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Thermal manipulation during embryogenesis improves certain semen parameters in layer breeder chicken during hot climatic conditions

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ABSTRACT

Thermal manipulation during incubation has been shown to improve post hatch performance in poultry. The aim of the present experiment was to evaluate thermal manipulation on semen quality of roosters during hot climatic conditions. Eggs obtained after artificial insemination from Dahlem Red layer breeders were randomly divided into two groups control (C) and heat exposed (HE). C group eggs were incubated at 37.5°C throughout the incubation period while the HE group eggs were exposed to higher temperature 40.5°C from 15-17th day of incubation for 3 hours each day. The relative humidity was maintained at 65% in both the groups throughout incubation. The chicks hatched were reared separately under standard husbandry conditions. During high ambient temperature semen from roosters (45 weeks of age) was collected and evaluated for different gross parameters, sperm chromatin integrity and sperm *HSP27* and *HSP70* gene expression by real-time PCR. The seminal plasma was evaluated for lipid peroxidation, ferric ion reducing antioxidant power (FRAP), triiodothyronine (T_3) and matrix metalloproteinase-2 (MMP-2) activity. The shed average Temperature Humidity Index (THI) during the experiment period was 78.55. The percent live sperm and FRAP level were significantly ($P<0.05$) higher and sperm gene expressions were significantly ($P<0.05$) lower in the HE group. No differences in other parameters were observed between the groups. Thus from the results it could be concluded that thermal manipulation during incubation improves certain semen parameters of roosters at high ambient temperature.

Key words: Chicken, Semen, heat shock protein, thermal manipulation, MMP, heat stress.

1.Introduction

The environmental temperature is an important factor affecting the semen quality and fertility of rooster in tropical countries. The semen quality deteriorates during summer or on exposure to high ambient temperature (Boone and Huston, 1963; Joshi et al., 1980). Even if the sperm motility was not affected by heat treatment of broiler males, the fertility and sperm egg penetration declined (McDaniel et al., 1995). It was suggested that heat exposure might have resulted in nuclear abnormality resulting in declined fertility. Furthermore, it has been shown that heat stress depresses the number of sperm penetrating the perivitelline membrane (McDaniel et al., 1996). Heat stress will lead to oxidative stress in semen (Rao et al., 2015) due to high levels of free radicals, more particularly reactive oxygen species (ROS). This

higher free radical level will lead to damage of different sperm components. Chicken sperm are rich in polyunsaturated fatty acids and are readily susceptible to oxidation by free radicals. There exists an enzymatic and non-enzymatic defence mechanism to counter the damage due to oxidative stress. The non-enzymatic defence consists of dietary components and metabolites such as vitamin E, vitamin C, uric acid, bilirubin etc. These non-enzymatic compound activities in biological fluids can be measured and expressed as ferric ion reducing antioxidant power (FRAP) (Benzie and Strain1996). Mild or transient scrotal heat stress for shorter duration of 30 minutes in lab animals has been shown to cause DNA damage in the developing sperm (Banks et al., 2005; Paul et al., 2008). Furthermore, determination of the sperm chromatin integrity offers valuable information on the male fertility potential (Agarwal and Said, 2003). Sperm Chromatin Dispersion (SCD) testis a simple and inexpensive method for the analysis of sperm DNA fragmentation (Fernández et al., 2003) and the test was optimized for chicken sperm in our lab (Shanmugam et al., 2014). This test is based on the principle that sperm with DNA fragmentation fails to produce a halo of dispersed DNA loops when mixed with agarose followed by acid denaturation and nuclear protein removal.

Heat Shock Proteins (HSPs) are a set of highly conserved proteins that act under physiological conditions as molecular chaperones and are also induced by cytotoxic stressors including temperature (Neuer et al., 2000). HSPs are classified based on their molecular weight and among them HSP70 and HSP27 seems to be closely associated with heat tolerance (Samali et al., 2001; King et al., 2002). In response to heat stress differential expressions of HSP70 and HSP27 gene or protein in different tissues of chicken has been reported (Yahav et al., 1997; Wang and Edens, 1998).

