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Age-related difference changes semen quality and seminal plasma protein patterns of Thai native rooster

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Abstract The association between age and semen quality of Thai native roosters (Leung hang khao) in relation to patterns of seminal plasma proteins were determined. Eighteen of the native roosters were grouped according to the ages 7-8, 10-11 and 23-24 months. Semen was collected by abdominal massage and evaluated for different physical parameters. Results showed that semen volume, color, pH and sperm concentration were not significantly different among the different age groups. In contrast, sperm viability was found to be significantly different. Sperm viability was significantly higher in the 7-8 ($93.34^{a} \pm 2.17$) than the 23-24 months age category ($71.38^{b} \pm 7.44$). Seminal plasma proteins were separated using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). Based on our findings, proteins with molecular weight fractions of 72, 90, 140, 220, and 260 kDa showed patterns of expression in 7-8, 10-11, and 23-24 month old roosters, respectively. Moreover, proteins with molecular weight fractions of 72, 90, and 140 kDa were shown sharply patterns of expression among age of roosters. These results indicate that sperm vitality (percentage of live sperm or dead sperm) and proteins with molecular weight fractions of 90, and 140 kDa are associated with age. Proteins with molecular weight fractions of 90 and 140, kDa may serve as markers to determine semen quality in Thai native roosters.

Keywords: seminal plasma proteins, semen quality, Thai native rooster

Introduction

In poultries and mammals, semen is a complex fluid containing spermatozoa and seminal plasma (Marzoni *et al.,* 2013). The precise observation of semen quality is required for successful artificial insemination

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(Almahdi *et al.,* 2014). Semen quality may be affected by various environmental factors such as feed, temperature, age, and strain thereby influencing fertilization rate (Karaca *et al.,* 2002; Shanmugam *et al.,* 2014; Zhang *et al.,* 1999). Aging playing an essential role in male fertility in all species of animals appears to have a significant impact on reproductive behavior (Avital-Cohen *et al.,* 2013). Fertility of domestic roosters is highest at 32 weeks of age and declines at about 45 weeks of age. Previous studies showed that older gobblers exhibit poor semen quality characterized by low sperm volume, number, motility, viability, and membrane integrity (Iaffaldano *et al*., 2008). Broiler breeders have been reported to produce the maximum number of sperm at 36 weeks of age and progressively declined until 55 weeks of age (Sarabia Fragoso *et al.,* 2013). Another findings in Iranian indigenous broiler breeders showed that spermatozoa concentration between 26 and 34 weeks of age maintain at the basal level. However, significant reductions were found in these broiler breeders at 45 weeks of age (Tabatabaei *et al.,* 2010). Semen volume, sperm concentration, and total sperm per ejaculation of Indian red jungle fowl were significantly higher at 43-54 months of age followed by the age groups of 31-42, 19-30, and 6-18 months (Rakha *et al.,* 2017).

The properties of proteins, nutrients, and buffer systems in seminal plasma connect with several sperm parameters, such as motility, capacitation, sperm transport, survival and longevity, protection against damages, and the formation of the sperm reservoir inside the female reproductive tract, all of which determine the semen quality (Novak *et al.,* 2010; Pilch and Mann, 2006; Troedsson *et al.,* 2005), (Al-Aghbari *et al.,* 1992; Brandon *et al.,* 1999; Marzoni *et al.,* 2013; Slowinska *et al.,* 2008). Several studies research seminal plasma proteins with fertility in various species (Borziak *et al.,* 2016; Marzoni *et al.,* 2013). The protein profiles of bovine seminal plasma have been investigated by using two-dimensional polyacrylamide gel electrophoresis, and two proteins (26 and 55 kDa) are indicators for bulls with high fertility (Killian *et al.,* 1993). Examining canine seminal plasma proteins by using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), two proteins (67 and 58.6 kDa) were correlated with sperm motility and other semen properties (de Souza *et al.,* 2007). In addition, studies involving seminal plasma of south Indian jersey and hybrid bulls showed that protein group 1 (205 and 97.4 kDa) and protein group 2 (22.0 and 14.3 kDa) were positively and negatively correlated with sperm concentration, respectively (Sundaram *et al.,* 2016). In this context rare data is available on semen quality and seminal plasma protein expression pattern of Thai native rooster at different ages. Therefore, the present study was performed to investigate the properties of semen and seminal plasma proteins in Thai native roosters, Leung hang khao.

