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Supplement Effects of Vitamin C, Vitamin E and the Combinations in Semen Extenders of Kub Chicken Quality

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Abstract | The study aims to determine the effects of vitamin C, vitamin E, and the combinations of vitamin C and E supplements on sperm quality of KUB Chicken for 4, 8, 24, and 48 hours in 4 °C at cold storage. A total of twelve KUB chickens were used in this study. The semen was collected by the dorso-abdominal massage method twice a week and diluted with Ringer's modified solution. The treatments were divided into four different treatment actions (P), the control group (P0) as neutral without supplement added, and the treatments group. The treatments were divided into three different groups, added vitamin C 200 µg/ml (P1), the vitamin E 5 µg/ml (P2), and combinations of vitamin C 200 µg/ml + vitamin E 5 µg/ml (P3). Evaluation rate included sperm motility, recovery rate, viability, membrane integrity, and DNA fragmentation after 2 (T1), 4 (T2), 8 (T3), 24 (T4), and 48 (T5) hours stored at 4°C. Whereas, the evaluation of motility, viability, and membrane integrity of T1, T2, and T3 storage showed no significant difference between treatment groups (P> 0.05), T4 and T5 in treatment group P2 were significantly higher (P<0.05) and P3 were lower (P<0.05) than the other treatment groups. DNA fragmentation examination on fresh semen at T5 storage showed no significant difference between treatment groups (P >0.05). This study concluded that vitamin E 5 µg/ml supplement bests to improve and extend the motility, viability, membrane integrity, and the combination of vitamin C and vitamin E may decrease the sperm quality KUB chickens up to 48 hours storage at 4°C.

Keywords | DNA Fragmentation, KUB chicken, Semen Quality, Vitamin C, Vitamin E.

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INTRODUCTION

Native chicken brings benefits to most households in developing countries and participates in economic growth (Padhi, 2016). The KUB Chicken is a moderate local chicken breed by Indonesian Research Institute for Animal Production (IRIAP) with high-productivity eggs and better weight gain ability compared to ordinary native chickens (Erwan, 2020). Artificial Insemination (AI) can be administered in the process of mating to increase the KUB Chicken population, but spermatozoa of KUB chicken contains high polyunsaturated fatty acids (PUFA) that are easily oxidized by reactive oxygen species (ROS) and caused lipid peroxidation (Khan, 2011). Lipid peroxidation can produce toxic biological substances that cause permanent damage to the sperm (Wen et al., 2020). Lipid peroxidation can lead to decreased motility, damaged the DNA spermatozoa, and reduced fertility (Peruma et al., 2013).

In the storage, spermatozoa usually expose to a cold shock and oxidative damage which results in decreased motility and fertility. The production of ROS is a normal physiological process in spermatozoa. However, the over-produced ROS may cause structural damage to the sperm membrane (De Lamirande et al., 1997). Oxidative-stressed is a factor that increases cell damage due to reactive oxygen species (ROS). The excessive ROS production in sperms is dangerous due to its negatives effect on counting the active spermatozoa (Prihantoko et al., 2020a). To prevent peroxidative damage as a result of ROS, the membrane must be layered by a highly effective antioxidant system. Catalase (CAT), superoxide dismutase (SOD), and glutathione peroxidase (GPx) are examples of the enzyme with roles as natural inhibitors which are similar to the antioxidant mechanism in sperm: vitamin A, C, E, uric acid, glutathione, and carotenoids (Bréque et al., 2003; Cerolini et al., 2006; Partyka et al., 2012). ROS production and the antioxidant system have to be balanced to maintain the viability and fertility of spermatozoa. Several studies have examined the effect of antioxidants on variable chicken sperm after cryopreservation (Amini et al., 2015a; Amini et al., 2015b; Moghbeli et al., 2016). Other several antioxidant studies applied to different species, for example, the additions of genistein antioxidant to Ongole grade bull semen (Prihantoko et al., 2020a).

Vitamin C is naturally a free radical exposure with its impact on optimizing sperm performance by reducing cell damage include the lipid peroxidase (Hu et al., 2010). The ascorbic acid will bring the best effect on the spermatozoan quality performance through its radical-scavenging by reducing cell injury (Amini et al., 2015a). Vitamin E is a fat-soluble compound with a membrane-stabilized inhibitor that performs effectively on cell membranes (Tabatabaei et al., 2011). According to Jeong et al. (2009), vitamin E can break the covalent bond of ROS that is formed within the fatty acids, besides, it is the major protectant against ROS and lipid peroxidation. Vitamin C and vitamin E functioned as antioxidants may decrease lipid peroxidation and increase the quality of semen. The combination of both vitamins in dietary has been observed to provide a marked increase in better protection against spermatozoa damage (Uzochukwu et al., 2020), therefore this study aimed to evaluate the supplementation effects of vitamin C, vitamin E, and the combinations in semen extenders to the variable of KUB chicken semen quality for 48 hours stored at 4°C.

