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Evaluation of semen quality in roosters of different age during hot climatic condition

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ABSTRACT

The present experiment was conducted to evaluate the semen quality of roosters of different ages during hot climatic condition. Semen from roosters (n=8 / age group) of 23, 42 and 65 weeks of age was collected and evaluated for different physical parameters. The sperm membrane integrity was evaluated by hypo-osmotic swelling test, whereas sperm DNA fragmentation was assessed by Sperm Chromatin Dispersion (SCD) test. The seminal plasma cortisol level was assessed by EIA. The shed average Temperature Humidity Index (THI) during the experiment period was 79.32. Semen volume and sperm DNA fragmentation were significantly different ($P \leq 0.05$) between the age groups tested. Roosters of 42 weeks age had higher semen volume and lower sperm DNA fragmentation during study period. None of the other parameters were influenced by the age of the birds. The results indicated that semen quality was affected by the age of the birds. The extreme heat condition also appears to exert a negative influence on the sperm chromatin in roosters.

Key words: Age, Chicken, Semen, Sperm chromatin dispersion test.

1. Introduction

The semen quality of the rooster is affected by different factors like breed, age, feed and environmental stressors like temperature and humidity (Zhang et al., 1999; Karaka et al., 2002; Shanmugam et al., 2012). The semen volume in broiler breeder males increased from 24 to 48 weeks of age (Shanmugam et al., 2012), decreased in White Leghorn roosters with advancing age (46 weeks and above) (Clark and Sarakoon, 1967) or fluctuated between the weeks of collection (Kelso et al., 1997). Similarly the sperm concentration decreased with age of the bird (Sexton et al., 1989; Hocking and Bernard, 1997; Zhang et al., 1999) or remained constant up to 53 weeks of age and afterwards started declining (Wilson et al., 1971, 1987; Cerolini et al., 1997). The sperm activity assessed through 3-[4,5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide (MTT) dye reduction test and number of live sperms was shown to be increased from early age to mid age in broiler breeder males (Shanmugam et al., 2012).

The environmental temperature is an important factor influencing the semen quality and fertility of rooster in tropical countries where the birds are mostly raised in open sided poultry house. It has long been reported that semen quality is decreased during summer or on exposure to high ambient temperature (Boone and Huston, 1963; Joshi et al., 1980). Though the sperm motility was not affected by heat treatment of broiler males, fertility and sperm egg penetration declined, however, the underlying mechanism is not known (McDaniel et al., 1995). The authors had suggested that heat exposure might have resulted in nuclear abnormality leading to declined fertility. It is well documented in lab animals that mild or transient scrotal heat stress for shorter duration of 30 minutes causes DNA damage in the developing sperm (Banks et al., 2005; Paul et al., 2008). There is no report that examines the effect of high ambient temperature on sperm nuclear status in chicken. Determination of the sperm chromatin integrity provides valuable information on the male fertility potential (Agarwal and Said, 2003). Different tests are available for detection of sperm chromatin/DNA damage like COMET assay (Gliozzi et al., 2011), TUNEL assay (Martins et al., 2007), SCSA (Partyka et al., 2010) and Sperm Chromatin Dispersion (SCD) test (Fernández et al., 2003). Among these assays the SCD test is a simple and inexpensive method for the analysis of sperm DNA fragmentation. This method is based on the principle that sperm with DNA

fragmentation fail to produce halo of dispersed DNA loops when mixed with agarose followed by acid denaturation and nuclear protein removal (Fernández et al., 2003). The halos can be visualised using bright-field microscopy after staining with Wright's stain (Fernández et al., 2005).

The aim of the present study was to determine the semen quality of different age roosters during hot summer conditions. Further in the present study the status of sperm chromatin damage of the different age birds was assessed using SCD test.

2. Materials and methods

2.1. *Experimental birds and husbandry*

The experiment was carried out at the experimental poultry farm of Directorate of Poultry Research located at Hyderabad, India. Dahlem Red, a brown tinted layer pure line chicken maintained at the institute was used in the experiment. Roosters (n=8 / age group) of three different ages 23, 42 and 65 weeks were housed in individual breeder cages from 18 weeks of age in an open-sided elevated house under natural photoperiod and climatic conditions. The birds had free access to both feed and water. They were trained for semen collection from 20 weeks of age and semen collected and discarded weekly unless used. The trial was conducted following the approval of the Institute Animal Ethics Committee.

