EFFECTS OF SUPEROXIDE DISMUTASE AND CATALASE SUPPLEMENTATION ON SEMEN QUALITY OF MULTI-SPURS CHICKEN IN LIQUID STORAGE

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ABSTRACT

This study aimed to investigate the effects of enzymatic antioxidants superoxide dismutase (SOD) and catalase supplementation on semen quality of multi-spurs chicken during liquid storage up to 48 h at 4°C. Pooled semen of twelve Vietnamese multi-spurred roosters was randomly divided into 7 experimental groups: control without supplement; SOD supplemented at 100 UI/mLor 200 UI/mL; catalase supplemented at 200 UI/mLor 400 UI/mL, and combination of 100 SOD + 200 catalaseUI/mL or 200 SOD +400 catalaseUI/mL.The highest quality treated semen was selected base on the semen motility, viability, abnormality at 0 h, 24 h, and 48 h of storage, and evaluated the fertility in comparison with control group. The results showed that the motility and viability of stored sperm were increased by the addition of 200 SOD + 400 catalaseUI/mL. Moreover, the fertility of stored chicken sperm was increased ($p<0.05$) compared to that of control treatment. In conclusion, the supplementation of 200 SOD +400 catalaseUI/mL improved the motility, viability, and fertility of multi-spurs chicken sperm during liquid preservation after 24 h and 48 h.

Keywords: *chicken semen; liquid storage; antioxidant; reactive oxygen species*

INTRODUCTION

Multi-spurs chicken, a Vietnamese native breed chicken was found in the mountainous areas of Xuan Son national park of North-Eastern Vietnam. Both rooster and hen have more than eight toes. Such physical traits obstruct the natural mating ability of roosters, which induce very low fertilizing and hatching ratesin this chicken breed. However, the market demand forthis chicken meat has been increasing because it involves the traditional culture and increased incomeoflocal people. Thus, the number of the multi-spurs chicken herd has significantly decreased, and the breed was listed as endangered livestock in Vietnam. The semen preservation and artificial insemination technique were expected to overcome the inefficient natural mating of multi-spursroosters, consequently enhance low fertilizing ability in natural reproduction and finally improve the population of this chicken breed.

In avian, liquid storage semen has been widely used because this technique has simpler process, higher fertility rate and lower cost than cryopreservation, which is largely used in mammals (Cardoso et al., 2020). However, the semen quality and fertility of stored semen were significantly lower than fresh semen after 24 hours of storage. The decrease of semen quality relates to the lipid peroxidation in membrane cells (Blesbois et al., 1999, Donoghue and Wishart, 2000). The lipid peroxidation is a result of the reactive oxygen species (ROS) generation during dilution semen with extender and storage at low temperatures (Chatterjee and Gagnon, 2001; Agarwal et al., 2003). However, lipid peroxidation could be inhibited by the supplementation of antioxidants which can scavenge, remove and suppress the formation of ROS.

The SOD and catalase enzymes that would decrease in the ROS formation and the plasma lipid peroxidation. Therefore, the supplementation of these antioxidants to extender may improve semen quality during liquid storage (Donoghue and Donoghue, 1997; Partyka et al., 2012). The respective supplementation of SOD or catalase could enhance the liquid storage time and semen quality of cat (Thuwanut et al., 2008), ram (Zeitoun and Al-Damegh, 2015) and chicken semen (Partyka et al., 2012; Rosato et al., 2012). However, the effects of the combination of SOD and catalase supplementation on the quality of chicken semen have been limited. Thus, the objectives of the study were to evaluate the effects of enzymatic antioxidants (SOD and catalase) on the quality of multi-spurschicken semen during the 48-hour liquid storage.

MATERIALS AND METHODS

Time and location

The experiment was conducted at Hung Vuong University, Viet Tri, PhuTho, Viet Nam from June to December, 2020.

Animals and experimental design

Twelve Vietnamese multi-spurred roosters with similar ages (28 weeks) and body weight (2.5 kg), were used to collect semen. All birds were numbered (from 1 to 12) and kept in an individual cage under room condition. The birds were given *ad libitum* feeding (CP: 17%, ME: 2700 Kcal/kg DM) and clean drinking water. Semen was collected by the abdominal massage method (Quinn and Burrows, 1936) twice a week for 3 months.

