EFFECTS OF CREATINE ON THE QUALITY AND FERTILITY OF CHICKEN SEMEN DURING LIQUID STORAGE

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ABSTRACT

Creatine plays the role as adenosine triphosphate synthesis substrate and antioxidant factor of sperm metabolism. Thus, it was hypothesised that the addition of creatine (0.1 mM, 1.0 mM, 10.0 mM) on diluent could enhance chicken semen quality in liquid storage. Semen collected from twelve Vietnamese native roosters was used in the experiment. The pooled semen of all roosters was diluted in Lake diluent at a ratio of 1:3 (v/v) and stored in 4^0C for 24 and 48 h. Each stored semen was inseminated into five Leghorn hens to evaluate the fertility. The results show that the chicken sperm mass activity, motility, and viability of 24 and 48 h stored semen was improved ($P < 0.05$) by the addition of 1.0 mM creatine. However, the sperm abnormality of 24 and 48 h stored semen was not different among treatments. The fertility of 24 and 48 h stored semen was highest for 1.0 mM creatine treatment ($P < 0,05$). The addition of 1.0 mM creatine on diluent could improve chicken semen quality in liquid storage.

Key words: ATP, antioxidant, fowl, preservation, sperm

INTRODUCTION

Artificial insemination (AI) and semen preservation can be useful to enhance the male breeding value in both commercial and native chicken breeds. However, semen quality during liquid storage depends on extender composition, dilution, temperature, pH, and osmolality (Hudson et al. 2015; Giesen and Sexton 1983; Sexton 1977; Siudzińska and Łukaszewicz 2008). The quality and fertility of semen stored for 24 h were significantly reduced compared to those of fresh semen (Douard et al. 2000). The decrease in semen quality relates to the reactive oxygen species (ROS) generation (Pagl et al. 2006) and the depletion of adenosine triphosphate (ATP) in spermatozoa during liquid storage (Delamirande and Gagnon 1992).

When sperms are in a cooling process during liquid storage, the ROS increases gradually and reaches the maximum level at 5^{o}C (Alexei et al. 2014; Chatterjee and Gagnon 2001; Wang et al. 1997). The high quantity of ROS promotes lipid peroxidation and changes in sperm membrane functions such as membrane fluidity and integrity, consequently decreased semen quality (Engel et al. 1999; Halliwell and Chirico 1993; Nimse and Pal 2015). Endogenous antioxidants, including enzymatic and non-enzymatic antioxidants, could protect sperms from peroxidative damage (Surai et al. 1998). However, the natural antioxidants in semen may be insufficient due to the increase in ROS generation and lipid peroxidation during liquid storage (Breque et al. 2003). Supplementation of antioxidants such as vitamin E, A, C, glutathione, superoxide dismutase (SOD) could improve semen quality during liquid storage (Al-Daraji 2000; Dalvit et al. 1998; Zhang et al. 2016; Zhang et al. 2017). Creatine has been

reported a significant ability to act as an antioxidant scavenger radical on reactive species (Lawler et al. 2002; Sestili et al. 2011), consequently may decrease the lipid peroxidation in sperm (Chatterjee and Gagnon 2001; Tiwari et al. 2002).

In general, sperms require an ATP–energy source for their functions, including the regulation of ionic flux, homeostasis maintenance, movement, and fertilization process (Harayama 2013; Silver and Erecinska 1997). The prolongation of storage duration results in the depletion of ATP, subsequently decreasing semen quality and fertility during liquid storage (de Lamirande et al. 1997; Nguyen et al. 2016; Strzeżek and Dziekońska 2011; Tiwari et al. 2002). Therefore, enhancing available ATP level for sperm may improve semen quality and fertility during liquid storage. Sperm contain a high level of creatine kinase, which plays an important role in energy production from phosphagen in avians (Kamp et al. 1996; Wallimann and Hemmer 1994). Creatine kinase participates in energy transfer from mitochondria to flagella (Auyeung et al. 2016; Moore 2000). Phosphagen has an important role in the synthesis and transfer of energy in bird sperm. Thus, avian sperm motility is inhibited by creatine kinase blockers (Wallimann et al. 1986). Therefore, supplementing creatine as the substrate of phosphagen process may enhance ATP synthesis for sperm activities during storage (Moore 2000). The effects of creatine addition on storage culture in human, buck, and mice semen were well-documented (Fakih et al. 1986; Farshad and Hosseini 2013; Umehara et al. 2018). However, there was no report about the effects of creatine on chicken semen in liquid storage.

