

The effect of L-carnitine in Tris egg yolk-based diluent on the quality of Pasundan bull semen preserved in chilled condition

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ABSTRAK

Penelitian ini dilakukan untuk mengevaluasi suplementasi L-Carnitine dalam pengencer Tris Kuning Telur (TKT) untuk mengoptimalkan kualitas semen cair sapi Pasundan. Semen dikoleksi dari tiga ekor sapi Pasundan (6 - 7 tahun) menggunakan vagina buatan. Semen dievaluasi secara makroskopis dan mikroskopis, semen yang memiliki $\geq 70\%$ motilitas progresif, konsentrasi sperma $\geq 500 \times 10^6 \text{ ml}^{-1}$, dan $\leq 20\%$ sperma yang abnormal, dibagi menjadi lima bagian. Masing-masing diencerkan dengan TKT dan ditambah dengan 0 mM (kontrol), 1 mM, 2 mM, 3 mM dan 4 mM L-Carnitine. Semen cair disimpan pada suhu 5 °C. Motilitas sperma dievaluasi secara objektif menggunakan *Computer Assisted Sperm Analysis* (CASA) setiap 12 jam sampai motilitas progresif mencapai 40%. Pengencer terbaik ditunjukkan oleh motilitas progresif (PM) pada pengencer TKT yang disuplementasi 1 mM L-Carnitine, hingga jam ke-108 ($43,08 \pm 0,49\%$) dibandingkan dengan kelompok kontrol ($43,63 \pm 0,70\%$) hanya sampai 72 jam ($P < 0,05$). Kesimpulan penelitian ini adalah 1 mM L-Carnitine dalam pengencer TKT merupakan konsentrasi terbaik untuk penyimpanan semen cair sapi Pasundan.

Kata kunci : L-Carnitine, semen cair, sapi Pasundan, pengencer kuning telur Tris

ABSTRACT

The study was conducted to investigate the effect of L-Carnitine supplementation in Tris-egg yolk (TEY) diluents to optimize the quality of Pasundan bull liquid semen. Semen samples were collected from three Pasundan bulls (6 – 7 years old) using an artificial vagina. Semen samples were evaluated macroscopically and microscopically, semen having $\geq 70\%$ progressive motility, $\geq 500 \times 10^6 \text{ ml}^{-1}$ sperm concentration and $\leq 20\%$ sperm abnormalities were divided into 5 equal part. Each part was diluted with TEY and supplemented with 0 mM (control), 1 mM, 2 mM, 3 mM and 4 mM L-Carnitine. Liquid semen was stored at 5 °C. Sperm motility was evaluated every 12 h objectively using *Computer Assisted Sperm Analysis* (CASA) until progressive motility reached 40%. The best diluent was demonstrated by progressive motility (PM) by TEY supplemented with 1 mM L-Carnitine ($43.08 \pm 0.49\%$) that remained up to 108 h compared to the control group ($43.63 \pm 0.70\%$) that remained up to 72 h ($P < 0.05$). In conclusion, 1 mM L-Carnitine in TEY was the best concentration for

preservation of Pasundan bull semen stored in the liquid form.

Keywords: L-Carnitine, liquid semen, Pasundan bulls, Tris-egg yolk diluents

INTRODUCTION

Pasundan cattle is one of the Indonesian local breeds. Population of Pasundan cattle at 2013 is estimated about 50.000 heads and mostly maintained by communities (cattle farmers) living along the northern and southern coastal areas of West Java with intensive and semi-extensive systems (Dwitresnadi *et al.*, 2015). As local genetic resources, population of Pasundan cattle needs to be improved and conserved continuously. The current breeding program to improve Pasundan cattle is using artificial insemination (AI) technique with the use of frozen or liquid semen.