MATERIALS AND METHODS

Research Animals

The study used a total of 12 male KUB chickens aged 1.5-2 years. The KUB chickens were housed in individual cages at 32°C in the Chicken Breeding Cage, Faculty of Veterinary Medicine, Gadjah Mada University in Yogyakarta, Indonesia. The chickens were fed by a commercial feed containing 19% crude protein without antioxidant supplementation and ad libitum water. The chickens were adapted for two weeks and at the same time trained to collect the semen.

Semen Collection

Semen samples were collected by dorsal-abdominal massage method every twice a week for four weeks. The sampling with semen volume of 0.2-0.6 mL, a concentration of ≥ 3×109 sperm/mL, motility ≥ of 80%, and abnormal morphology ≤ 10% were used in this study. Then the sampling was thawed in a water bath (37°C) and analyzed macroscopically and microscopically.

Extender preparation

The base extender used in this study contained ringer lactate, egg yolk, penicillin, and streptomycin. Then the base was added with vitamin E 5µg/mL (Sigma, St. Louis, MO, USA), vitamin C 200µg/mL (Merck KGaA, Darmstadt, Germany), and the combinations (vitamin E 5µg+vitamin C 200µg/mL). Control diluent was used the base extenders composition only.

Semen storage

The semen mixed with the extenders fluids, the solution was diluted to a final concentration of 5×106 sperm/mL and placed in a 0.25 ml straw (Minitube, Slovakia) according to the control label (P0), vitamin C (P1), vitamin E (P2). The combinations (P3) were placed on a cooling shelf at 4°C. Spermatozoa quality was evaluated at 2 (T1), 4 (T2), 8 (T3), 24 (T4), and 48 (T5) hours after being stored. Then the straws were warmed in a water bath at 37°C for 15 seconds.

Semen Evaluation

Sperm Motility: Sperm motility evaluation used a sample of 10 µL (400×104 sperm cells). Then dropped the semen in a warm glass slide with the coverslip. Then the semen was observed using a light microscope (400x) under five different fields of glass slide view. The motility evaluation was subjectively assessed by percentages on a scale range of 0-100%.

Sperm Viability: Sperm viability was assessed by the eosin-nigrosin staining method (Akhlaghi et al., 2014). A mixture of 10 µl semen and 20 µl eosin nigrosine staining was placed on a warm slide object-glass and smear within 15 seconds, dried. The dried smear was observed by counting 200 cells under a light microscope with 400x magnification. Living spermatozoa were characterized by an intact membrane structure and undyed body and dyed body to the dead sperm due to the damaged membrane sperm.

Table 1: Percentages of sperm motility KUB chicken spermatozoa stored at 4°C in various diluent treatments within 48 hours of observation

Treatment	Observation Time				
	T1 (%)	T2 (%)	T3 (%)	T4 (%)	T5 (%)
P0	86.50 ± 2.646^{x}	83.00 ± 2.160^{x}	79.00 ± 3.367^{x}	63.50 ± 10.755^{x}	31.00 ± 8.400^{x}
P1	87.75 ± 1.258^{x}	84.75 ± 2.062^{x}	79.50 ± 4.796^{x}	62.00 ± 7.071^{x}	38.50 ± 10.59^{x}
P2	90.75 ± 1.708^{x}	85.50 ± 4.203^{x}	83.50 ± 2.887^{x}	76.25 ± 5.058^{y}	64.75 ± 4.113^{y}
P3	87.50 ± 2.380^{x}	84.00 ± 1.414^{x}	79.25 ± 3.686^{x}	48.75 ± 8.539^{z}	16.25 ± 4.787^{z}

x, y, z: different superscripts in the same column indicate significant differences (P≤0,05)

Table 2: Percentages of sperm viability KUB chicken stored at 4°C in various diluent treatments within 48 hours of observation

Treatment	Observation Time				
	T1 (%)	T2 (%)	T3 (%)	T4 (%)	T5 (%)
P0	91.75 ± 2.630^{x}	85.25 ± 3.862^{x}	83.75 ± 4.573^{x}	64.00 ± 8.832^{x}	49.25 ± 8.694^{xy}
P1	91.50 ± 3.697^{x}	87.75 ± 2.986^{x}	82.75 ± 4.031^{x}	63.75 ± 2.630^{x}	46.00 ± 6.683^{y}
P2	95.50 ± 2.082^{x}	91.25 ± 1.500^{x}	88.50 ± 3.873^{x}	76.75 ± 2.986^{y}	63.50 ± 2.380^{z}
P3	92.25 ± 1.708^{x}	88.00 ± 2.160^{x}	84.75 ± 2.986^{x}	61.00 ± 13.292^{x}	27.75 ± 10.372^{x}

x, y, z : different superscripts in the same column show significant differences (P≤0.05)

