

The effect of adding vitamin c and e in native chicken semen extender stored at temperature 4 °c on semen quality and egg fertility

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ABSTRACT: The objective of this research was to verify the addition of vitamin E (α -tocopherol) and vitamin C (ascorbic acid) as antioxidants and the storage at 4°C on semen quality and fertility. Semen was collected and pooled once time every 3 days from 10 cockerels aged 56 weeks. It was diluted into Buffer phosphate and divided into seven (7) treatments, i.e ; control; vitamin E (α -tocopherol 0.1 mM, 0.3 mM, 0.5 mM), and vitamin C (ascorbic acid 2,500 μ g/mL; 5,000 μ g/mL; 7,500 μ g/mL), and then was stored at 4°C for 72 hours. Semen quality was evaluated after 0 hour, 6 hour, 24 hour, 48 hour, and 72 hours storage. Data were analyzed by analyses of variance split subject, and Duncan's Multiple Range Test (DMRT). A total of 24 hens of Lohman Brown commercial layers were inseminated with 400 x 10⁶ sperm for chicken fertility trials. The result showed that diluents treatment gave significantly effect ($P \leq 0.05$) on pH and sperm abnormality, but not different on motility and viability. Stored sperm gave significantly effect ($P \leq 0.05$) on motility, pH, viability, and sperm abnormality. Vitamin E improved the fertilizing ability until 93.10 \pm 5.98 % of semen stored for 6 hours at 4°C at the dose of 0,3 mM of semen diluent. It can be concluded that addition of vitamin E to extended chicken semen keeps motility, viability, and sperm normal morphology after 72 hours storage at 4°C. In contrast, vitamin C decrease motility, pH, and sperm viability.

Key words: native chicken, buffer phosphate, vitamin C, vitamin E, semen quality, fertility

INTRODUCTION

Major problem faced by the technology of artificial insemination is the viability spermatozoa after ejaculation, due to losing its integrity and ability to fertilize of the a few months of collection (Tri-Yuwanta *et al.*, 1998). Lowering of sperm quality which is store in vitro will affect to degradation of fertility in a short time.

Proportion Polyunsaturated fatty acids (PUFA) in fowl spermatozoa membrane keep the stability and membrane flexibility during the fertilization process. Long and Kramert (2003) argued that the damage caused by lipid peroxidation in poultry sperm includes damage to morphology, degradation of motility and lower fertility. In addition, Blesbois (2007) reported damage of membrane spermatozoa caused degradation of motility and differences in metabolic factor including ATP concentration in spermatozoa. The aim of present study was to evaluate the influence of ascorbic acid and vitamin E in phosphate buffer extender stored at temperature 4 °C on sperm quality and egg fertility.

MATERIALS AND METHODS

Materials Research

Sperm was obtained from ten male native chicken aged 56 weeks which were reared in individual cages. Males were consumed commercial feed for native chicken in ad libitum, that was R-19 produced by PT Japfa Comfeed Surabaya. Twenty-four Lohmann Brown layer hens aged 92 weeks used for artificial insemination (AI), were also reared in individual cage.

Material utilized was Na- phosphate monobasis, Na- phosphate dibasis, aquadest, alcohol, gentamicine, vitamin E (α -tocopherol) produced by E-merck, vitamin C (L(+)) ascorbic acid produced by E-merck with purity rate 99,7%), ethanol, hayem solution and eosin.

Research Methods

Semen was collected according to Burrows and Quinn method (1937) that was dorso-abdominal massage (Donoghue and Wishart, 2000). Each chicken was collected once for every replication.

Semen extender used Buffer phosphate (BP) with Gentamicin 0.5 mg/100 mL and pH adjusted to 7.2 (Anonimus, 2004). Buffer phosphate divided into seven different tubes. One tube for extender without vitamins addition, whereas the next three tubes added by vitamin C ; 2500 µg/mL, 5000 µg/mL, and 7500 µg/mL, respectively. Then the last of three tubes added by vitamin E to produces final concentration 0.1 mM, 0.3 mM, and 0.5 mM respectively. Vitamin E was added in the form of stock solution in ethanol.