The use of frozen semen for AI in cattle is very common, however, liquid semen can be applied as an alternative method when the availability of liquid nitrogen is limited (Arifiantini and Purwantara, 2010). Liquid semen can minimize the metabolism of sperm. The condition of sperm during storage process will be exposed to reactive oxygen species (ROS). ROS play a pivotal role on several sperm functions through activation of different intracellular mechanisms such as sperm capacitation associated-events (Martin-Hidalgo *et al.*, 2019). Therefore, sperm motility and viability in liquid semen maintained not over a long time (Fattah *et al.*, 2017a).

The quality of liquid semen is affected by various factors including storage techniques and the type of diluents. Tris-egg yolk (TEY) is one of diluents commonly used in bull semen. TEY contains Tris-based material which is functioning as a buffer solution, fructose as an energy source and egg yolk containing phospholipid and lecithin to protect sperm from cold shock during cooling or freezing process. However liquid storage or the freeze-thawing process also produced the formation of ROS, such as H₂O₂ which results in damage to the sperm motility, viability and DNA integrity (Sariozkan *et al.*, 2012; Perumal *et al.*, 2013). Therefore, it is necessary to add an additive to the diluents in order to protect the sperm during cool-storage.

L-Carnitine (3-hydroxy-4-trimethylaminobutyrate) is a quaternary ammonium compound (Bieber, 1988) and water-soluble amino acid naturally biosynthesized in the kidney

and liver of animal from lysine and methionine (Bremer, 1983). It plays a key role in reducing the availability of lipids for peroxidation by facilitating fatty acids transport into mitochondria for β -oxidation to generate Adenosine Triphosphate (ATP) energy (Neuman *et al.*, 2002). Therefore, adding L-Carnitine in diluents medium will increase sperm motility (Agarwal and Said, 2004). L-Carnitine is found in seminal plasma and has a positive correlation with the quality of stallion semen (Stradaoli *et al.*, 2004) and humans (Ahmed *et al.*, 2011).

Antioxidant characteristics and anti-apoptotic activity in L-Carnitine are able to stabilize the mitochondrial membrane and prevent damage to the DNA structure (Deon *et al.*, 2015). L-Carnitine increases the activity and level of antioxidant enzymes such as catalase (CAT) and glutathione peroxidase (GPO) as well (Koberska and Yermishev, 2017). These enzymes play an important role in preventing oxidative damage (Bucak *et al.*, 2010). The use of L-Carnitine in semen diluents have shown its beneficial effects on maintaining the quality of frozen semen in human (Banihani *et al.*, 2014), chicken (Tabatabaei and Aghaei, 2012; Fatah *et al.*, 2017a), cat (Manee-in *et al.*, 2014), stallion (Gibb *et al.*, 2015), sheep (Souza *et al.*, 2019), buffalo (Longobardi *et al.*, 2017) and bull (Bucak *et al.*, 2010). However, the effects of addition of L-Carnitine to semen diluents on the quality of liquid stored of bulls semen have not yet been investigated. Therefore, this study was conducted to investigate the efficacy of different L-Carnitine concentrations in the TEY diluents to improve the quality of Pasundan bulls semen stored in liquid form.

MATERIAL AND METHODS

Animals

Semen were obtained from three mature Pasundan bulls maintained at Beef Cattle Breeding Development Center and Artificial Insemination Institute, Cijeungjing, Ciamis., West Java, Indonesia. Age of the bulls was between 6 to 7 years with body weight of 380 to 430 kg. Bulls were managed in similar fed of *Pennisetum purpureum* grass up to 10% of body weight and commercial concentrate up to 1% of body weight

containing 16% crude protein. Fed is given twice a day and water is given *ad libitum*. Approval for the current study was given by the Animal Care and Use Committee (ACUC) at IPB University No. 143 – 2019 IPB.

Chemicals

All chemicals used in this research were purchased from Merck (Darmstadt, Germany) unless it was stated.