2.2. *Temperature data*

Mean ambient temperature (Ta) in Celsius and percent relative humidity (RH) in the shed during the week of the experiment was used to calculate the Temperature Humidity Index (THI), according to the formula: THI = (0.8 × Ta) + [(RH / 100) × Ta – 14.3)] + 46.4) (Mader et al., 2010). The average THI during the week of semen evaluation was 79.32.

2.3. *Semen collection and evaluation*

Semen was collected in a glass funnel by abdominal massage method (Burrows and Quinn, 1937). The semen collected once during the week of the experiment was used for analysis. The samples were immediately diluted four times using high temperature (HT) diluent (NaCl 0.8g; TES 1.374g; 1M NaOH 2.75ml; glucose 0.6g, dissolved in 100 ml of double distilled water, pH 7.4) (Chaudhuri and Lake, 1988). The four fold diluted semen samples were brought to the lab and used for laboratory evaluation of seminal parameters.

The ejaculate volume was assessed by drawing the sample in to 1 ml syringe with an accuracy of 0.02 ml. Sperm motility was assessed as percentage of progressively motile sperms and a drop of the diluted semen was kept on a clean glass slide and cover slip was applied to examine under high power magnification (40×) and was subjectively assessed. The concentration of sperm was estimated by the method described by Taneja and Gowe (1961) using a colorimeter at 540 nm of wavelength. Tetrazolium dye 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction test was carried out in duplicate tubes and absorbance was measured with a colorimeter (CL 157, Elico Ltd, India) at 570 nm according to Hazary et al. (2001).

The live and dead sperm percent was calculated by differential staining technique using eosin–nigrosin (Campbell et al., 1953). The slides were used for estimating the percent abnormal sperms on the basis of observable abnormalities. A minimum of 200 sperms were counted in each slide for calculating live, dead and abnormal sperm per sample.

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2.4. *Hypo-osmotic swelling test*

The plasma membrane integrity was determined through hypo-osmotic swelling test as described for chicken spermatozoa (Santiago-Moreno et al., 2009). In a glass tube 25 µl of diluted semen was taken and 500 µl of hypo-osmotic solution (100 mOsm/kg; 1 g of sodium citrate in 100 ml distilled water) was added and incubated for 30 min at 37°C. The samples fixed in 25 µl of 2% gluteraldehyde were examined at 400× under phase-contrast microscope by counting 200 sperm. The percentage of spermatozoa having coiled mid-pieces and tails were calculated.

2.5. *SCD test*

The test was performed according to the procedure of Fernández et al. (2003), with minor modifications. Equal volumes of diluted semen sample and 1% low-melting agarose were mixed at 37°C. An aliquot of 30 µl of the mixture was pipetted onto glass slides precoated with 0.65% normal melting agarose, covered with cover slip and kept on ice pack for 4 minutes to solidify. Immediately after careful removal of cover slip the slides were immersed horizontally in a tray containing acid denaturation solution (0.08N HCl) for 3 minutes at 22°C in the dark to generate restricted single-stranded DNA (ssDNA) motifs from DNA breaks. Then the denaturation was stopped and proteins were removed by transferring the slides to a tray with neutralising and lysis solution (0.4 M Tris, 0.8 m DTT, 1% SDS, 50 mM EDTA, pH 7.5) for 5 minutes at room temperature. The slide was then washed in Tris-borate-EDTA buffer (0.09 M Tris-borate and 0.002 M EDTA, pH 7.5) for 2 minutes, dehydrated in sequential 70%, 90% and 100% ethanol (2 minutes each) and air dried. The prepared slides may be stored in tightly closed box in dark if not immediately stained. For bright-field microscopy slides were horizontally covered with a mix of Wright's stain and buffer solution (380 mg Na_2 PO_4, 547 mg KH_2 PO_4 in 100 ml distilled water) for 10 minutes with continuous air flow. The stain was poured off and slides were washed briefly in tap water and dried.

The stained slides were evaluated manually by counting 500 sperm on each slide for halo size and dispersion pattern at 1000× magnification as described by Fernández et al. (2003). The nuclei with large to medium size halo were considered sperms with nonfragmented DNA while nuclei with small size halo or without halo or without a halo and degraded were taken as sperms with fragmented DNA.

2.6. *Cortisol estimation*

The ejaculated native semen samples were centrifuged at 3000 rpm for 10 min twice to separate seminal plasma and stored until analysis. The seminal plasma was analyzed for cortisol using commercial EIA kit (DSI S.r.l. Italy) with a sensitivity of 1.81ng/ml. The kit was used according to the specifications of the manufacturer. The intra assay coefficient of variation was 14%.