To evaluate the fertility of stored semen, 42 Leghorn hens (42 weeks old) were used to collect eggs. The birds were grouped 6 hens per cage and given *ad libitum* feeding (CP 17%, ME: 2900 Kcal/kg DM) and clean drinking water.

Lake diluentcontained 1,35g sodium glutamate, 0,128g potassium citrate monohydrate, 0,51g sodium acetate, 0,08g magnesium acetate tetrahydrate, 0,8g glucose in 100 mL of distilled water, 300 osmolality/kg of water (Lake, 1960).

To evaluate semen quality during liquid storage,pooled semen was divided into seven experimental treatments consisting: Control include Lake extender without additive; superoxide dismutase (SOD) supplement at100 and 200 UI/mL;catalase supplement at 200 and 400 UI/mL; SOD and catalase supplement at 100 and 200 UI/mLrespectively, and 200 and 400UI/mLrespectively. Semen was diluted with extender at the ratio 1: 3 (vol/vol) and stored at refrigerator (4°C) directly. The stored semen quality was evaluated for 0 h, 24 h and 48 h of preservation.

To evaluate the fertility of stored semen, 42 Leghorn hens were divided into seven groups, six hens pereach group. The hens were artificially inseminated by treated stored semen after 24 h and 48 h of preservation.

Measurements

The mass activity and progressive motility of sperm were evaluated following the description of Herman and Madden (1987). The mass activity of the spermatozoa was determined by the phase-contrast microscope observation (100 × magnification) (Olympus CX41, Japan) at 37°C in a hanging drop, three visual microscopic fields each sample were observed. The mass activity was expressed as scores by a scale from 0 to 5 as:

0 = no activity

1 = 1 to 20% of motile spermatozoa

2 = 20 to 40% of motile spermatozoa

3 = 40 to 60% of motile spermatozoa

4 = 60 to 80% of motile spermatozoa

5 = near 100% of motile spermatozoa

The progressive motility was estimated by microscopic observation (400 × magnification) (Olympus CX41, Japan). Semen was diluted in the phosphate-buffer saline (PBS) solution, then placed on the slide and covered with a coverslip. Progressive motility was assessed as the percentage of rapidly progressive movement spermatozoa in a total of motile spermatozoa. At least five visual microscopic fields were observed per sample. The progressive motility of sperm was assessed as a percentage of the total of rapidly progressive movement sperm per total of motile sperm.

The sperm viability was investigated via the assessment of alive sperm proportionby the 0.4% (wt/vol) Trypan blue (Nacalai, Kyoto, Japan) in PBS solutionstaining procedure described by Mortimer (1994). Five µL of diluted semen was placed on the slide, then 5 µL of Trypan blue 0.4% was added and gently mixed with semen. The stained sperm slide was covered by a coverslip and waited for several minutes (up to 5 minutes) for coloring. The stained sperm were observed under the phase-contrast microscope (400 × magnification) (Olympus CX41, Japan) to distinguish the death sperm based on spermatozoa color. The blue-stained sperm was distinguished as the death sperms. Three replicated stained sperm with approximately 300 spermatozoa per each preservation treatment samples were examined. The viability of sperm was expressed as

$$\text{Sperm viability (\%)} = 100 - \frac{\textit{total of blue sperm}}{\textit{total of observed sperm}} \times 100$$

The sperm abnormality was evaluated by 3% (vol/vol) buffered glutaraldehyde (Nacalai, Kyoto, Japan) solution under the phase-contrast microscope (400 × magnification) (Olympus CX41, Japan). Five µL of stored semen was diluted by 100µL of buffered glutaraldehyde solution (3%) then placing a drop of semen on a slide and covering with a coverslip. If sperms have a double tail, no tail, or a head that is crooked, misshapen, double heads, or too large, they are considered to be abnormal. The sperm abnormality was assessed as a percentage of the total of abnormal morphology sperm per total of observed sperm.

Artificial insemination was performed at 3 p.m. with a volume of 0.4 mL stored semen. Semen was immediately placed 2 cm depth into the vaginal orifice of hens following the description of Quinn and Burrows (1936). Eggs were collected daily until two weeks after insemination day, preserved at 15°C and 60% relative humidity (RH), and then incubated after one week of collection. For estimating fertility, the cracking was done at day 5 of incubation. The fertilization rate was assessed as a percentage of the total of fertilized egg per total of egg incubated.