Preparation of diluents

The basic diluents used for this study is Tris-egg yolk (TEY). Tris buffer consists of 3.03 g Tris (hydroxymethyl) aminomethane, 1.78 g citric acid, 1.25 g fructose in 100 ml of aquabidest. Tris-egg yolk consisted of 80% Tris buffer and 20% of egg yolk, well mixed and centrifuged at 3000 rpm for 10 minutes, the supernatant was taken as a diluents. The TEY diluent was divided into five equal part and each part was supplemented with different concentrations of L-Carnitine (Sigma, Cat no: C0158), 0 (control) 1, 2, 3, and 4 mM (Table 1).

Semen Collection and Evaluation

The ejaculates from each bull were collected using an artificial vagina once a week, in the morning at 07.30 AM to 10.00 AM according to the Operational Standard Procedure of the AI centre. Immediately after collection, semen samples were evaluated both macroscopically and microscopically according to Arifiantini (2012). Macroscopic evaluation includes volume, color, pH and consistency. Volume (ml) was determined from the scale of graduated conical test tube attached to the artificial vagina. Color and consistency were determined visually and pH was measured by indicator paper (Merck, scale 6.4–8, 0.2 sensitivity).

Microscopic evaluation includes mass activity, sperm progressive motility, sperm viability, sperm morphology and sperm concentration. Mass activity was evaluated by dropping 10 μ l of semen on warm object glass and evaluated under light microscope (Olympus CX23) at 100 \times magnification. Evaluation of sperm motility was conducted by mixing 10 μ l of semen with 40 μ l of saline solution on a clean glass object then examined objectively using Computer Assisted Sperm Analysis (CASA; Androvision, Germany).

The percentages of sperm viability and

morphology were evaluated with the same slide using eosin-nigrosin staining technique. Ten microlitre of semen and 40 μ l of eosin-nigrosin were placed on a clean glass object, homogenized, smeared, and dried above heating table. Sperm concentration was evaluated using a photometer (SDM 6, Minitub, Germany). Semen that having $\geq 70\%$ progressive motility of sperm, concentration of sperm $\geq 500 \times 10^6$ ml⁻¹ and morphologically abnormal sperm $\leq 20\%$ were used for the experiment.

Processing of Liquid Semen

Fresh semen that match with criteria that used for this experiment were diluted based on the treatment (Table 1). The final sperm concentration was approximately 10×10^6 sperm ml⁻¹. The diluted semen was then packaged in a corning tube (15 ml). The samples were then placed in a beaker glass with water jacket and cooled from 37 °C to 5 °C and then stored at 5 °C (Arifiantini and Purwantara, 2010).

Evaluation of Sperm Parameters during Chilled-storage

Sperm Motility. Twenty μ l of semen was dropped on the warm object glass and then covered using a cover slip. Samples were then placed in heating table for thirty second and then observed in seven visual fields with 200 \times magnification. Liquid semen were evaluated every 12 hour. The variables tested were sperm motility for total motility (TM) and progressive motility (PM). The movement speed of the sperm were divided into fast motility (FM), slow motility (SM), local motility (LM) and immotile (IM). The evaluation were done until the sperm PM values reach 40%.

Sperm Viability. Twenty μ l of semen with 40 μ l of eosin-nigrosin solution were homogenized, smeared, and dried above heating plate. The stained slide was evaluated using a light microscope at 400 \times magnification and at least 200 sperm cells were counted in ten different microscopic fields (Arifiantini and Yusuf, 2010). Sperms which did not take up the eosin-nigrosin stain were considered alive, while those that did take up the stain were considered dead. Value is expressed in percentage (%).