2.7. *Statistical analysis*

Data were analysed using SAS 9.2 software and $P \leq 0.05$ was considered significant. Statistical analyses of semen parameters and cortisol were performed by one way ANOVA with Tukeys post hoc test. Percent value data were arc sine transformed before analysis.

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3. Results

The semen quality parameters and seminal plasma cortisol level did not differ between the three age groups studied except for semen volume and sperm chromatin fragmentation (Table). The semen volume was significantly higher ($P \leq 0.05$) in the 42 weeks age roosters than at 23 weeks of age. The sperm DNA fragmentation (Fig) was significantly lower ($P \leq 0.001$) in the 42 weeks age roosters.

4. Discussion

The ejaculated semen volume was highest at 42 weeks of age and lowest at 23 weeks of age. This may be due to the reason that the birds started giving semen on stimulation from 20 weeks of age only. This was similar to that reported by Shanmugam et al. (2012) where the semen volume increased with age till 48 weeks of age. Semen volume in White Leghorn chicken was found to decline from 46 weeks of age (Clark and Sarakoon, 1967). The volume was found to fluctuate between ages with highest volume at 24 weeks and lowest at 72 weeks of age (Kelso et al., 1997). The semen volume, sperm concentration and percentage of males in semen production declined linearly with age and were lowest in broiler breeders at 52 weeks of age during the experimental period (Zhang et al., 1999). In a recent report also broiler breeders were shown to produce maximum number of sperm at 36 weeks of age and declined progressively until 55 weeks of age (Fragoso et al., 2013). Among other reasons, declining testis weight and testosterone level may form partial explanation for lower sperm production with advancing age (Fragoso et al., 2013). Overall, with advancing age of roosters the semen production capacity decreased but with a varying magnitude. The motility of sperms was not different between the ages in this study and is similar to that reported by Kelso et al. (1997). The functional membrane integrity of sperm assessed by hypo-osmotic swelling test was similar between the ages. From the results it seems that age of the birds did not influence membrane functionality though changes in lipid and fatty acid composition of sperm membrane have been reported (Cerolini et al., 1997; Kelso et al., 1996). The sperm concentration was not significantly different between the different ages of the birds. However, there was high numerical difference between the ages. The sperm concentration linearly declined with age (Hocking and Bernard, 1997; Zhang et al., 1999), or different between the ages of the roosters, peaking at 39 weeks of age and lowest at 72 weeks of age (Cerolini et al., 1997; Kelso et al., 1996, 1997). Though similar trend of peaking at middle age was observed in the present study the non significant difference may be due to high variability between the birds in that age group. The metabolic activity assessed by the MTT dye reduction test was not different between the age groups. The result is in variance with an earlier report where older aged birds had significantly lower metabolic activity (Kelso et al., 1996). Similarly in the same study a significant reduction in live sperm percentage was reported, but in this study there was no difference between the age groups. The variation in the results may be ascribed to difference in the type of chicken used in the studies. Further the differences in different semen parameters due to age might have been compromised due to high ambient temperature. A THI threshold of 70 was reported for chicken, above which production was affected in layers and broilers (Tao and Xin, 2003; Karaman et al., 2007). The THI during the present experiment was 79.32 and the daily average ambient temperature was 32.12°C which might have produced heat stress in the experimental birds. White Leghorn roosters of 60 week old were affected by ambient temperature of 32.2°C for 40 days (Joshi et al., 1980) but in a fluctuating temperature of 21 to 38°C had no detrimental effects on semen characteristics of 34 to 55 week old White Leghorn roosters (Clark and Sarakoon, 1967). The sperm quality index and fertility was found to decline and dead sperm percent increased by heat exposure

(32°C) of broiler breeders (McDaniel et al., 2004). Further it was observed that the body temperature was also increased in the exposed males.