Pooled semen was assessed, then divided into seven equal parts. Each parts was diluted in the ratio of sperm: extenders was 1: 2. Treatment in tube I for sperm with BP/ Buffer phosphate (control); tube II for sperm with BP which added vitamin C 2500µg/mL (C1); tube III for sperm with BP which added vitamin C 5000µg/mL (C2); tube IV for sperm with BP which added vitamin C 7500µg/mL (C3); tube V for sperm with BP which added vitamin E 0,1 mM (E1); tube VI for sperm with BP which added vitamin E 0,3 mM (E2); and tube VII for sperm with BP which added vitamin E 0,5 mM (E3). Every treatment stored in refrigerator (temperature 4°C) for 72 hours. Observations were carried out for five times at 0, 6th, 24th, 48th and 72th hours. Each treatment was replicated five times from five different storage times.

Artificial insemination was performed to assess fertility rate. Only BP, E1, E2, and E3 were tested for fertility because C1, C2 and C3 produce lower semen quality than control. Each treatment tested by artificial insemination with three times a replication and each replication consisted of two hens.

Hens were inseminated with liquid semen which had been stored at temperature 4 °C during 6 hours. Artificial insemination performed with intravaginal method at 2.00 to 3.00 PM or 4 to 3 hours before dark. Artificial insemination in the manner and the same dose (200×10^6) was repeated again the next day for each chicken treatment.

Eggs were collected on days 2 to 7 after first artificial insemination. The fertility was determined by candling the eggs 7 days after the incubation started.

Analysis Data

Data obtained from fresh sperm (includes volume, color, consistency, concentration, motility, viability, abnormalities and fertility) was analyzed with a mean and standard deviation. Motility, pH, viability, and abnormality after in vitro treatment were analyzed with Split design subject, followed by DMRT test (Astuti, 1980).

RESULTS AND DISCUSSION

Fresh Sperm of Native Chicken

Fresh sperm characteristic of native chicken after storage is presented at Tables 1 below.

Table 1. Fresh sperm characteristic of native chicken

Parameter	Result (average ± SD)
Volume (mL)	0.25±0.09
Colour	White
Consistency	Viscous
pH	7.07±0.08
Concentration ($\times 10^9$ spermatozoa/mL)	369±1.10
Motility (%)	86.67±8.16
Viability (%)	96.80±3.35
Abnormality (%)	5.26±3.18

The results showed fresh sperm of native chicken had average of volume 0.25±0.09 mL; white colour; consistency viscous; pH 7.07±0.08; concentration $3.69 \pm 1.10 \times 10^9$ /mL; motility 86.67±8.16%;

viability 96.80±3.35%; abnormality 5.26±3.18%. Results of this research within normal limits, which was volume ranges from 0.1 to 0.9 ml (Etches, 1996); viscous white color (Utomo, 1997); pH between 7.1 to 7.25 (Tri-Yuwanta *et al.*, 1998); concentrations between 3.10⁹ to 7.10⁹ spermatozoa/mL (Hafez, 2000); viabilities with average 96,64% and abnormalities around 8% (Blesbois *et al.*, 2005).

Native Chicken Sperm After Diluted Using Buffer Phosphate with Addition Vitamin C or E and Kept in Temperature 4 °C for 72 hours

Motility

Motility of stored sperm in vitro at temperature 4 °C during 72 hours with treatments of diluent BP, C1, C2, C3, E1, E2, and E3 can be seen at Table 2 below.

Table 2. Average of sperm motility stored at temperature 4 °C in various treatments of diluent for 72 hours