Materials and methods

Animals and Semen collection

Eighteen Thai native roosters, Leung hangs khao, were divided into three groups at three different age, 7-8, 10-11, and 23-24 months of age. They were housed in individual cages in an open-sided house under natural photoperiod and climatic conditions. Each rooster was daily fed with 130 g of commercial feed, and water was provided *ad libitum*. Prior to the experiment, the roosters were accustomed to the environment. The semen was collected by using abdominal massage method.

Semen evaluations

Semen samples were collected in 1.5 mL microtubes and evaluated for total volume, color, pH value, concentration, and percentage of live and dead sperm. Semen color was evaluated based on a visual scoring scale of 1 - 5 (1 = watery semen, 2 = watery semen with white streaks, 3 = medium white semen, 4 = thick white semen, and 5 = very viscous chalky white semen sample) (McDaniel and Craig, 1959). The pH value was investigated by using a p-Hydrion test paper (pH value ranging from 0.0 to 13.0) and a color chart meter. The concentrated sperm was diluted 1:1000 in 4% Sodium chloride solution and was loaded into hematocytometer. The count was done at 400x magnification under bright-field microscopy. The percentage of live and dead sperm was determined by using eosin-nigrosin staining (Campbell *et al.*, 1953) Figure 1. A minimum of 200 live or dead sperms were counted on each slide (Shanmugam *et al.*, 2014).

Seminal plasma preparation

The seminal plasma from the native rooster in each group was harvested and centrifuged at 8,000 rpm for 10 min at 4 ℃. The supernatant was transferred into 2 mL microtubes and was centrifuged at 12,000 rpm for 20 min at 4 ℃. Total protein was determined by Bradford method (Bradford, 1976). The seminal plasma was stored at -80 ℃ until electrophoresis was performed.

Sodium dodecyl polyacrylamide gel electrophoresis (SDS-PAGE)

The seminal plasma proteins from the native roosters in each group were subjected to SDS-PAGE. The total proteins were denatured by using the sample

buffers (10% SDS; 0.5 M Tris, pH 6.8; 20% v/v glycerol; 0.2% w/v bromophenol blue). After boiling at 100 ℃ for 5 min, proteins (20 µg) from each sample were analyzed on a stacking gel containing 4.5% and 10% polyacrylamide gel. To stain proteins, the PAGE gels were placed in a coomassie brilliant blue R250 dye solution for a few hours and were de-stained until the protein molecules was separated and clearly visualized. Each sample was normalized by divided the density of the band by Image J Software (Version 1.6; NIH).

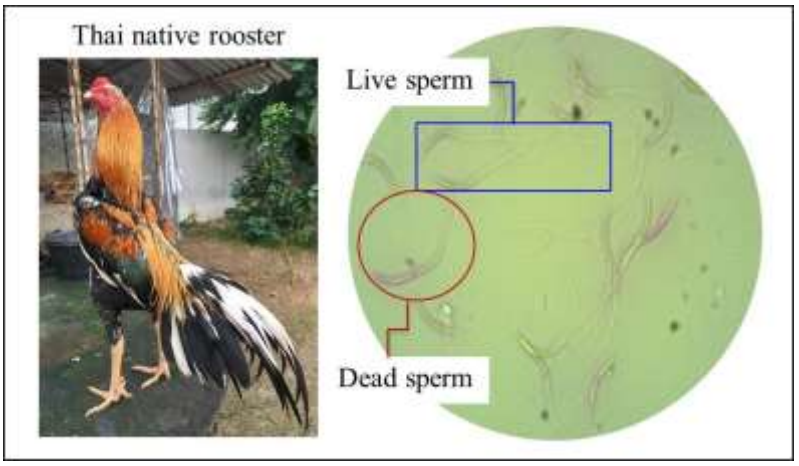

Figure 1. Using eosin-nigrosin staining to determine sperm viability. The live sperms were colorless and the dead were pink

Statistical analysis

Data were analyzed by using the Statistical Package for the Social Sciences (SPSS, version 16). One way analysis of variance (ANOVA) was used to compare semen parameters between the various groups of roosters. Duncan's multiple comparison test was performed to evaluate differences in the semen quality parameters among the different age groups. Results were reported as Mean ± SEM. P values less than 0.05 were considered statistically significant.