This is the first study to report the use of SCD test in chicken sperm and the test was used to analyse the state of sperm chromatin of different age roosters. This is a simple technique requiring no sophisticated instruments when Wright stain is used for visualization of the halos. Gross semen parameters were not affected in broiler breeder males exposed to 32.2°C ambient temperature for 8 weeks but sperm-egg penetration was decreased by 67% when semen from exposed group males were used with no contribution from the heat stressed females leading to conclusion that only males contributed to decreased fertility (McDaniel et al., 1995). These results lead the authors to hypothesise that nuclear abnormality due to heat stress might be the reason for lowered fertility. Since then no study has explored the chicken sperm nuclear status as such or during heat stress. When chicken semen from different ages was inseminated peak fertility was obtained from 39 weeks males and progressively declined till 72 weeks in broiler breeders (Cerolini et al., 1997). Similarly, the fertility was highest by using semen from 39 weeks roosters than that of other ages (Kelso et al., 1997). Thus the previous results indicate that heat exposure and possibly age of the male have negative effect on sperm nuclear status but not supported by experimental evidence. This lack of information has been addressed in the present study where sperm chromatin fragmentation at different ages of roosters was assessed during summer. High levels of reactive oxygen species (ROS) resulting in oxidative stress condition is correlated with increase in sperm DNA damage (Agarwal and Said, 2003). Heat stress in rats lead to increased scrotal heat and resulted in oxidative stress which in turn caused damage to sperm DNA (Ikeda et al., 1999). Though damaged sperm cells may be removed by apoptosis during development, many cells complete the process and appear as motile sperms but with damaged DNA (Banks et al., 2005). Even a single transient heat stress is reported to produce DNA damage in the sperm of mice (Paul et al., 2008). In chickens exposed to 32°C the body temperature increased significantly compared to the control (McDaniel et al., 1996, 2004). Similarly in the present study because of the higher ambient temperature the bird's body temperature would have increased and resulted in heat stress and sperm chromatin damage. The heat stress may form a partial explanation for the observed sperm chromatin damage effects in the present study and the age of the birds is another contributor as clearly observed from the results of this study. Age of human subjects had been shown to have strong effect on sperm chromatin defects. Aged men had significantly higher DNA fragmentation index; the damage was found to be doubled between 20 and 60 years of age (Wyrobeck et al., 2006). In our study apart from the 65 week age roosters the 23 week age roosters were also found to have higher DNA fragmentation. It may be speculated that the 23 week birds that were at the start of semen production were more affected by severe heat condition resulting in the higher DNA fragmentation.

There is no previous report on cortisol level in chicken seminal plasma. Age of the birds had no effect on the cortisol level in the present study. Therefore it may be assumed that all the three age groups undergo similar level of stress due to high ambient temperature. The level of cortisol in seminal plasma was higher compared to the blood plasma level of broilers reported earlier (Sohail et al., 2010). The potential role of this hormone at higher level in seminal plasma is unclear. Earlier it was reported that the level of corticosterone and fertility are unrelated and it was concluded that circulating corticosterone does not provide information about the male reproductive potential (Brougher et al., 2005). It is believed that corticosterone is the major adrenal corticosteroid and not cortisol in birds (Schmidt and Soma, 2008). The circulating glucocorticoids levels need not be indicative of local glucocorticoids levels and organ specific differences may occur in the glucocorticoids levels (Schmidt and Soma, 2008). Further the immune organs were found to produce more cortisol than corticosterone in zebra finches (Schmidt and Soma, 2008). Extra adrenal production of

corticosteroids is well reported (Davies and MacKenzie, 2003), however, presence of enzyme machinery for cortisol synthesis in the testis is not known. The sperm is stored in the sperm storage tubules in chicken up to 3 weeks and it may be hypothesized that cortisol in the semen may play an immunosuppressive role. Thus the significance of higher level of cortisol in seminal plasma and its role in sperm storage in female and fertility needs to be investigated.

In conclusion semen volume and sperm DNA integrity were different during different ages of chicken roosters. Sperm DNA fragmentation was higher at both young and old age indicating the probable reason for lower fertility generally observed by use of semen obtained from these age birds. The role of higher seminal plasma cortisol in sperm storage in hen and fertility needs to be elucidated.

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Legend for Fig: Sperm chromatin dispersion (SCD) test in roosters. (A) sperm with small halo, (B) sperm with medium halo, (C) sperm with large halo.