Table 1. Composition of Tris Egg Yolk-Based Diluents

Ingredients	Total of Additions				
Tris buffer (mL)	80	80	80	80	80
Egg yolk (mL)*	20	20	20	20	20
<i>L-Carnitine</i> (mM)	0	1	2	3	4
Penicilline (IU/mL)**	1000	1000	1000	1000	1000
Streptomycine (mg/mL)**	1	1	1	1	1

*Fresh hen egg yolk

** Meiji Seika Pharma Co., Ltd, Japan

Table 2. Characteristics of Pasundan Bull Fresh Semen

Semen Characteristics	Mean±SEM
Volume (ml)	4.66±0.32
Colour	Milky white
pH	6.44±0.02
Consistency	Moderate
Mass motility	++ to +++
Progressive motility (%)	80.38±0.92
Sperm concentration (×10 ⁶ sperm ml ⁻¹)	1016.95±50.45
Sperm viability (%)	81.17±1.62
Sperm abnormality (%)	11.92±0.81

Research Design and Data Analysis

The study was designed using a completely randomized design (CRD) in time. Each treatment consists of four replications. Data were analyzed using analysis of variance (ANOVA) with SAS program version 9, followed by Duncan Multi Range Test if differences were found within and between treatments. Data are expressed as means ± SEM.

RESULTS

Quality of Pasundan Bull Fresh Semen

The results demonstrated macroscopically Pasundan bulls semen shows a volume of 4.66 ± 0.32 ml, milky white in colour, pH of 6.44 ± 0.02 with moderate consistency. Microscopic

evaluation showed mass movement of ++ to +++, progressive motility 80.38 ± 0.92% with sperm concentration of 1016.95 ± 50.45×10⁶ ml⁻¹. Sperm viability and abnormalities value, in this study were 81.17 ± 1.62% and 11.92 ± 0.81%, respectively (Table 2).

Effect of Various L-Carnitine Concentration in Tris-egg Yolk Diluents on the Motility Parameters of Pasundan Bulls Sperm

The computer-assisted sperm analysis demonstrated a decrease in TM and PM of Pasundan bulls sperm during storage. The decrease in TM did not quick as decrease in PM (Figure 1a and Figure 1b), and only after 84 h of storage we can see the effect of L-Carnitine. At 84 h of storage the TM of sperm without addition of L-Carnitine (88.19 ± 0.30%) was lower (P<0.05) than that of TM of sperm supplemented with L-Carnitine 1, 2, 3 and 4 mM that is 91.99 ± 0.25%, 90.98 ± 0.37%, 91.24 ± 0.79%, 90.54 ± 0.67%, respectively. At 108 h of storage, TM of sperm in TEY diluent with the addition of 1 mM L-Carnitine (89.64 ± 0.15%) was found to be higher (P<0.05) than that of TM of sperm the addition of 2 mM and 3 mM L-Carnitine (88.60 ± 0.49% and 88.64 ± 0.74%, respectively; Figure 1a). Thereafter, when time of storage reached 120 h, only TM of sperm in diluent with 1 mM L-Carnitine remained high (87.83 ± 0.47%).

In contrast with TM, the value of PM sperm in TEY diluents decreased gradually as storage time increase from 0 to 84 h. the value of PM sperm in TEY diluents given with 1 mM L-Carnitine reached a value of 49.44 ± 0.92% at 84 h of storage, this was higher (P<0.05) than sperm in TEY diluents given with 0 (control), 2, 3 and 4 mM L-Carnitine showed the value of 39.40 ± 0.49%, 46.11 ± 0.67%, 44.27 ± 1.24%, 44.13 ±

0.55% respectively. Afterward, when time of storage reached 120 h, only PM of sperm in diluent with 1 mM L-Carnitine remained high (Figure 1b).

No differences were found in FM of sperm at 84 h of storage in TEY diluents with different L-Carnitine concentration (Figure 2a). The FM were between $5.85 \pm 1.02\%$ to $6.82 \pm 0.72\%$ higher ($P < 0.05$) than control ($3.64 \pm 0.20\%$). The speed of sperm with SM, LM and IM category in TEY with 1 mM L-Carnitine have similar pattern with

PM category that was significantly at 84 h of storage (Figure 2b, Figure 3a and Figure 3b). It was clear from the data that addition 1 mM L-Carnitine in TEY diluents give the best result for sperm motility parameter.