The matrix metalloproteinase-2 (MMP-2) is a zinc dependant endopeptidase that hydrolyses a variety of extracellular matrix and non-extra cellular matrix proteins. MMP-2 has been detected in seminal plasma and other reproductive tissue fluids of human, dog, ram, boar and stallion (Metayer et al., 2002; Tentes et al., 2007; Saengsoi et al., 2011; Warinrak et al., 2015). MMP-2 has been shown to be correlated with sperm concentration and motility (Baumgart et al., 2002; Saengsoi et al., 2011). The presence or activity of MMP-2 in chicken seminal plasma is not known. Thyroid hormones play a role in the regulation of metabolic rate. Chronic heat stress has been shown to reduce plasma T_3 concentration in layer hens (Decuypere and Kuhn, 1988; Melesse et al., 2011).

The procedure of thermal manipulation during embryogenesis has been shown to improve thermotolerance in poultry (Yahav et al., 1997; Piestun et al., 2008; Al-Zghoul et al., 2013). The epigenetic thermal adaptation described as changes that occur in a short critical developmental period during pre- or early postnatal ontogeny affects physiological control systems for a lifelong adaptation to an expected post-natal environmental condition (Tzschentke and Plagemann, 2006). Thermal manipulation during broiler embryo development has been shown to improve post-hatch performance under hot conditions (Piestun et al., 2011). The aim of the present study was to investigate the effect of thermal manipulation during embryonic development on rooster sperm characteristics associated with sperm function during hot climatic conditions.

2. Materials and methods

2.1. Experimental birds and husbandry

The experiment was carried out at the experimental poultry farm of the institute following the approval of the Institutional Animal Ethics Committee. Eggs obtained after artificial insemination from Dahlem Red layer breeder were randomly divided into two groups control (C) and heat exposed (HE). C group eggs were incubated at 37.5°C and 65% relative humidity throughout the incubation period whereas the HE group eggs were exposed to higher temperature of 40.5°C during 15-17th days of incubation for 3 hours each day with relative humidity maintained at 65%. The hatchability on fertile eggs set in C and HE group were 87% and 83% respectively. The chicks hatched were wing banded and reared group wise in battery brooders and starting from 18 weeks of age the roosters were housed in individual breeder cages in an open-sided house under natural photoperiod and climatic conditions. The birds had free access to feed and water. The roosters were trained for semen collection from 22 weeks of age and semen collected and discarded periodically unless used.

2.2. Temperature data

The semen collection and evaluation was carried out during the middle of summer when the maximum environmental temperature will reach up to 45°C. Mean ambient temperature (Ta) in Celsius and percent relative humidity (RH) in the shed during the week of the experiment was used for calculation of 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 shed average THI, temperature and relative humidity during the week of semen evaluation were 78.55, 31.08 and 43.4 respectively.

2.3. Semen collection and evaluation

All chemicals used in the experiment were purchased from Sigma-Aldrich (USA) unless otherwise specified. Semen sampling in nineteen randomly selected roosters (45 weeks of age) from each group was done twice during the week of the experiment in an interval of five days. The initial semen collected was used for analysing gross semen parameters, biochemical parameters and SCD test and the second sampled semen was used for gene expression in sperm and MMP activity determination in seminal plasma. Semen samples collected by abdominal massage (Burrows and Quinn, 1937) after assessing for ejaculate volume and appearance, were diluted four times with 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). These four-fold diluted semen samples were used for laboratory evaluation of seminal parameters.