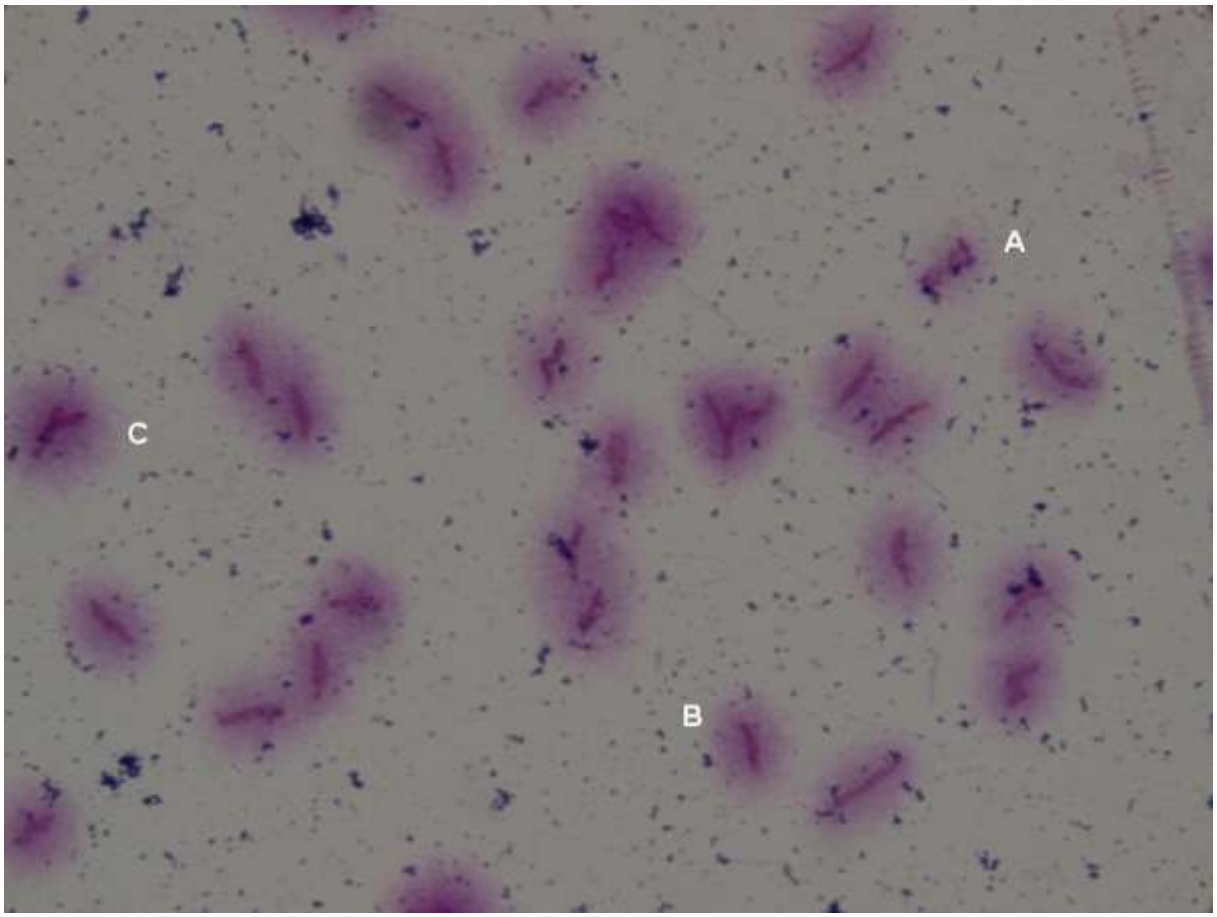

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Table Mean ± SE semen quality parameters of different age roosters.

Semen quality parameters	23 weeks	42 weeks	65 weeks
Volume (ml)	0.29 ± 0.05^{b}	0.48 ± 0.06^{a}	0.36 ± 0.05^{ab}
Progressive motile sperm (%)	46.25 ± 2.45	45.0 ± 2.5	45.0 ± 2.67
Sperm concentration (million/ μl)	3.32 ± 0.54	4.8 ± 0.41	3.57 ± 0.54
MTT dye reduction test (nM of MTT Formazan /min/million sperm)	23.76 ± 0.85	25.55 ± 0.95	23.64 ± 1.49
Live sperm (%)	93.01 ± 2.39	91.21 ± 1.49	92.12 ± 1.90
Dead sperm (%)	6.99 ± 2.39	8.79 ± 1.49	7.88 ± 1.90
Morphologically abnormal sperm (%)	1.61 ± 0.93	5.58 ± 2.82	6.13 ± 2.76
Hypo-osmotic sperm swelling (%)	86.07 ± 1.48	87.54 ± 1.75	89.65 ± 1.60
Sperm DNA fragmentation % (SCD Test)	69.05 ± 3.74^{a}	24.62 ± 1.89^{b}	62.72 ± 3.71^{a}
Cortisol (ng/ml)	13.82 ± 1.35	11.63 ± 1.27	13.16 ± 1.58

Means with different superscripts in a row differ significantly ($P \leq 0.05$)