Creatine is not only a free radicals scavenger, but also supplies energy precursor for sperms during liquid preservation (Cosson 2013; Sestili et al. 2011). The addition of creatine may have further positive effects on semen quality during storage in comparison with antioxidant supplement. Thus, this study sought to evaluate the effects of different creatine levels in diluent on the quality and fertility of multi-spurred chicken semen during storage at 4^{o}C up to 48 h. The results of study may be helpful to improve the efficiency of artificial insemination in chicken as well as in the reproductivity in multi-spurred chicken breed.

MATERIALS AND METHODS

Animals and experiment design. The experiments were conducted at Hung Vuong University, Phu Tho province, Vietnam. Twelve multi-spur roosters (native chicken breed of Tan Son, Phu Tho, Vietnam) had similarly initial age (28 weeks) and body weight (2.5 kg). Each bird was kept in an individual cage (50x50x50 cm) under room conditions. The experimental chickens were fed *ad libitum* layer commercial feed (CP: 17%, ME: 2700 Kcal/kg DM) and clean water.

The roosters were allowed four weeks for adaptation and semen collection training by the abdominal massage method following the description of Burrows and Quinn (1937). The rooster was restrained and gently stroked on the back, from behind the wings towards the tail, with firm rapid strokes. The rooster responded with tumescence erection of the phallus, at which time the handler gently squeezed the cloaca extracting semen through the external papillae of the ductus deferens collecting the semen into a container.

The Lake diluent, containing 1.35 g sodium glutamate, 0.128g potassium citrate monohydrate, 0.51g sodium acetate, 0.08g magnesium acetate tetrahydrate, 0.8g glucose in 100mL of distilled water, 300 mosM/kg H_2O of osmolality, was used as a basic medium (Lake 1960). The Lake diluent was supplied at 0.1 mM, 1.0 mM and 10 mM creatine. To prepare the Lake diluent containing 10 mM creatine, 152.2 mg creatine monohydrate 98% (Product No: 09630-22, Nacalai, Kyoto, Japan) was put into 100 mL volumetric glass and filled up by Lake diluent. 10 mL and 1.0 mL of Lake diluent containing 10 mM creatine was taken and filled up to 100 mL with 90 mL and 99 mL Lake diluent to form Lake diluent containing 1.0 mM and 0.1 mM creatine.

The semen was collected twice a week (Monday and Thursday) from all birds for 6 weeks. Individual semen was collected from each bird and pooled as a representative sample after eliminating contaminated feces, dust, and blood samples. The pooled semen was randomly separated into 4 experimental treatments with various level of creatine, including Lake diluent without creatine (T0), Lake diluent containing 0.1 mM creatine (T1), Lake diluent containing 1.0 mM creatine (T2), and Lake diluent containing 10 mM creatine (T3). Semen was diluted with the Lake diluent at the ratio of 1: 3 (v/v) (Parker and McDaniel 2006). The diluted semen samples of each treatment were divided into three sub-samples to evaluate semen quality at 0, 24, and 48 h of preservation and were stored immediately in a refrigerator (4ºC) (Donoghue and Wishart 2000).

Semen quality evaluation

Mass activity. The mass activity of sperm was evaluated following the description of Tarif et al. (2013). A sample drop was placed on a pre-heated slide at 37ºC without coverslip and determined by the phase-contrast microscope observation (100 × magnification) (Olympus CX41, Japan). The mass activity was expressed as scores by a scale from 1 to 5 as:

1+ = no perceptible motion
2+ = few spermatozoa move without forming any waves
3+ = small slow moving waves
4+ = vigorous movement with moderately rapid waves and eddies
5+ = dense, rapidly moving waves and eddies

Three visual microscopic fields were observed on each sample for each treatment semen. All measurements were assessed by the same researchers throughout the trial to ensure consistency, accuracy, and repeatability.