Effect of Various L-Carnitine Concentration in Tris-egg Yolk Diluents on the Viability of Pasundan bulls sperm

Viability of Pasundan bull sperm in this study shows a similar trend to PM of sperm.

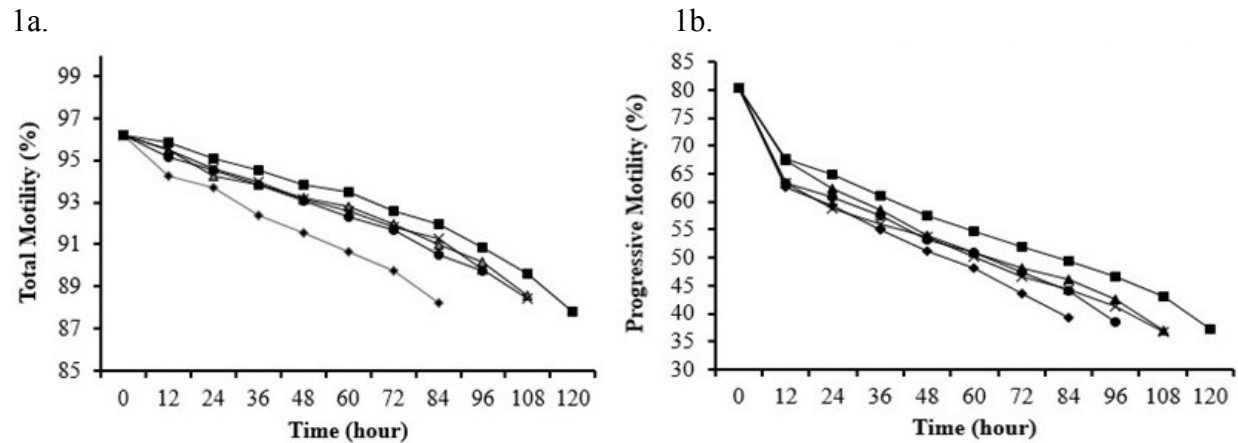


Figure 1. Total motility (TM) and progressive motility (PM) of Pasundan bull sperm in Tris-egg yolk diluents with a various concentrations of L-Carnitine (LC). The symbol represent diluents with 0 mM LC (\square), diluents with 1 mM LC (\blacksquare), diluents with 2 mM LC (\circ), diluents with 3 mM LC (\times) and diluents with 4 mM LC (\triangle).

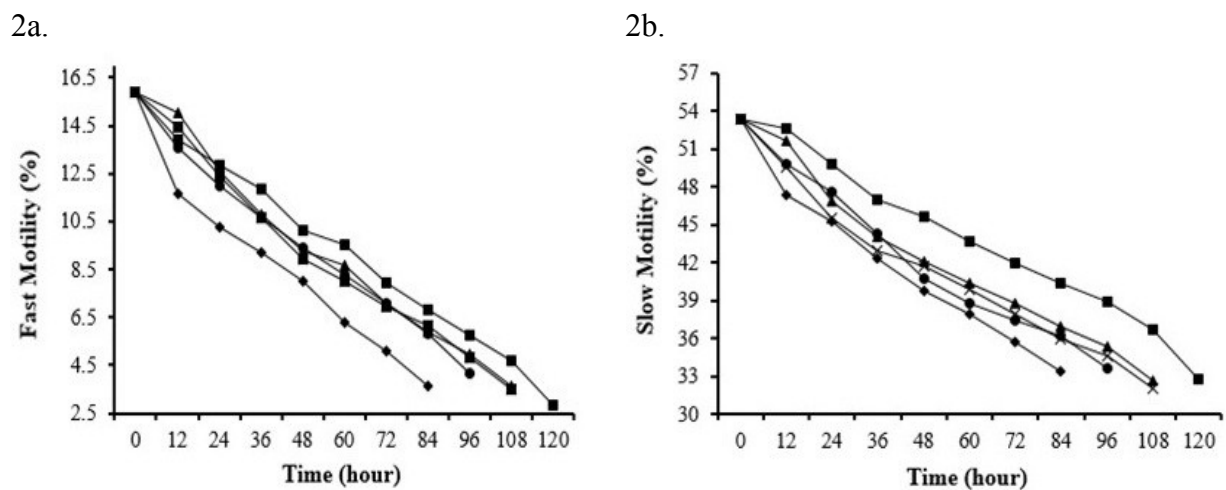
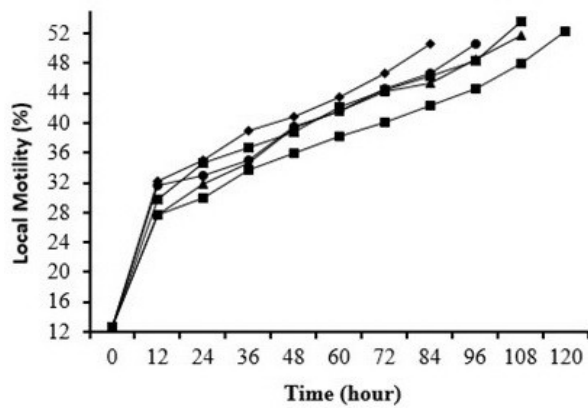


