans.** 21(4):323-330.
- Zhang, Y.Q. 2002. Applications of natural silk protein sericin in biomaterials. **Biotechnol. Adv.** 20:91-100.