Statistical analysis

The data were statistically analyzed by thegeneral linear model (GLM) procedure of SAS

software version 9.2 (SAS Institute, Cary, NC, USA). The Tukey's procedure was applied for multiple comparisons among the experimental treatments at each preservation time. The model used in the present study was as follows:

$$Y_{ij}=\mu+T_i+e_{ij}$$

Which:

Y_{ij} stands for observed depended variables including sperm parameters,

μ is the mean of the population,

T_i is the effect of treatment,

e_{ij} is the random residual error.

The original data were shown as Mean ± Standard errors. The significance was declared at $p<0.05$.

RESULTS AND DISCUSSION

Liquid storage semen quality

The effects of superoxide dismutase (SOD) and catalase supplementation on the chicken semen quality during liquid storage at 4°C were shown in Table 1. The sperm motility, consisting of mass activity and progressive motility, was gradually decreased with the increasing of preservation time ($p<0.001$). The sperm mass activity at 0 h of storage was not different among all treatments, while it tended to be higher for 100 SOD, 200 catalase and 200 SOD + 400 catalase supplement treatments at 24 h of storage ($p<0.05$). At 48 h of preservation, the mass activity was highest for200 catalase and 200 SOD + 400 catalase supplement treatments, and lowest for the control group ($p<0.05$). In addition, the progressive motilities of sperm at 24 h and 48 h of preservation were the highest for the 200 SOD + 400 catalase supplement treatment ($p<0.05$). The viability of sperm was decreased with the increasing of the preservation time ($p<0.001$). Even respective supplement SOD and catalase enhanced the sperm viability, the combined supplement of 200 SOD + 400 catalase significantly improved the sperm viability ($p<0.05$) at 24 h and 48 h of preservation. The sperm abnormality was increased with increasing of preservation time ($p<0.001$). However, either respective or combined supplement SOD and catalase did not affect the abnormality of chicken sperm during liquid storage.

Table 1. The effects of SOD and/or catalase supplementation on chicken semen quality in liquid storage

Storage time (h)	**Control**	**SOD (UI/mL)**		**Catalase (UI/mL)**		**SOD + Catalase (UI/mL)**		**P_{time}**
		100	***200***	***200***	***400***	***100 + 200***	***200 + 400***	
Mass activity (scale)								
0	4.28 ± 0.15	4.30 ± 0.13	4.35 ± 0.14	4.30 ± 0.16	4.37 ± 0.16	4.32 ± 0.14	4.35 ± 0.14	
24	3.70 ± 0.67	4.00 ± 0.56	3.70 ± 0.83	4.00 ± 0.54	3.80 ± 0.66	3.60 ± 0.55	4.10 ± 0.42	<0.001
48	2.40 ± 0.41^{b}	2.80 ± 0.4^{ab}	2.70 ± 0.27^{ab}	2.90 ± 0.4^{a}	2.60 ± 0.51^{ab}	2.70 ± 0.47^{ab}	3.00 ± 0.48^{a}	
Progressive motility (%)								
0	90.30 ± 1.02	91.40 ±1.03	90.80 ±1.10	90.80 ± 1.07	91.30 ± 1.16	91.80 ± 0.94	90.80 ± 1.04	
24	65.00 ± 3.60^{b}	73.60 ± 2.80 ^{ab}	63.30 ± 4.87^{b}	73.00 ± 2.95 ^{ab}	68.60 ± 3.97 ^{ab}	63.90 ± 3.08 ^{b}	75.60 ± 1.76 ^{a}	<0.001
48	41.40 ± 2.03^{b}	47.10 ± 2.47 ^{ab}	41.50 ± 2.11^{b}	49.10 ± 3.17 ^{ab}	42.70 ± 3.12 ^{b}	43.90 ± 3.01^{ab}	51.40 ± 2.87 ^{a}	
Sperm viability (%)								
0	89.7 ± 0.93	89.3 ± 0.84	90.3 ± 0.71	89.3 ± 1.01	89.9 ± 0.87	88.4 ± 0.91	89.8 ± 0.55	
24	81.2 ± 1.31^{b}	83.4 ± 1.45^{ab}	84.5 ± 1.13^{ab}	84.9 ± 1.31^{ab}	84.3 ± 1.27^{ab}	82.9 ± 1.12^{ab}	86.4 ± 0.87^{a}	<0.001
48	78.6 ± 1.63^{b}	81.4 ± 1.56^{ab}	81.1 ± 1.15^{ab}	82.1 ± 1.55^{ab}	81.6 ± 1.29^{ab}	81.1 ± 1.00^{ab}	83.5 ± 0.93^{a}	
Sperm abnormality (%)								
0	9.1 ± 0.98	7.5 ± 0.49	7.3 ± 0.51	7.2 ± 0.58	7.3 ± 0.62	8.0 ± 0.66	8.0 ± 0.70	
24	16.7 ± 0.90	16.1 ± 0.77	16.2 ± 0.96	16.4 ± 0.69	15.8 ± 1.01	18.4 ± 1.32	15.5 ± 0.95	<0.001
48	18.0 ± 0.79	20.1 ± 1.34	18.1 ± 0.77	18.6 ± 0.81	18.8 ± 1.32	21.9 ± 2.84	17.5 ± 0.78	