Sperm motility. The motility was observed by optical microscope (Olympus CX41, Japan). One drop of diluted semen was placed on the pre-heated at 37ºC and covered with a glass coverslip. The number of immotile sperm and total observed sperms (at least 200 sperms) were counted. The sperm motility was assessed using the formula of Mineshima et al. (2000):

$$\text{Sperm motility (\%)} = \frac{total\ number\ of\ observed\ sperm - number\ of\ immotile\ sperm}{total\ number\ of\ obsserved\ sperm} x\ 100$$

The final motility score is the mean of three estimations of each semen treatment.

Sperm viability. The sperm viability was investigated via the assessment of alive sperm proportion by the eosin-nigrosin stain containing 1.67g of eosin, 10g of nigrosin, and 2.9g sodium citrate dissolved in 100 mL distilled water). The procedure was described by Tarif et al. (2013). Ten µL of diluted semen was mixed with 200 µL of eosin-nigrosin stain and placed on the slide. The sperm were observed under the phase-contrast microscope (400 × magnification) (Olympus CX41, Japan) to realise the dead sperm based on spermatozoa color. The pink-stained sperm (stained with eosin) was recognised as dead sperm. The sperm which appeared without any color was regarded as alive sperm. Three replicated stained sperm with approximately 300 spermatozoa per each preservation treatment samples were examined. The viability of sperm was calculated as:

$$\text{Sperm viability (\%)} = \frac{\text{total number of observed sperm} - \text{number of pink sperm}}{\text{total number of observed sperm}}\ x\ 100$$

Sperm abnormality. The sperm abnormality was evaluated in formol saline-fixed (34.7mM disodium hydrogen phosphate, 18.7mM potassium 92.6.mM sodium chloride, and 1.54 mM formaldehyde in 1000 mL distilled water) under a phase-contrast microscope (400 × magnification) (Olympus CX41, Japan). Five µL of stored semen was diluted with 100µL of formol saline-fixed solution, then a drop of

semen was placed on a slide and covered with a coverslip. If a sperm has a double tail, no tail, or a head that is crooked, misshapen, has double heads, or too large, it is considered to be abnormal (Tarif et al. 2013).

The sperm abnormality was assessed as:

$$\text{Sperm abnormality (\%)} = \frac{\text{total number of abnormal sperm}}{\text{total number of observed sperm}} \text{ x } 100$$

The final motility score is the mean of three estimations of each semen treatment.

Stored semen fertility. Forty Leghorn hens (42 weeks old) were divided into eight groups to artificial insemination four treated semen samples which were stored in cool temperature for 24 and 48 h, respectively. The hens were kept individually in commercial layer cages (40x60x35 cm). The hens were fed 115 gram layer commercial feed (CP: 17%, ME: 2700 Kcal/kg DM) and freely accessed clean water by nipple drinkers. Artificial insemination was performed at 3 P.M. using 0.4 mL stored semen (containing about 1 billion sperm). Semen was placed immediately 2 cm depth into the vaginal orifice of hens following the description of Quinn and Burrows (1936). Eggs were collected daily on days 2 to 7 after first artificial insemination, preserved at 15^{o}C and 60% relative humidity (RH), and then incubated after one week of collection. For estimating fertility, the cracking was done on the fifth day of incubation. The fertilization rate was assessed by calculating fertilized eggs:

$$\text{Fertility (\%)} = \frac{\text{total number of fertile egg}}{\text{total number of incubated egg}} \text{ x } 100$$

Statistical analysis. The data were analysed statistically by the General Linear Model (GLM) procedure of SAS 9.1 (SAS Institute, Inc., Cary, NC). The percentage data were transformed initially by arcsine calculation. The GLM model includes the experimental treatment, storage time, and interaction effect of experimental treatments and storage time as the fixed factors, and the semen collection days as a random factor. Because the interaction effects of preservation time and experimental treatments on the semen quality were not detected in this study, Tukey's procedure was applied for multiple comparisons among treatments at 0, 24, and 48 h of storage, separately. The original data were shown as the least square means with standard errors of the mean. The significance was declared at $P < 0.05$.