The ejaculate volume was assessed by using 1 ml syringe with an accuracy of 0.02 ml. The appearance of raw semen was scored 1 to 5 visually (McDaniel and Craig, 1959). Sperm motility was subjectively assessed as percentage of progressively motile sperm by placing a drop of the diluted semen on a Makler chamber and observed under a microscope. The concentration of sperm was estimated as described by Taneja and Gowe (1961) using a colorimeter at 540 nm wavelength. The Tetrazolium dye 3-(4,5-dimethylthiazol-2-yl)-2,5-

diphenyltetrazolium bromide (MTT) reduction test was carried out and absorbance was measured with a colorimeter at 570 nm (Hazary et al., 2001). The percent live sperm was calculated by a differential staining technique using eosin–nigrosin (Campbell et al., 1953). The slides were used for estimating the percent abnormal sperm on the basis of observable abnormalities such as coiled, twisted, or knotted head, loose midpiece, broken sperm or tail defects, bent sperm, sperm coiling and with attached droplets. Around 200 sperm were counted in each slide for calculating live and abnormal sperm per sample.

The ejaculated native semen samples from 2 or 3 birds were pooled in the respective groups and centrifuged at 1500 x g for 10 min to separate seminal plasma and stored at -80°Cuntil analysis. The sperm pellet was washed twice in the diluent and final pellet for RNA isolation was stored at -80°C.

The seminal plasma parameters evaluated were lipid peroxidation (Hsieh et al., 2006), and antioxidant power by ferric reducing ability of plasma (FRAP) assay (Benzie and Strain 1996). The T_3 level was measured using commercial EIA kit (RFCL Ltd, India).The EIA kit had a sensitivity level of 0.04 ng/ml for T_3. The intra-assay coefficient of variation was 10%.

2.4. SCD test

The test was performed following the procedure of Fernández et al., (2003), with minor modifications for chicken sperm (Shanmugam et al., 2014).Briefly, equal volumes of diluted semen sample and 1% low-melting agarose (Invitrogen, India) 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 (Invitrogen, India), cover slipped and kept on an ice pack for 4 minutes. The cover slips were carefully removed and immediately the slides were kept 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. The denaturation was stopped and proteins were removed by transferring the slides to a tray with neutralising and lysing solution (0.4 M Tris, 0.8 M DTT, 1% SDS, 50 mM EDTA, pH 7.5) for 5 minutes at room temperature. The slides were then washed in Tris-borate-EDTA buffer (0.09 M Tris-borate and 0.002 M EDTA, pH 7.5) for 2 minutes and dehydrated sequentially in 70%, 90% and 100% ethanol (2 minutes each) and air dried. The slides thus prepared were stored in a tightly closed box in the dark until staining. For bright-field microscopy, slides were horizontally covered with a mix of Wright's stain (HiMedia, India) and normal saline for 10 minutes with continuous air flow. The stain was poured off and slides were washed briefly in tap water and dried.

The stained samples were evaluated by counting manually 500 sperm on each slide for halo size and dispersion pattern under oil immersion as described by Fernández et al. (2003). The nuclei with large to medium size halo were considered sperm with non fragmented DNA whereas nuclei with small size halo, without halo or without a halo and degraded were taken as sperm with fragmented DNA.

2.5. Gelatin zymography

Gelatin zymography of seminal plasma was carried out according to Saengsoi et al., (2011) with minor modifications. Seminal plasma was mixed with sample buffer (0.5 M Tris–HCl with pH 6.8, 10% sodium dodecyl sulfate (SDS), 20% glycerol, and 0.05% bromophenol blue). The samples and human MMP-2 marker (Sigma–Aldrich, USA) were loaded in the wells of casted gel. The zymography gel used was 4% stacking polyacrylamide gel (PAGE) and 8% resolving gel, containing 4% (w/v) porcine gelatin and 10% (w/v) SDS. Gels were run at 100 V for the first 30 min and at 150 V till the dye reached the bottom of gel. The SDS was removed from the zymograms by washing in a water solution with 2.5% (w/v) Triton X-100 for 1 h on a shaker. The water solution was discarded and gels were incubated for 19 h at 37°C in an incubation solution (50 mMTris–HCl, 5 mM $CaCl_2$, 150 mM $NaCl_2$ and distilled water). Gels were stained for 1 h in a staining solution (0.2% Coomassie Brilliant Blue R-250 dye in 50% methanol and 10% acetic acid) on a shaker, and then destained in the same solution without the dye until clear bands were visible. The destained gels were scanned and the image subjected to densitometric analysis of the zymograms using the Image J program (v 1.48v).