Results

Age affected sperm live percentage in Thai native roosters, Leung hang khao

Semen samples from three different ages, 7-8, 10-11, and 23-24 months of age. of Thai native roosters were collected and evaluated for semen quality

parameters. The semen quality parameters of roosters in the each group were shown in Table 1. The parameters of volume, color, and pH value and sperm concentration of the semen derived from younger or older roosters did not show significant differences. However, the age had significant effects on live and dead sperm ($P < 0.05$).

Table 1. Mean ± SE of semen volume, color, pH and sperm concentration of Thai native rooster, Leung hangs khao at different ages

Semen parameters	Age of rooster (months)		
	7-8	10-11	23-24
Volume (mL)	0.255 ± 0.09	0.208 ± 0.06	0.167 ± 0.04
Color	3.80 ± 0.18	3.80 ± 0.18	3.80 ± 0.18
PH value	7.00 ± 0.00	7.0 ± 0.00	7.5 ± 0.27
Concentration ($x10^9$/mL)	2.28 ± 0.47	2.17 ± 0.63	2.14 ± 0.63

Mean within a row with different superscripts differ significantly ($p < 0.05$)

The percentage of live sperm in the group of 7-8 months of age (93.34^a ± 2.17) was significantly higher than that in the group of 23-24 months of age (71.38^b ± 7.44) ($P < 0.05$). However, the results in the group of 10-11 months of age (88.95 ± 2.11) showed no significant differences in the groups of 7-8 months and 23-24 months of age (Fig 2.). The percentage of dead sperm in the group of 7-8 months of age (6.66^a ± 2.17) was significantly lower than 23-24 months of age (28.62^b ± 7.44).

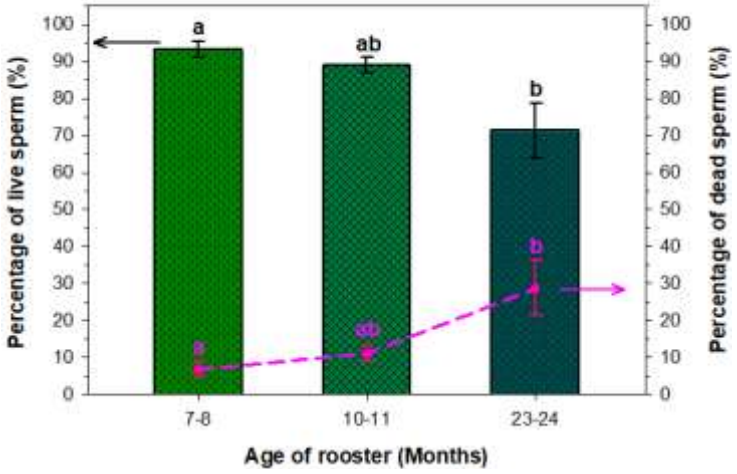

Figure 2. The effect of age on the percentage of live and dead sperm of Thai native rooster (Leung hangs khao)

Younger rooster expressed high level of high molecular weight proteins

The analysis of seminal plasma protein derived from native roosters was performed by SDS-PAGE. A total of 12 protein bands with the molecular weight ranging from 34 to 260 kDa were identified in Thai native rooster seminal plasma. According to the results, the expressions of proteins with molecular weights of 72, 90, and 140 kDa were more predominant than other proteins. The protein bands with molecular weight of 72 kDa was high at 7-8 months of age and slightly decreased at 10-11 months of age but non-significant different among the different age groups. The expression of 140 kDa proteins at 7-8 and 10-11 months of age were higher than that at 23-24 months of age. However, the expression of 90 kDa proteins at 7-8 months of age was significantly lower than that at 23-24 months of age (Fig. 3). Therefore, sperm live and dead was correlated to two bands (90 and 140 kDa) and the protein bands with molecular weight of 72 kDa were correlated with semen volume and sperm concentration.