RESULTS AND DISCUSSION

Liquid storage semen quality. The effects of creatine on semen quality after storage are shown in Table 1. The mass activity of sperm was similar among treatments at 0 h of storage, range from 4.35 to 4.45 ($P > 0.05$). However, the creatine significantly enhanced the sperm mass activity in the treatment using Lake diluent containing 1.0 mM creatine (treatment T2) after 24 h and 48 h storage. The mass activity of sperm was not different among T0 (Lake diluent base), T1 (Lake diluent containing 0.1 mM creatine), and T3 (Lake diluent containing 10 mM creatine) treatments for both 24 and 48 h stored semen. Even the sperm motility at 0 h of storage was similar among experimental treatments ($P > 0.05$), the sperm motility at 24 and 48 h of storage was the highest for the T2 treatment ($P < 0.05$). Nevertheless, the sperm motility was not different among T0, T1, and T3 treatments. The sperm viability of T1 treatment and T2 treatment for 24 and 48 h of storage were significantly higher than those of T0 and T3 treatments. The sperm viability was the highest for T2 treatment, 83.85% and 77.94% for 24 and 48 h of storage, respectively. On the contrary, the sperm viability was similar among T0 and T3 treatments, range from 72.61 – 73.42% for 24 h of storage and 70.10 – 71.91% for 48 h of storage. The abnormality of sperm for 24 and 48 h of storage ranged from 15.72% - 17.36% and 16.30 % to 18.16%, respectively. The supplementation of creatine in semen diluent did not affect the sperm abnormality of chicken semen in this study.

Table 1. The effects of creatine supplementation on sperm quality while in liquid storage.

Storage time	Group			
	T0	**T1**	**T2**	**T3**
	Mass activity (+)			
0h	4.40 ± 0.13	4.40 ± 0.11	4.35 ± 0.15	4.45 ± 0.16
24h	3.60 ± 0.19^{b}	3.80 ± 0.15^{ab}	4.10 ± 0.20^{a}	3.60 ± 0.28^{b}
48h	2.40 ± 0.34^{b}	2.60 ± 0.32^{ab}	3.10 ± 0.27^{a}	2.30 ± 0.43^{b}
	Motility (%)			
0h	85.00 ± 2.25	83.60 ± 2.85	84.91 ± 2.20	84.12 ± 3.63
24h	62.30 ± 2.15^{b}	65.30 ± 3.35^{ab}	70.62 ± 2.56^{a}	63.10 ± 1.78^{b}
48h	45.70 ± 3.80^{b}	48.80 ± 3.61^{ab}	59.92 ± 2.26^{a}	46.45 ± 2.49^{b}
	Viability (%)			
0h	86.81 ± 3.62	87.30 ± 2.85	88.72 ± 1.29	88.15 ± 2.15
24h	72.61 ± 2.30^{c}	79.70 ± 1.27^{b}	83.85 ± 1.23^{a}	73.42 ± 1.06^{c}
48h	70.10 ± 2.40^{c}	74.72 ± 1.66^{b}	77.94 ± 1.61^{a}	71.91 ± 2.94^{bc}
	Abnormality* (%)			
0h	12.21 ± 1.27	11.82 ± 0.68	12.18 ± 1.54	12.42 ± 1.14
24h	17.36 ± 4.19	16.33 ± 4.54	15.72 ± 2.83	15.83 ± 3.20
48h	18.16 ± 2.37	17.20 ± 2.27	16.30 ± 2.88	17.90 ± 1.56

Data are shown Least Square Mean ± SE.

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

T0: The treatment using Lake diluent without creatine.

T1, T2, T3: The treatment using Lake diluent containing 0.1, 1.0 and 10 mM creatine addition respectively.

* The sperms that have a double tails, no tail, double heads or the head is crooked, misshapen, or too large were considered abnormal.