Figure 2. Fast motility (FM) and slow motility (SM) of Pasundan bull sperm in Tris-egg yolk diluents with a various concentrations of L-Carnitine (LC). The symbol represent diluents with 0 mM LC (\square), diluents with 1 mM LC (\blacksquare), diluents with 2 mM LC (\circ), diluents with 3 mM LC (\times) and diluents with 4 mM LC (\triangle).

3a.



3b.

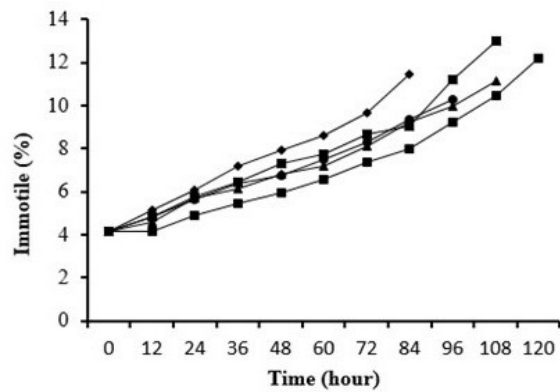


Figure 3. Local motility (LM) and Immotile (IM) of Pasundan bull sperm in Tris-egg yolk diluents with a various concentrations of L-Carnitine (LC). The symbol represent diluents with 0 mM LC (□), diluents with 1 mM LC (□), diluents with 2 mM LC (□), diluents with 3 mM LC (×) and diluents with 4 mM LC (□).

Viability of sperm in TEY with 1 mM L-Carnitine showed the best concentration. A value of $43.95 \pm 0.42\%$ was shown at 108 h of storage, while given with L-Carnitine 2, 3 and 4, the same value was only up to 96 h, even the control was only up to 84 h (Figure 4).

DISSCUSION

The value of the semen volume of Pasundan bulls was higher than that of Baharun *et al.* (2017) who recorded volume of 3.80 ± 0.58 ml collected from the same bulls. The volume of Pasundan bulls semen is still in the normal range, according to Arifiantini (2012) the volume of bulls semen ranges from 2-15 ml. The semen colour is still normal according to Garner and Hafez (2000) that observed the colour of normal ejaculate of bull semen is creamy to milky white. The semen pH is still normal according to Arifiantini (2012) normal range semen of mammals from 6.0-7.5.