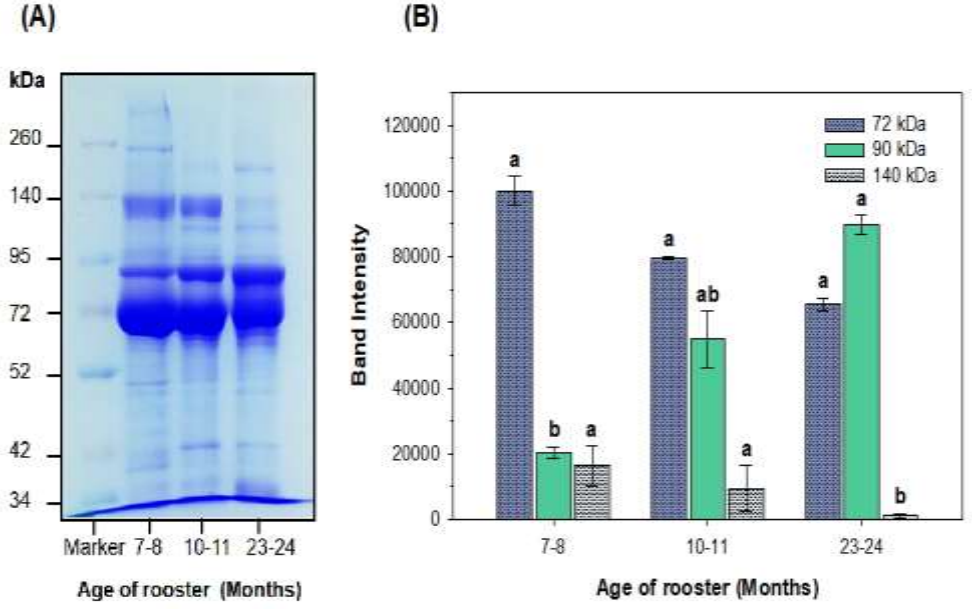

Figure 3. (A) Separation of seminal plasma proteins derived from Thai native roosters by using polyacrylamide electrophoresis gel (SDS-PAGE). Lane 1; protein marker, lane 2; the group at 7-8 months of age, lane 3; the group at 10-11 months of age, and lane 4; the group at 23-24 months of age.(B) Intensity of protein bands (72, 90, and 140 kDa) by using ImageJ software analysis

Discussion

The relation between age and semen quality in avian species has been extensively discussed (Shanmugam *et al.,* 2012). Generally, the low sperm viability and motility will occur between 31 and 52 weeks of age (Douard *et al.,* 2003), and age shows negative correlation with sperm concentration, motility, and viability (Tabatabaei *et al.,* 2010). In the present study, age did not significantly effect on volume, color, sperm concentration, and pH value of semen (Table 1). The previous study showed that sperm concentration of naked neck at 24 weeks of age (5.18 $\pm$ 0.55) was higher than that at 48 weeks of age (3.81 $\pm$ 0.48) but non significantly different between these ages (Shanmugam *et al.,* 2012). In the present study, the highest concentration of rooster sperm was found in 7-8 months of age (2.28 $\pm$ 0.47) but no significantly different with 10-11 months of age (2.17 $\pm$ 0.63) and 23-24 months of age (2.14 $\pm$ 0.63). Another study found that semen concentration in white leghorn cocks was higher at 2 years of age (2.92 $\pm$ 0.85[a]) than that at 1 year of age (2.85 $\pm$ 0.72[a]) although the results showed no significant differences (Elagib *et al.,* 2012). Moreover, we also found that the percentage of live sperm decreased as age increased. This was consistent with the results reported by Tabatabaei and his colleagues (2010). Older age of indigenous roosters also results in low sperm viability rates (that is, 90.64 $\pm$ 1.47, 82.30 $\pm$ 1.62, and 74.11 $\pm$ 1.35% for roosters at 26, 34, and 45 weeks of age, respectively) (Tabatabaei *et al.,* 2010).

Moreover, previous studies have reported that seminal plasma plays a role in the regulation of semen quality such as sperm morphology, motility, sperm concentration, acrosome reaction, and fertility (Mann, 1981). This is because it contains many proteins that originate from the testis, the rudimentary epididymis, and the ductus deferens which provide an optimal environment for cell survival while others serve in the defense against oxidative or microbiological damage and assist in sperm interactions with different microenvironments in the female genital tract (Atikuzzaman *et al.,* 2017; Marzoni *et al.,* 2013). Therefore, we assessed the relation between age and semen quality of roosters at different ages, and found that their seminal plasma protein pattern changed with age. The apparent molecular masses were 72, 90, and 140 kDa. Protein bands at 72 and 140 kDa were abundant in the group at 7-8 months of age and slightly decreased with age increased. However, protein bands at 90 kDa were low at 7-8 months of age and slightly increased with age increased (Fig. 3). Therefore, seminal plasma proteins with molecular weights of 90 and 140 kDa might be associated with live sperm or viability. It could be concluded that the semen quality of Thai native roosters (Leung hangs khao) is affected by age of the bird. The roosters in different age groups contain specific

seminal plasma proteins that can be used as valuable markers of semen quality. This might serve as a better alternative for other qualitative methods.

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