Data are shown Mean ± SE.

P_{time}: the probability of storage time effects.

Mass activity of sperm scale of 0 (no motility) to 5 (vigorously swirling motion).

^{ab} Means with different superscripts within a row are significantly different ($p<0.05$).

In semen, ROS is originated from leukocytes, spermatozoa with residual cytoplasm and mitochondrial respiration of normal sperm (Pagl et al., 2006). ROS is required to regulate sperm functions such as moving, capacitation, acrosome reaction (Sharma and Agarwal, 1996). When sperm undergo chilled injury in liquid storage, the high quantity of ROS was generated (Wang et al., 1997). Thus, the ROS increased gradually during the cooling process and reached max at 5°C (Chatterjee and Gagnon, 2001; Alexei et al., 2014) which accounted for the decreasing of semen quality during cool storage. ROS are reactive molecules containing oxygen in the cell include peroxyl radicals (ROO), hydroxyl radical (OH), and superoxide anion (O_2^-). These molecules must be shared or transferred unpaired electron to another molecule to achieve a stable configuration. Polyunsaturated fatty acids (PUFAs) are major component of the sperm membrane that contains multiple double bonds especially being the priority target of ROS, causing lipid oxidative degradation (Nimse and Pal, 2015). Lipid peroxidation results in the change of many functions of the sperm membrane such as membrane fluidity, plasma membrane integrity, subsequently decrease the semen quality (Halliwell and Chirico, 1993; Engel et al., 1999). Moreover, avian spermatozoa is rich in polyunsaturated fatty acids (PUFAs) that make sperms vulnerable to lipid peroxidation (Cecil and Bakst, 1993). Therefore, the semen quality was gradually decreased with increasing preservation time ($P<0.001$).

Enzymatic antioxidant factors including SOD and catalase have been well known as the first antioxidant defense system by the ability to scavenge, remove and suppress the formation of ROS. SOD and catalase could reduce the production of hydrogen peroxide and protect sperm against the effects of ROS (La Falci et al., 2011; Dak et al., 2015). SOD responds to scavenge superoxide anion – aprecursor for H_2O_2 production which is more harmful to sperm than superoxide anion. Afterward, catalase quickly converts hydrogen peroxide to water and oxygen which does not damagesperm cells (Surai et al., 2001). Therefore, the supplementation of SOD or/and catalase could have positive effects on storage semen quality (Maxwell and Stojanov, 1996, Moghbeli et al., 2016). Individual supplement of SOD at 100UI/mL and 200 UI/mL improved mithun and boar semen quality during cool storage, respectively (Perumal, 2014; Zhang et al., 2017). The catalase supplementation could enhance rat and human semen quality in liquid storage (Maxwell and Stojanov, 1996; Moghbeli et al., 2016). However, current study did not show the significant effect of separate SOD and catalase supplement on the chicken semen quality in liquid storage after 24 h and 48 h. These results were relevant with the report of Aurich et al. (1997) and Rossi et al. (2001) that showed no significant effects of separate supplement SOD and catalaseon semen quality during liquid storage. SOD and catalase may have simultaneous action on the chain reaction of anion super oxide and hydrogen peroxide and only contribute to part of antioxidant function (Nimse and Pal, 2015). Therefore, the combined supplement of SOD and catalase can protect sperm against the negative effects of ROS and improve semen quality (Maxwell and Stojanov, 1996; Rossi et al., 2001).