2.6. RNA Extraction, cDNA synthesis and RT- PCR

The sperm pellets obtained after centrifugation was used for total RNA extraction using TRIzol reagent (Invitrogen, USA) according to the manufacturer's protocol. The purity of the RNA was determined by measuring absorbance in Genova Nano (Jenway, UK) at 260 and 280 nm. Briefly, 1.5µg of RNA was reverse transcribed with high capacity cDNA reverse transcription kit (Applied Biosystem, USA). The expression of mRNA was quantified by SYBR green method using Step One Real time PCR system (Applied Biosystem,USA). The glyceraldehyde-3-Phosphate dehydrogenase (*GAPDH*) and hypoxanthine phosphoribosyl-transferase (*HPRT*) were used as endogenous controls. The first-strand cDNA were used as a template to amplify gene specific primers for *HSP70* and *HSP27*; reference gene *GAPDH* and *HPRT*. These reactions were performed in a 25 µl volume of SYBR green master mix (KAPA Biosystem, USA) with 10 pM of each primer (Table 1). The amplification protocol used was as follows: initial denaturation at 95°C for 10 min, followed by 40 cycles of cyclic denaturation at 94°C for 15 s, annealing at 60°C for 1min and extension at 72°C for 15 s. Real time quantification of HSP genes was normalized to the threshold number of cycles (ct) *GAPDH* and *HPRT*. The difference in *HSP70* and *HSP27* mRNA expression relative to *GAPDH* and *HPRT* expression was calculated using $2^{-\Delta\Delta ct}$ formula. Primer amplification efficiency was assessed from the standard curve generated by using a serial tenfold dilution of transcribed RNA.

2.7. Statistical analysis

Data were analysed using SAS 9.2 software and $P<0.05$ was considered significant. Statistical analyses of semen parameters were performed by Student's *t* test. The percent value data were arc sine transformed before analysis.

3. Results

The percent live sperm and seminal plasma FRAP level were significantly higher ($P<0.05$) in the HE group roosters (Table 2). There was no difference in other gross semen parameters between the groups. There was no difference in sperm DNA fragmentation between the groups (Fig. 1). In the seminal plasma samples of both the C and HE groups MMP-2 activity in the form of two bands at 72 and 62 kDa molecular weight was observed corresponding to pro-MMP-2 and MMP-2 respectively (Fig. 2). However, the densitometric analysis did not reveal any difference in activity between the groups. The mRNA expressions of *HSP27* and *HSP70* genes in sperm were significantly lower ($P<0.05$) in the HE group roosters (Fig. 3).

4. Discussion

To the authors' knowledge there is no report in the literature on thermal manipulation for improving semen quality in chicken. Earlier, we did not find any beneficial effects of 2°C increased *in ovo* incubation temperature during 16-18th days of incubation on rooster semen quality (unpublished data). This 2°C increased *in ovo* incubation temperature during 16-18th days of incubation has been shown to improve thermotolerance in chicks (Yahav et al., 2004). In the present experiment, a protocol of 3°C increased *in ovo* incubation temperature was used and the results reported. Environmental manipulation during 'critical period' of prenatal embryonic development influences the physiological control system. Thus, the actual incubation temperature applied for the eggs brings about life long imprinting in the thermoregulatory system (Tzschentke and Plagemann, 2006). The hypothesis is that application of thermal manipulation during embryogenesis the set point of the controlling system could be altered during the development/maturation of hypothalamus-hypophysis-thyroid axis and the hypothalamus-hypophysis-adrenal axis that occurs during 10-16th day of egg incubation (Wise and Frye, 1975). In chicken, epigenetic temperature adaptation has been shown to be induced by changes in incubation temperature during the end of embryonic development (Loh et al., 2004). The long lasting effect of a change in incubation temperature in chicken has been shown through altered c-Fos expression in the hypothalamus (Janke and Tzschentke, 2010). In chicken embryonic development, gonadal differentiation and appearance of first spermatogonia occurs on the 7th day and 13th day of incubation respectively (Kirby and Froman, 2000). Based on the above reasons the present protocol of thermal manipulation was adopted.