Table 3: Percentages of sperm membrane integrity of KUB chicken stored at 4°C in various diluent treatments within 48 hours of observation

Treatment	Observation Time				
	T1 (%)	T2 (%)	T3 (%)	T4 (%)	T5 (%)
P0	85.50 ± 1.291^{x}	80.25 ± 3.500^{x}	74.75 ± 3.594^{x}	62.25 ± 2.872^{x}	44.50 ± 1.29^{x}
P1	86.25 ± 2.500^{x}	80.75 ± 2.062^{x}	75.50 ± 3.416^{x}	60.75 ± 2.217^{x}	41.75 ± 1.258^{x}
P2	88.50 ± 1.732^{x}	84.25 ± 1.708^{x}	80.25 ± 2.217^{x}	72.75 ± 3.202^{y}	61.25 ± 2.380^{z}
P3	88.25 ± 2.062^{x}	83.50 ± 1.732^{x}	78.00 ± 3.162^{x}	57.50 ± 3.697^{x}	18.50 ± 3.109^{y}

x, y, z : different superscripts in the same column show significant differences (P≤0.05)

Membrane Integrity: Membrane integrity examination used the HOST test (hypoosmotic swelling test) which was previously determined by Santiago-Moreno et. (2009) with modification. HOST solution consists of 0.49 g sodium citrate, 0.9 g fructose, and 100 ml distillate water. A 20 µl semen was diluted to 200 µl HOST solution and briefly incubated for 45 minutes at 25°C. The solutions were smeared, fixated with methanol for 10 minutes, washed, and dried. The smear was observed under the light microscope by counting 200 cells of spermatozoa with 400 x magnification. An intact membrane of spermatozoa had swollen and coiled tails while the damaged spermatozoa characterized with straight tails.

DNA Fragmentation: Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) Kit, TMR Red version 12 (Merck, Australia) were being used to evaluate the DNA fragmentation. The spermatozoa smear was fixated 60 minutes, added the permeability solution 0.1% of Triton X-100 in 0.1%, sodium citrate for 2 minutes in ice at 2-8°C, washed with PBS saline (phosphate buffer saline) twice, and dried. A mixture of 50 µl TUNEL was reacted to the smeared, incubated for 60 minutes at 37°C in the dark, and after that gently rinsed with PBS three times. The labeling was performed under a laser confocal microscope with a wavelength of 470-495 nm. Fragmented DNA of spermatozoa will be seen as fluorescent green.

Statistical Analysis

Data were analyzed using one-way analysis of variance (ANOVA) followed by Duncan's multiple range test to determine differences between the treatments. A difference of P<0.05 was considered statistically significant. SPSS version 25.0 was used in this study (SPSS Inc., Chicago, IL, USA).

RESULT AND DISCUSSION

The total average of fresh semen examination on volume, pH, concentration, motility, viability, and abnormalities were 0.26 ± 0.06 ml, 7.18 ± 0.54, 4.24 ± 0.90 x 109 sperm/mL, 92.00 ± 2.47%, 95.00 ± 1.59% and 5.31 ± 1.62%. Otherwise, evaluation of the motility, viability, and membrane

integrity after T1, T2, and T3 stored, there were no significant differences between the treatments (P> 0.05), T4 and T5 of the P2 treatment group were significantly higher (P<0.05) while the P3 treatment group was lower (P<0.05) than the other treatment groups (Tables 1, 2, and 3).

Results found that the decreasing KUB chicken sperm motility correlates with time at the semen storage. The reaction between the spermatozoa and diluents in cooling storage would be affecting metabolic activity (Lukman et al., 2014). The cold storage was set at 3-5°C and a certain temperature, the motility would decrease as it was caused by the sperm's utilized energy which derived from glyceryl-phosphorylcholine, fructose, and sorbitol in semen that can be gradually lowered (Iswati et al., 2018). Oxidative-stressed might occur in cold-stored leads to motility decrease and increase the dead sperm (Khan, 2011). Moreover, the level of vitamin C antioxidant in diluent would not affect the motility due to high levels of vitamin C may lead to inhibit the oxidation. However, it could not directly react to the free radicals in a membrane, as vitamin C works outside the cell (Castelini et al., 2000). According to Gecha and Fagan (1992), vitamin C's protecting ability is at the beginning of fat peroxidation occurred. Furthermore, it does not have a significant effect in the long term of use.