The fertility of stored semen

The fertility of stored semen for 24 h and 48 h of storage were shown in Table 2. After 24 h of storage, the fertility of stored semen during the first week after insemination was high for both the control and the treatment group (from 76.59% to 88.73%). There is no significant difference between the fertility of stored semen with or without supplementation. However, the fertility of stored semen was decreased in the second week after insemination (40.95% and 62.86% in the control and treatment group, respectively). Until the second week after

insemination, the fertility of semen at 200 SOD + 400 catalase supplement treatments was higher than that of the control and other treatments ($p<0.05$). The fertility of semen after 48 h of storage was reduced in all groups due to the reduction of semen quality. For 48 h of storage, the fertility of semen in 200 SOD + 400 catalase supplement treatment was higher than control and other treatment groups either the first week or second week after insemination ($p<0.05$). In the first week after insemination, the fertility of semen in 200 SOD + 400 catalase supplement treatment (63.97%) was higher than the control group (46.19%), respectively. In the second week, the rate of fertilized egg in the control group (19.76%) was lower than that of 200 SOD + 400 catalase supplement treatment (38.33%). The results demonstrates that the supplementation of combined SOD and catalase improved stored semen fertility.

Table 2. The effects of SOD and/or catalase supplementation on the fertility of semen (%) during liquid storage

Period of insemination	Control	SOD (UI/mL)		Catalase (UI/mL)		SOD + Catalase (UI/mL)	
		100	*200*	*200*	*400*	*100 + 200*	*200 + 400*
24h of storage							
1st week	76.59±5.30^{c}	84.99±3.07^{ab}	79.29±2.68^{b}	84.36±5.30^{ab}	81.18±2.09^{b}	83.57±0.86^{ab}	88.73±3.64^{a}
2nd week	40.95±0.60^{b}	48.33±3.07^{b}	42.22±2.68^{b}	47.78±4.28^{b}	43.41±3.50^{b}	48.33±2.49^{b}	62.86±1.76^{a}
48h of storage							
1st week	46.19±2.97^{b}	52.38±1.51^{b}	48.81±2.83^{b}	52.38±1.51^{b}	48.33±3.07^{b}	52.38±1.51^{b}	63.97±1.75^{a}
2nd week	19.76±1.88^{b}	25.87±2.72^{b}	20.32±1.78^{b}	25.87±2.72^{b}	21.11±2.53^{b}	24.29±1.92^{b}	38.33±2.69^{a}

Data are shown Mean ± SE.

^{ab} *Means with different superscripts within a row are significantly different (P<0.05)*

The effects of supplement of ROS and catalase on semen quality during storage depended on their concentration in the extender (Sisy et al., 2007, La Falci et al., 2011, Moghbeli et al., 2016; Zhang et al., 2017). In current study, the chicken semen quality and fertility were not affected by the combined supplement at 100 SOD + 200 catalase UI/mL, while it was significantly improved at the 200 SOD + 400 catalase treatment. In natural, seminal plasma and spermatozoa themselves contain endogenous antioxidants SOD and catalase in order to protect spermatozoa against peroxidative damage (Surai et al., 1998). The antioxidant capacity of these endogenous enzymes is deficient when the ROS generation increases during storage (Breque et al., 2003). The optimal concentration of antioxidants supplemented may depend on the endogenous antioxidants, cooling process, temperature and time of storage (Wang et al., 1997; Chatterjee and Gagnon, 2001; Alexei et al., 2014). In this study, the supplementation of 100 SOD UI/mL would encourage the H_2O_2 production that was excess the catalysis of endogenous and 200 UI catalase additive. In addition, the supplement SOD at 200 UI/mLseemed to be over the O_2^- radial source. Hence, catalase additive at 400 UI/Ml was consistent with H_2O_2 availability. Consequently, the free radical was minimized in the 200 SOD + 400 catalase supplement treatment and responded to the highest semen quality and fertility.

CONCLUSSION

In conclusion, the results of the study demonstrated that the addition of antioxidant enzymes SOD and catalase improved the mass activity, progressive motility, viability and fertility of stored multi-spurs chicken semen. The stored semen quality and fertility were higher with the addition of 200 SOD + 400 catalase UI/mLafter 24 h and 48 h of storage.

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