A THI threshold of 70 has been reported for layer chicken above which egg production was affected (Karaman et al., 2007). The THI formula used for calculation in this experiment gave values similar to that reported by Karaman et al. (2007). Furthermore, there is no reported THI threshold value for roosters and therefore the threshold value of layer chicken was applied in this study. The THI during the present experiment was 78.55 and the daily average ambient temperature was 31.08°C and might have caused heat stress in the experimental birds as observed by the panting in the birds during the study. An ambient temperature of 32.2°C for 40 days affected the semen quality of 60 week old White Leghorn roosters (Joshi et al., 1980) however, in an environment of fluctuating temperature (21 to 38°C) no detrimental effects on semen characteristics of 34 to 55 week old White Leghorn

roosters (Clark and Sarakoon, 1967). In heat exposed (32°C) broiler breeders the body temperature increased and the sperm quality index and fertility declined and dead sperm percent increased (McDaniel et al., 2004). Our results are in concurrence with that of Clark and Sarakoon (1967) and except for live sperm percent none of the other gross semen parameters were affected though the minimum shed temperature has remained above 25.5°C during the experimental period. The beneficial effect of *in ovo* heat exposure on gross semen parameters has been observed in the HE roosters in terms of higher live sperm.

The gross semen parameters remained unaffected in broiler breeder males exposed to 32.2°C ambient temperature for 8 weeks but sperm-egg penetration declined leading to hypothesise that nuclear abnormality due to heat stress might be the reason for the declined sperm-egg penetration (McDaniel et al., 1995). Heat stress, even of a transient nature, in lab animals has been shown to produce DNA damage in the sperm (Ikeda et al., 1999; Paul et al., 2008). In the present study, no difference could be observed in sperm DNA fragmentation between the roosters of the two groups. The causes for sperm DNA fragmentation are apoptosis, chromatin immaturity and oxidative stress (Muratori et al., 2015). The lipid peroxidation was numerically higher in the C group and should have caused damage to the sperm. With the limited information in this study, it is difficult to put forth a convincing explanation for no difference in the DNA fragmentation between the groups. Chicken sperm is composed of a high proportion of polyunsaturated fatty acids predisposing it to peroxidative damage due to oxidative stress. Stress of different origin, including heat stress will lead to oxidative stress and results in lipid peroxidation (Ayo et al., 2011). In the present study there was only numerically lower lipid peroxidation level in the HE group of birds, however, the group had significantly higher seminal plasma FRAP. This indicates that the levels of non-enzymatic antioxidants (glutathione, uric acid, lipoic acid, ascorbic acid, ubiquinol etc.) are at higher level in the HE group birds that can protect the sperm cells from free radical damage. Under high ambient temperature the digestibility and retention of nutrients are reduced in chicken (Bonnet et al., 1997). Chicken raised in chronic elevated environmental temperature or short term heat exposure have decreased villi height in the jejunum, reduced feed intake, innate immunity, egg production and lower circulating T_3 level (Mitchell and Carlisle, 1992; Franco-Jimenez et al., 2007; Quinteiro-Filho et al., 2010). Thus it can be assumed that the C roosters might have evinced stress due to high ambient temperature and the HE roosters due to thermal manipulation was tolerant to the heat, the metabolic pathways or feed utilization was largely unaffected and could accumulate the non-enzymatic antioxidants. The thyroid hormone T_3 plays an important role in metabolism. During heat stress the metabolism and resultant heat production should be less so that the birds are not further burdened by heat load. Thus, the level of T_3 in peripheral circulation remains low during high ambient temperature (Melesse et al., 2011). The chicks that were thermal manipulated during embryonic stage had lower or similar T_3 level compared to that of control chicks (Piestun et al., 2008; Pietsun et al., 2009). In the present study the T_3 level was similar between the treatments.