Vitamin E and diluent reaction compared to the control group was significantly higher and affecting the sperm. This study found a similar finding that was conducted by Donoghue and Donoghue (1997), vitamin E addition to sperm diluent in turkey able to maintain the spermatozoa motility up to 75% for 48 hours through in vitro storage. Vitamin E can directly bind to free radicals such as peroxyl and alkoxyl so that free radicals will break the bond reaction and form tocopheroxyl radicals at a stable compound. Vitamin E also could interrupt the peroxyl and alkoxyl radicals during the conversion of lipid hydroperoxides that may lead to trigger the peroxidative chain reactions, it prevents damage through the membrane plasm (Bansal et al., 2009).

The vitamin E viability showed similar results of Donoghue and Donoghue (1997) on turkey which was stored for 24-48 hours and Cerolini et al. (2000) in pigs who demonstrated that adding α-tocopherol (Vitamin E) to semen extender helped in maintaining the sperm viability by in vitro storage for 48 hours. The longer the semen is being stored, then the higher possibility of spermatozoa mortality increase due to membrane plasm damage. It might disrupt the spermatozoa energy supply and reduce motility. The total amount of dead spermatozoa will affect the living spermatozoa during the storage process (Solihati et al., 2006). Adding Vitamin E to semen extenders tends to improve semen quality in chickens by increasing the viability (Sarica et al., 2007). Vitamin E directly reacts to cells providing a protective effect against free radicals that can maintain the spermatozoa, which is different from vitamin C reacts to the outside cells (Donoghue and Donoghue, 1997). The sperm membrane plasm contains high amounts of unsaturated fatty acids. Thus, it is susceptible to peroxidative damage which is shown by the subsequent loss of membrane integrity. A failure of sperm cell functions will decrease sperm motility (Tabatabaei et al., 2011).

Lipid peroxidation tends to continuously happen (autocatalyst) since the reaction releases different reactive oxygen species (ROS) which could lead to a new reaction of lipid peroxidation. Eventually, it affects the entire membrane plasm of sperm. Lipid peroxidation can alter the membrane function results in metabolism, morphology, motility, and sperm fertility reduction (Prihantoko et al., 2020b). Vitamin E in semen extender roles as an effective membrane-stabilized antioxidant, a natural component of male gametes, and protecting the sperm cell membranes from damage (Tabatabaei et al., 2011), furthermore, vitamin E inhibits oxidative damage through action binding against toxic free radicals, particularly the ROS (Uzochukwu et al., 2020). The result study of vitamin C and the combinations showed no significant effect to maintain the membrane integrity.

Table 4: Percentages of fresh sperm DNA fragmentation KUB chicken and stored at 4°C at 48 hours of observation

Treatment	Observation Time	
	Fresh	T5 (%)
P0	0.875 ± 0.25	2.625 ± 0.478^{ns}
P1	0.875 ± 0.25	2.125 ± 1.030^{ns}
P2	0.875 ± 0.25	2.000 ± 0.816^{ns}
P3	0.875 ± 0.25	2.375 ± 1.108^{ns}

ns: different, not significant (P≥0,05)

Evaluation of DNA fragmentation in spermatozoa after 48 hours of storage at 4°C did not show significant DNA damage (Table 4). It is in accordance with the opinion of Gliozzi et al. (2011) who stated that spermatozoa DNA, both the proportion of cell DNA and the proportion of tail DNA did not change after the equilibration time at temperature 4°C, and there was a negative correlation between damaged sperm DNA and sperm motility and membrane integrity. It indicates that the decrease in the value of mortality and integrity of the spermatozoa membrane along with the length of storage time will not affect the DNA damage of the spermatozoa. The main factors involved in the etiology of DNA damage in spermatozoa include oxidative stress, deficiency in

natural processes such as chromatin packaging, and failure of apoptosis (Sharma et al., 2004). Oxidative stress provides exposure to DNA which causes more than 20 types of damage to DNA bases resulting in oxidized and fragmented nitrogen bases (Slupphaug et al., 2003).

CONCLUSION

This study has shown that the level of vitamin E supplement 5 µg/ml to the semen extender would increase the motility, viability, and integrity to the membrane up to 48 hours at 4°C stored. Additions of vitamin C did not affect any sperm quality parameters and the combination of vitamin C and vitamin E had negatives effect on the quality of spermatozoa in KUB chicken at the storage.

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CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest in the study publication include the funding and data research.

AUTHOR CONTRIBUTION

All authors participate from the beginning of this study until the publications are released.

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