Microscopic evaluation showed that the motility value of sperm was lower when compared with Baharun *et al.* (2017), that showed the motility value of $82.41 \pm 2.97\%$ in the same type of bulls. The sperm concentration in this study was slightly lower than Baharun *et al.* (2017) in Pasundan bulls, that was $1355.85 \pm 6.06 \times 10^6$ m^{-1} . The sperm concentration in this study was still in the normal range according to Garner and Hafez (2000). Normal sperm

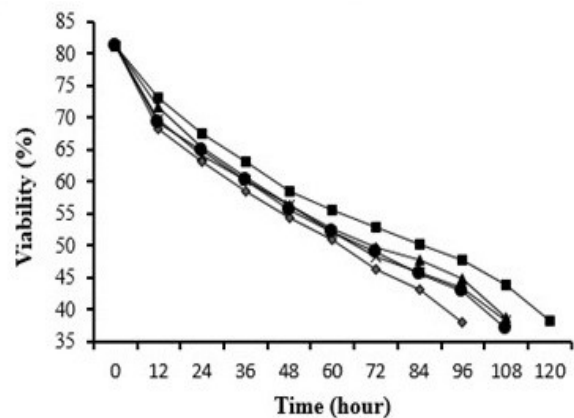


Figure 4. Viability of Pasundan bull sperm in Tris-egg yolk diluents with a various concentrations of L-Carnitine (LC). The symbol represent diluents with 0 mM LC (□), diluents with 1 mM LC (□), diluents with 2 mM LC (□), diluents with 3 mM LC (×) and diluents with 4 mM LC (□).

concentration in bulls ranges from $800-2000 \times 10^6$ sperm ml^{-1} . Sperm viability and abnormalities, obtained in this study almost the same value as Baharun *et al.* (2017) that indicate the viability and abnormalities of $84.37 \pm 1.05\%$ and $11.13 \pm 0.39\%$ respectively. The quality of fresh semen from this study qualifies to be processed as liquid semen.

Under physiological conditions, sperm produce small amounts of ROS, which are needed for capacitation and acrosomal reaction (Agarwal *et al.*, 2003). Since mammalian sperm plasma membrane and cytoplasm contain large amounts of polyunsaturated fatty acids; are vulnerable to excessive amount of ROS that produced from dead, abnormal sperm cell as well as leukocytes content (Agarwal and Prabakaran, 2005). The longer periods of storage, sperm may lead to an increased level of dismutation of the superoxide anion generating higher levels of oxidative stress within the sperm sample (de Lamirande and O'Flaherty, 2008). As expected, the TM and PM score on all treatments declined with respect to time. However, semen supplemented with 1 mM of L-Carnitine retained a PM score of at least 40% up to h 84 of the study.

In this study, the addition of L-Carnitine proved to be better than controls. Concentration of 1 mM L-Carnitine in TEY diluents showed the best effect in all variables. The high PM in TEY diluents supplemented by L-Carnitine is related to the function of L-Carnitine which can increase mitochondrial activity (Fattah *et al.*, 2017a). The mitochondria contained in the middle part of the sperm was responsible to produce ATP which is used for the sperm movement.

The addition of 1 mM L-Carnitine also proved to be better than controls in of motile sperm (Figure 2a and b). Figure 3a and b (local and immotile sperm) in liquid semen added with 1 mM L-Carnitine demonstrated lower value than control or other concentration of L-Carnitine. This is related with the functions of L-Carnitine to facilitate the transport of fatty acids to the mitochondrial membrane to produce ATP through the process of β -oxidation so it can increase sperm motility (Agarwal and Said, 2004). Supplementation of L-Carnitine in liquid stored diluents according to Longobardi *et al.* (2017) can increase ATP production by up to twice when compared to controls. Supplementation of L-Carnitine in the diluents leads to a partial removal of Na from diluents (Gibb *et al.*, 2015) to maintain isotonicity (Silver and Erecinska, 1997). Na increases the depletion of ATP via activation of Na-ATPase pumps (Silver and Erecinska, 1997).