Extender	Perception hour					Average ^{ns}
	0 hour ^{ns}	6 hours ^{ns}	24 hours ^{ns}	48 hours ^{ns}	72 hours	
BP	80.00±6.12 ^x	78.00±6.71 ^x	69.00±18.51 ^{xy}	57.00±28.64 ^x _y	41.00±31.70 ^y _a	65.00±24.37
C1	71.00±18.17 ^x	68.00±17.54 ^x	62.60±16.70 ^x	38.60±17.95 ^y	14.00±13.87 ^z _b	50.84±26.97
C2	74.00±13.87 ^x	74.00±13.87 ^x	62.40±19.27 ^x	37.00±24.40 ^y	16.00±12.94 ^y _b	52.68±28.17
C3	77.00±7.58 ^x	75.00±11.73 ^x	66.00±16.73 ^x	37.40±22.23 ^y	13.60±8.44 ^{z,b}	53.20±28.72
E1	78.00±8.37 ^x	77.00±7.58 ^x	73.00±7.58 ^x	61.40±15.16 ^x _y	49.00±20.12 ^y _a	67.68±16.24
E2	78.00±8.37 ^x	75.00±10.61 ^x	65.60±17.44 ^{xy}	58.00±23.61 ^x _y	46.00±24.34 ^y _a	64.52±20.35
E3	79.00±6.52 ^x	75.00±10.61 ^x	69.60±14.26 ^{xy}	59.00±23.29 ^x _y	52.00±19.56 ^y _a	66.92±17.80

^z: Data with different superscripts within each row were significantly different (P≤0.05)

^{a, b}: Data with different superscripts within each column were significantly different (P≤0.05)

^{ns}: Non significant

Diluted treatment on sperm motility at various observation hours (0, 6, 24, and 48 hours) did not significantly different, except at 72 hours. Motility observation of spermatozoa indicated treatment C1, C2, and C3 were significantly different with BP, E1, E2, and E3 (P≤0.05). Whereas, addition of vitamin C and E did not increase sperm motility.

In vitro storage at temperature 4 °C in the treatment BP, C1, C2, C3, E1, E2, and E3 showed significant differences (P≤0.05) for motility. Motility decreased over storage time, especially after 72 hours in all treatment. Castellini *et al.* (2000) stated that vitamin E provides oxidative stability of stored sperm, while sperm were given additional vitamin C produce ROS in higher level that instantaneously drops fast of motility and motion speed of spermatozoa.

Sperm pH

Observation result of pH sperm stored in vitro at temperature 4 °C during 72 hours with treatments of diluent BP, C1, C2, C3, E1, E2, and E3 can be seen at Table 3 below. Results showed the treatment of diluents in various observation hours were significantly different (P≤0.05). It's due to addition of vitamin C into diluent results degradation of pH. More and more vitamin C level given will decline pH sperm.

Table 3. Average of pH of sperm stored at temperature 4 °C in various treatments of diluent during 72 hours

Extender	Perception hour					Average
	0 hour	6 hours	24 hours	48 hours	72 hours	
BP ^{ns}	7.10±0.07 ^a	7.12±0.04 ^a	7.06±0.26 ^b	7.08±0.33 ^{ab}	7.16±0.11 ^a	7.10±0.18 ^a
C1 ^{ns}	6.84±0.05 ^b	6.84±0.05 ^b	6.88±0.90 ^b	6.90±0.10 ^b	6.84±0.13 ^b	6.86±0.09 ^b
C2 ^{ns}	6.64±0.05 ^c	6.60±0.10 ^c	6.68±0.16 ^{ab}	6.66±0.17 ^c	6.70±0.20 ^{bc}	6.66±0.14 ^c
C3 ^{ns}	6.48±0.08 ^d	6.46±0.05 ^d	6.54±0.18 ^c	6.48±0.15 ^c	6.56±0.13 ^c	6.50±0.12 ^d
E1	7.04±0.09 ^{y,a}	7.06±0.09 ^{xy,a}	7.18±0.04 ^{x,a}	7.14±0.10 ^{xy,a}	7.12±0.11 ^{xy,a}	7.11±0.10 ^a
E2 ^{ns}	7.06±0.55 ^a	7.16±0.89 ^a	7.16±0.11 ^a	7.14±0.09 ^a	7.12±0.11 ^a	7.13±0.10 ^a
E3 ^{ns}	7.08±0.08 ^a	7.08±0.11 ^a	7.12±0.08 ^a	7.16±0.09 ^a	7.14±0.13 ^a	7.12±0.10 ^a

^{x,y} : Data with different superscripts within row were significantly different (P≤0.05)

^{a,b,c,d} : Data with different superscripts within column were significantly different (P≤0.05)

^{ns} : Non significant

In vitro storage treatment at temperature 4°C showed significantly different (P≤0.05) to sperm pH. All pH treatments tends to still after stored during 72 hours, but just in treatment E1 showed existence significantly different (P≤0.05). Motility and metabolic rate of spermatozoa convertible by diluent pH, low pH can reduce the motility, lactic acid production, and oxygen demand of chicken spermatozoa, whereas high pH increased metabolic rate in vitro (Donoghue and Wishart, 2000).