The MMPs main function is digesting components of extracellular matrix and its role in release and activation of growth factors and cytokines has also been reported (Nagase and

Woessne, 1999; Fowlkes and Winkler, 2002). MMPs are secreted as pro peptides and become active after cleavage. MMP-2 has been shown to be secreted by Sertoli cells and Leydig cells of the testis (Robinson et al., 2001; Le Magueresse-Battistoni, 2008). The present study has confirmed the presence of both latent and active form of MMP-2 in chicken seminal plasma. Their functional role in semen or female reproductive system is unclear. The thermal manipulation did not have any effect on the activity of this protein in semen, probably because enzyme activity is dependent on the actual conditions of reactions.

HSPs are expressed at higher levels under stress conditions to protect the cellular proteins from damage and bring back to their native state. Heat stress causes an increase in expression of *HSP27* and *HSP70* genes in chicken (Gabriel et al., 1996; Liu et al., 2014). Using a microarray study it was shown that, among other genes, *HSP70* gene was upregulated in heat stressed rooster testis (Wang et al., 2013). HSP27 has anti-apoptotic effect during thermal stress and protects cells from death. HSP27 in association with ubiquitin refold the damaged cellular proteins or inducts in to destruction. Thermal conditioning during embryonic stage makes broiler chicken thermotolerant and the *HSP70* mRNA expression was induced to a lesser extent than control birds under heat stress conditions (Yahav et al., 1997). Similar to this report in the present study the gene expressions of *HSP27* and *HSP70* were lower in HE rooster sperm than that of C rooster sperm. It indicates that *in ovo* thermal manipulation has induced thermotolerance in the mature sperm of the roosters. Our result is in variance with that of others (Wang and Edens, 1998; Al-Zhgoul et al., 2013) where thermal manipulated chicken was shown to have higher *HSP70* gene expression and were thermotolerant. Bovine breeds that are heat adapted produced lower HSP70 or showed better cellular survivability during heat stress than less well-adapted bovine breeds (Kamwanja et al., 1994; Lacetera et al., 2006). Thus *HSP70* an inducible gene will be upregulated only when there is damage to cellular proteins. In the present study the HE birds may be experiencing a lesser effect of heat stress and therefore, lesser the gene expressions.

From the results, it can be concluded that thermal manipulation of developing embryo imparts long-lasting effect on the reproductive tissue resulting in an improvement in certain semen parameters in adult roosters under hot climatic conditions. However, thermal manipulation studies that unravel the molecular mechanisms (methylation etc.) in the chicken reproductive tissues and protocols that effects better changes in other semen parameters are required so that improvement in overall semen quality of roosters and fertility during hot climate can be achieved.

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Table 1 Primers sequence utilized for amplification of gene fragments

Gene	Primer Sequence (5'…3')	Product size (bp)	Accession number
GAPDH	F- GTTGTTGACCTGACCTGCCG	130	K01458
	R- CCATCAGCAGCAGCCTTCAC		
HPRT	F- CCCAAACATTATGCAGACGA	66	AJ132697
	R- TGTCCTGTCCATGATGAGC		
HSP70	F-ATGAGCACAAGCAGAAAGAG	95	J02579
	R-TCCCTGGTACAGTTTTGTGA		
HSP27	F-GCGACCAGCCAGGAAGAAGAA	126	NM_205290
	R- GGGTCCGTGCTGTGCTTTGA		

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Table 2 Semen quality parameters of roosters that were incubated under control (C) conditions or under high incubation temperature between 15th to 17th days of incubation (HE). Values are presented as Mean ± SE.