Reactive oxygen species (ROS), such as superoxide anion（$O_2^{\cdot-}$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($^{\cdot}OH$), have deleterious effects on sperm function. ROS could cause sperm immobilization by removal of intracellular ATP, decrease in the phosphorylation of axonemal protein (de Lamirande and Gagnon 1995). In addition, ROS are known to attack on the cell membrane phospholipids generates fatty acid peroxides, hydroxy alkenyls, malonadehyde which have detrimental effects on sperm motility and fertilizing ability (de Lamirande and Gagnon 1995). 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, and acrosome reaction (Sharma and Agarwal 1996). When sperms are in the cooling process in liquid storage, a high quantity of ROS is generated (Wang et al. 1997), which reaches the maximum level at 5^{o}C (Alexei et al. 2014; Chatterjee and Gagnon 2001). The high quantity of ROS was accounted for the decrease of semen quality due to the lipid peroxidation (Engel et al. 1999; Halliwell and Chirico 1993). Moreover, avian spermatozoa are rich in polyunsaturated fatty acids (PUFAs) that make sperms vulnerable to lipid peroxidation (Cecil and Bakst 1993). High level of ROS causes a decrease in intracellular ATP due to the inhibition of ATP production (Armstrong et al. 1999; Delamirande and Gagnon 1995) which induce the sperm immobilization and low of fertilizing ability.

ROS have harmful effects on the spermatozoa, such as lipid damage, protein damage, DNA damage, carbohydrate damage (Saraswat et al. 2016). Antioxiant supplement could improve motility, viability, acrosomal integrity, and hypoosmotic swelling (HOS) of semen during storage (Perumal et al. 2013). Creatine was approved displays a potential to scavenge reactive oxygen species (ROS) such as 3-ethylbenzothiazolamine-6-sulfonic acid ($ABTS^{+}$), $O_2^{\cdot-}$, and $OONO^{-}$ (Chatterjee and Gagnon 2001,

Lawler et al. 2002). Moreover, creatine has an important role in energy production in chicken sperm. The concentration of creatine kinase in bird sperm was 700 μmol min^{-1} g^{-1} cell wet weight, which was higher than other species (rat: 1.2; stallion 1,9; bull and boar <0,1 μmol min^{-1} g^{-1}) (Kamp et al. 1996). Rooster sperm motility is inhibited by creatine kinase blockers (Wallimann et al. 1986). The presence of creatine may compensate for the limitation of energy diffusion to the distal axoneme in sperm (Kamp et al. 1996, Wallimann and Hemmer 1994). Creatine/creatine phosphate can fuel the transport of ATP from mitochondria to the tail of sperm and regeneration of ATP from ADP at the site of energy usage (Cosson 2013). Thus, creatine supplement may elevate APT production and decrease the effects of ROS, consequently improving sperm motility, viability, and fertility (Kamali Sangani et al. 2017, Kamp et al. 1996, Lawler et al. 2002, Wallimann and Hemmer 1994).

In this study, supplementation of 1.0 mM creatine in liquid chicken semen storage displayed higher sperm quality and fertilizing ability. Creatine concentration in avian seminal plasma range from 3.0 to 8.0 mM depending on the rooster age (Iaffaldano et al. 2018). Thus, the supplementation of 0.1 mM creatine may have insufficient antioxidant capacity as well as ATP precursor for chicken sperm at 24 and 48 h of storage. On the contrary, the creatine concentration in avian seminal plasma has a negative relationship with sperm motility and semen osmolality which may reduce sperm integrity and function during storage (Iaffaldano et al. 2018). It may be the reason for the decreased semen quality associated with high creatine supplement (10 mM).

Fertility of stored semen. The effects of creatine addition in semen diluent on fertility are shown in Figure 1. The fertility of semen stored for 24 and 48 h were the highest ($P < 0.05$) for the T2 treatment (89.64% and 61.43%, respectively), while it was similar with T0, T1, and T3 treatments ($P > 0.05$).