Viability

The results of spermatozoa viability which stored in vitro at temperature 4 °C during 72 hours with treatment of diluent BP, C1, C2, C3, E1, E2, and E3 is presented at Table 4 below.

Table 4 . Average of native chicken sperm viability stored at temperature 4 °C in various semen extenders for 72 hours

Extender	Perception hour					Average
	0 hour ^{ns}	6 hours ^{ns}	24 hours ^{ns}	48 hours ^{ns}	72 hours	
BP	96.12±3.63 ^x	93.40±3.85 ^{xy}	89.24±7.42 ^{xy}	77.34±8.24 ^{yz}	61.80±24.81 ^z _a	83.58±17.15 _{ab}
C1	94.32±5.09 ^x	93.43±3.24 ^x	87.46±10.27 ^{xy}	73.76±15.33 ^y	29.60±16.82 ^z _b	75.71±26.83 _b
C2	94.24±5.53 ^x	94.52±3.82 ^x	88.02±8.12 ^{xy}	75.50±14.87 ^y	31.00±15.12 ^z _b	76.66±26.19 _{ab}
C3	95.60±3.44 ^x	94.28±3.95 ^x	86.20±8.23 ^{xy}	75.86±11.87 ^y	30.60±14.45 ^z _b	76.51±25.97 _{ab}
E1	96.94±3.35 ^x	95.14±4.26 ^{xy}	88.94±10.99 ^{xy}	80.32±11.91 ^y	64.50±16.92 ^z _a	85.17±15.58 _a
E2	95.98±3.08 ^x	95.37±2.70 ^x	87.42±9.10 ^{xy}	77.11±10.50 ^{yz}	62.20±23.13 ^z _a	83.62±17.10 _{ab}
E3	97.20±2.95 ^x	95.80±2.59 ^x	88.48±7.46 ^{xy}	81.32±10.08 ^y	66.20±18.86 ^z _a	85.80±14.89 _a

^{x,y,z} : Data with different superscripts within row are significantly different (P≤0.05)

^{a,b} : Data with different superscripts within column are significantly different (P≤0.05)

^{ns} : Non significant

Dilution treatments on the sperm viability at various hours of observation showed no significant differences except at 72 hours. Observation at 72 hours showed significantly different (P≤0.05). C1 treatment showed lowest viability, while E3 had the highest viability after stored during 72 hours at temperature 4 °C. Similar to statement of Donoghue and Donoghue (1997) that addition of vitamin E 80 µg/mL into turkey semen diluent can maintain spermatozoa viability up to 68.6 % when stored in vitro during 48 hours.

In vitro storage at 4 °C on the spermatozoa viability of the treatment BP, C1, C2, C3, E1, E2, and E3 at various hours of observation were significantly different (P≤0.05). It was due to vitamin C only

works as an extracellular antioxidant, while vitamin E is an intracellular antioxidant (Donoghue and Donoghue, 1997). Castellini *et al.* (2000) also stated that free radical inhibitors on the surface membrane only done by vitamin E.

Spermatozoa Abnormality

The results of spermatozoa abnormality in the diluent treatments BP, C1, C2, C3, E1, E2, and E3 which stored in vitro at temperature 4 °C during 72 hours is presented at Table 5 below.