Semen quality parameters	C	HE
Volume (ml)	0.47 ± 0.04	0.56 ± 0.05
Appearance	3.63 ± 0.17	3.84 ± 0.21
Progressive motile sperm (%)	65.00 ± 2.83	61.05 ± 2.60
Sperm concentration (million/µl)	5.39 ± 0.25	5.39 ± 0.30
MTT dye reduction test (nM of MTT Formazan /min/million sperm)	18.36 ± 0.64	18.27 ± 0.68
Live sperm (%)	83.16 ± 2.29[b]	89.49 ± 1.41[a]
Morphologically abnormal sperm (%)	3.96 ± 0.72	3.58 ± 0.782
Sperm DNA fragmentation % (SCD Test)[x]	14.64 ± 2.19	11.92 ± 1.83
Seminal plasma parameters[y]		
Lipid peroxidation (nmol MDA/mg protein)	0.70 ± 0.17	0.54 ± 0.05
FRAP (µmol/L)	743.50 ± 55.33 [b]	1159.83 ± 189.44 [a]
T_3 (ng/ml)	0.95 ± 0.07	1.11 ± 0.11

[a,b]Means with different superscripts in a row differ significantly ($P<0.05$)

[x] n=10/group

[y] n=6/group

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Fig. 1. Representative picture of Sperm chromatin dispersion (SCD) test showing different size halos in rooster sperm. A-large halo; B-medium halo; C-small halo; D-no halo.

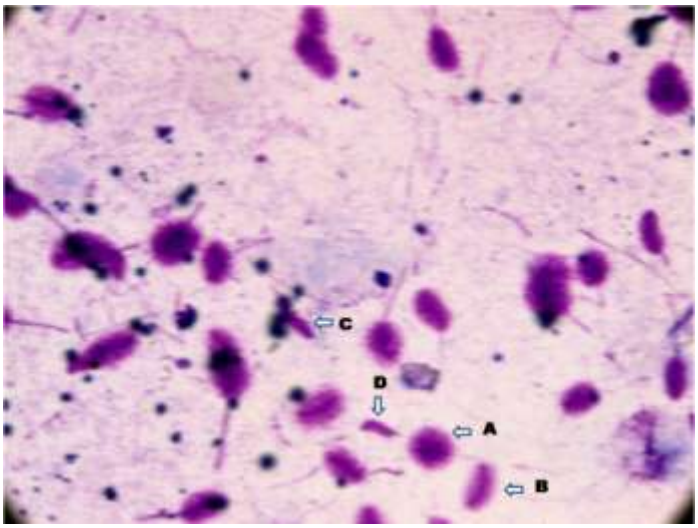

Fig. 2. MMP-2 proteolytic activity in seminal plasma samples detected by gelatin zymography. M – human MMP-2 marker; L – 72kDa ladder; 1-6 – seminal plasma of HE roosters; 7-12 – seminal plasma of N roosters. Recombinant MMP-2 was applied in a dose of 0.5µg/10µl.

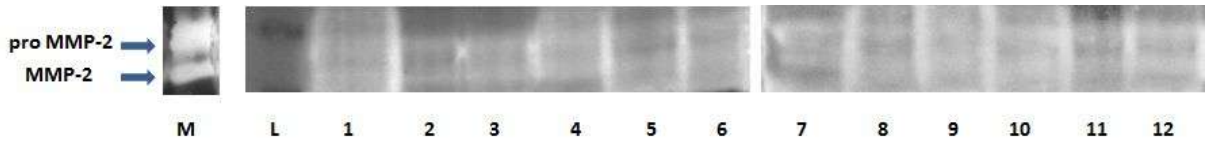

Fig. 3 Fold change in *HSP70* and *HSP27* mRNA expression in sperm samples of HE roosters. Means ± SE bearing * mark differ significantly from control (P<0.05).