The research on addition of L-Carnitine in bull liquid semen has never been reported. The addition of L-Carnitine to liquid semen was only reported in rooster semen (Tabatabaei and Aghaei, 2012; Fatah *et al.*, 2017a), rabbit (Sariozkans *et*

al., 2014) and horses (Gibb *et al.*, 2015). All researchers conclude that the addition of L-Carnitine to liquid semen can improve the quality of semen during preservation.

This research had found that adding of L-Carnitine (1 mM) to Pasundan bull semen diluent can enhance its sperm viability $43.95 \pm 0.42\%$ until 108 h by looking through Figure 4. L-Carnitine as anti-oxidant (Solarska *et al.*, 2010), may protect sperm plasma membrane with high level of unsaturated fatty acid content (Aitken *et al.*, 2010). The process of liquid semen causes the formation of ROS. ROS can induced the lipid peroxidation due to mammalian sperm membrane has high polyunsaturated fatty acids, thus resulted in the deterioration of sperm viability.

Antioxidant characteristics and anti-apoptotic activities of L-Carnitine have a protective role against damaging effects of ROS and may stabilize mitochondrial membrane and DNA structure (Qi *et al.*, 2006). L-Carnitine also increases the activity and levels of antioxidant enzymes such as superoxide dismutase and glutathione peroxidase (Neuman *et al.*, 2002). L-Carnitine arise from the scavenging of ROS, destroyed hydrogen peroxide and has a function on metal chelation as well as inhibition of xanthine oxidase activity by L-Carnitine (Surai, 2015).

The evidence of the decreasing in lipid peroxidation at 48 h of liquid semen testing with additional L-Carnitine by 1 and 2 mM was reported by Fattah *et al.* (2017a). In addition, according to Longobardi *et al.* (2017), total antioxidant capacity (TAC) increases and ROS production decreases when L-Carnitine was added to the semen diluents. On the other hand, L-Carnitine has a function to interact with membrane phospholipid, which modulates fluidity of plasma membrane (Fattah *et al.*, 2017b). The decreasing of ROS and cell membrane can be maintained, this will cause the enhancement of sperm viability. This mechanism proved that it is matched with the result of our study that sperm viability that supplemented with L-Carnitine was better than control.

Tabatabaei and Aghaei (2012) reported that the sperm viability of chicken liquid semen given with L-Carnitine supplementation as much as 1% at 24 h showed significant results compared with L-Carnitine 2% and 3%. Fattah *et al.* (2017a), reported that 2 mM L-Carnitine supplementation was the best results of chicken sperm viability at 48 h when compared with L-Carnitine

concentrations of 0.5, 1, 4 and 8 mM. The present study showed that 1 mM of L-Carnitine supplementation demonstrated the best results, it was probably due to the differences in physiology between bulls and chicken sperm.

L-Carnitine plays an essential role in β -oxidation (Gib *et al.*, 2015). Addition of L-Carnitine to diluents act as antioxidant and osmolyte. L-Carnitine assisting mitochondrial ATP production through the transportation of acetyl groups from pyruvate into the mitochondrial matrix and the buffering of free coenzyme-A (CoA). Production of ATP, thus maintained sperm motility. In the other hand L-Carnitine is powerful osmolyte (Fattah *et al.* 2017) and therefore in the present study its supplementation to diluents in higher concentrations of L-Carnitine (2, 3 and 4 mM) might related along with a high osmolality, and interrupt sperm plasma membrane thus decrease sperm motility.

CONCLUSION

The conclusion based on the best finding results demonstrated that the supplementation of L-Carnitine (1 mM) in the diluents medium not only preserve the sperm cell motility parameters but also preserving its viability of Pasundan bull liquid semen. Further applied reserach should be conducted either in the field using AI and or in the laboratory using in vitro fertilization (IVF) test to determine that supplemented 1 mM L-Carnitine in the diluents can enhanced the fertility.

CONFLICT OF INTEREST

There is no conflict of interest with any financial, personal, or other relationship with other people or organization related to the material discussed in the manuscript.

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