Table 5. Average of native chicken spermatozoa abnormality stored at temperature 4 °C in various treatments of diluent during 72 hours

Extender	Perception hour					Average
	0 hour ^{ns}	6 hours ^{ns}	24 hours	48 hours	72 hours	
BP	4.52±1.62 ^y	8.68±3.82 ^y	14.30±7.45 ^{x,ab}	16.90±2.50 ^{x,ab}	19.60±2.51 ^{x,abc}	12.80±6.76 ^a
C1	5.60±2.59 ^z	9.36±1.10 ^{yz}	9.68±3.82 ^{yz,bc}	15.00±5.48 ^{y,ab}	23.20±7.16 ^{x,a}	12.57±7.49 ^a
C2	4.60±2.57 ^z	8.66±2.92 ^y	11.60±3.12 ^{y,abc}	16.10±2.26 ^{x,ab}	19.60±3.29 ^{x,abc}	12.11±6.00 ^a
C3	6.07±2.74 ^z	7.46±2.92 ^z	15.28±2.40 ^{y,a}	17.98±2.76 ^{y,b}	20.42±1.06 ^{x,ab}	13.44±6.25 ^a
E1	5.12±1.73 ^z	5.16±1.92 ^z	9.06±1.82 ^{y,bc}	13.28±4.71 ^{x,ab}	16.62±2.53 ^{x,bcd}	9.85±5.27 ^b
E2	5.68±0.50 ^z	6.68±2.39 ^{yz}	8.00±2.55 ^{yz,c}	11.28±6.73 ^{y,b}	14.90±2.12 ^{x,cd}	9.31±4.72 ^b
E3	5.66±2.25 ^y	5.72±1.47 ^y	6.28±1.62 ^{y,c}	11.42±4.33 ^{x,b}	14.02±2.52 ^{x,d}	8.62±4.72 ^b

^{x, y, z} : Data with different superscripts within same row are significantly different (P≤0,05)

^{a, b, c, d} : Data with different superscripts within same column are significantly different (P≤0,05)

^{ns} : Non significant

The results showed that diluents treatments significantly different (P≤0.05) to sperm abnormality at various observation hours (0, 6th, 24th, 48th, and 72nd). Abnormality levels after storage for 72 hours at 4 °C in the treatments of BP, C1 C2, C3, E1, E2, and E3 were 19.60 ± 2.51%, 23.20 ± 7.16%, 19, 60 ± 3.29%, 20.42 ± 1.06%, 16.62 ± 2.53%, 14.90 ± 2.12%, and 14.02 ± 2.52% respectively. The treatments E1, E2, and E3 reduce more sperm abnormality level compared with BP, C1, C2, and C3. It means vitamin E can limit the occurrence of sperm damage by ROS, such as notice of Michael *et al.* (2008) that the addition 0.1 mM of vitamin E in canine sperm extender (yolk-tris glucose) can reduce ROS level.

In vitro storage treatment at temperature 4 °C for abnormalities of spermatozoa to various treatments showed significant differences (P≤0.05). Level of abnormality of each treatment in 0 hours was not significantly different, but after storage the abnormality level was rises. This is due to the occurrence of processes that cause lipid peroxidation, the formation of radicals lipid and lipid hydroperoxide, simultaneously causing a reaction among the radicals, cell dysfunction, decrease of membrane function and integrity (Sanocka and Kurpisz, 2004).

Eggs Fertility

The treatments BP, E1, E2, E3 tested by artificial insemination due to the better in vitro sperm quality than C1, C2, C3. The results of fertility percentage by artificial insemination can be seen in Table 6 below.

Table 6. Results of egg fertility percentage from artificial insemination with various extender treatments

Extender	Replication			Mean ± SD
	I	II	III	
BP	93.80%	85.70%	64.25%	81.25 ± 15.27%
E1	100.00%	75.00%	75.00%	83.33 ± 14.43%
E2	100.00%	89.30%	90.00%	93.10 ± 5.98%
E3	100.00%	90.00%	71.30%	87.10 ± 14.57%

Treatment E2 had the highest fertility (93.10±5.98%) then alternately followed by E3, E1, and BP. Extender with vitamin E showed improvement of egg fertility. According to Blesbois *et al.* (1993) that addition of vitamin E 80 µg/mL in chicken sperm extender increase fertility which was stored at 4°C during twenty-four hours. The addition of 40 µg/mL vitamin E in turkey sperm also increase fertility (Long and Kramert, 2003).

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