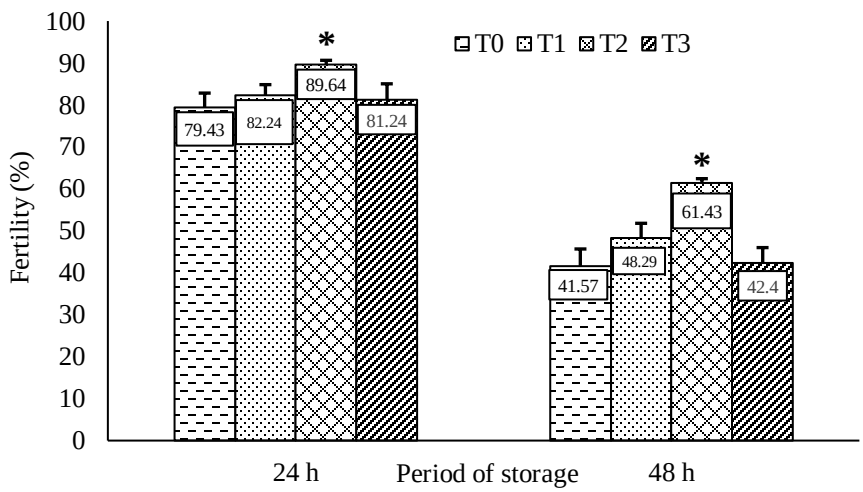

Fig. 1. Effects of creatine supplementation on fertility of chicken sperm after 24 h and 48 h of liquid storage. T0: The treatment using Lake diluent without creatine; T1, T2, T3: The treatment using Lake diluent containing 0.1, 1.0 and 10 mM creatine addition respectively. *P<0.05 values compared to other groups.

Multi-spur chicken is a native breed of Phu Tho provine, Northwest region of Vietnam. The hens have wild behavior and low egg performance (Thinh et al. 2015) which makes it difficult to collect enough fertilized eggs after insemination. On the contrary, Leghorn are commonly reared in Vietnam, due to their gentle behavior and high egg production. Thus, in the current study, we used the Leghorn hen to collect maximum number of eggs after artificial insemination. In addition, because the fertility of the chicken semen is the highest in the first week after artificial insemination (AI) and usually reduces

from day 8th after AI (Bui et al. 2018). Thus, we only collected the experimental eggs in the first week after AI to ensure the high fertility of all semen treatments.

The previous studies have shown that the addition of creatine enhanced the fertilizing capacity of human sperm during *in vitro* fertilization of gamete intrafallopian transfer (Fakih et al. 1986). The addition of 2.5 mM creatine could also increase significantly motility, progressive motility, viability, acrosome, and decrease abnormalities of buck sperm (Farshad and Hosseini 2013). The addition of 500 – 1000 µM creatine to *in vitro* fertilization (IVF) medium increases sperm ATP levels and sperm motility in mice, consequently, improve the efficiency of IVF and *in vivo* fertilization in mice (Umehara et al. 2018).

In the current study, the 1.0 mM supplement enhanced chicken semen mobility which is associated with increased fertilizing ability. Creatine supplement may significantly degenerate the chicken sperm membrane lipid peroxidation, consequently improve sperm cell metabolism and function, which may prolong the survivability of sperm in the sperm storage tubules (SSTs) after insemination (Bui et al. 2018; Zaniboni and Cerolini 2009). In addition, creatine supplement may elevate ATP production and decline ROS effects, consequence improve chicken sperm progressivity, and fertility (Kamali Sangani et al. 2017). The chicken spermatozoa creatine content supplement (> 8.0 mM) may have negative effects on the sperm mobility and semen osmobility, consequently decreasing chicken semen fertility (Iaffaldano et al. 2018).

CONCLUSION

The creatine supplementation to diluent at 1.0 mM could improve multi-spur chicken semen quality and fertility after liquid storage. The effects of creatine on the ATP production in sperms and the survivability of sperms in female reproduction tract should be confirmed in future studies.

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CONFLICT OF INTEREST

The authors declare no conflict of interest with any financial support, personal, or relationships with other people or organizations related to the material used and discussed in the manuscript.

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