Pakistan Veterinary Journal

ISSN: 0253-8318 (PRINT), 2074-7764 (ONLINE)
Accessible at: www.pvj.com.pk

RESEARCH ARTICLE

Effect of Protein, Probiotics and Vitamins Supplementation on Semen Quality and Immunohistochemistry of Pituitary Gland in Molted Male Layer Breeders

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ARTICLE HISTORY (15-498)

Received:	December 04, 2015
Revised:	March 01, 2016
Accepted:	March 09, 2016
Published online:	March 18, 2016

Key words:
Immunohistochemistry
Layer breeder
Molting
Pituitary gland
Probiotics
Semen quality
Vitamins

A B S T R A C T

In the current study, the effects of dietary supplementation of protein, probiotics and vitamins (C and E) on semen quality and immunohistochemistry of pituitary gland in zinc-induced molted male layer breeders were evaluated. For this purpose, male layer breeders (n=270) at the age of 59 weeks were used. After acclimatization of one week, all birds were subjected to forced molt by dietary supplementation of ZnO for a period of two weeks. After completion of molting, the birds were divided into six equal groups, keeping one group as control. The other groups were fed diet supplemented with protein (12%), probiotics (50 mg/kg feed), vitamin C (500 mg/kg feed), vitamin E (100 mg/kg feed) and combination of all above treatments, respectively. The birds took about 5 weeks to produce semen after molting. The trial continued for next 5 weeks during which semen and pituitary samples were collected from 5 and 3 birds of each group respectively, once a week. The results indicated that semen volume and sperm motility increased, while %age of dead sperm decreased, significantly in vitamin C and E treated groups. The results of immuno-histochemistry also showed that the size of FSH gonadotrophs, LH gonadotrophs and lactotrophs were significantly higher in vitamin E supplemented group which ultimately caused an increase in semen quality. Hence, the above results advocate the use of vitamin C and E in post molt male layer breeders to improve their reproductive performance.

To Cite This Article: Muzaffar H, Khaliq T, Khan JA, Rahman ZU, Mahmood A, Iftikhar A, Rahman SU and Mahmood F, 2016. Effect of protein, probiotics and vitamins supplementation on semen quality and immunohistochemistry of pituitary gland in molted male layer breeders. Pak Vet J, 36(2): 149-152.

INTRODUCTION

Dramatic decline in male fertility, predominantly after the age of 50 weeks, is one of the major problems faced by commercial breeder industry. Some of the major causes of poor fertility in broiler breeder male flock in old age are decreased semen quality and testosterone production, lack of *libido*, poor physical condition, leg problems and reduction in nutrients (Romero-Sanchez *et al.*, 2008).

In poultry industry forced molting has become a cost-effective process to increase productive and reproductive life span of birds (Anwar *et al.*, 2015; Iftikhar *et al.*, 2015). Various techniques are used to induce molting such as fasting, reduction of photoperiod or combination of both, feed supplementation of dietary salts of zinc, copper and aluminum (Khan *et al.*, 2013a). Rejuvenation of reproductive organs is the most significant benefit of Zn-induced molting, which results from increased efficiency of tissues and organs, loss of adipose tissue, development of gonads (Berry, 2003) and hence performance of birds after molting is improved (Khan *et al.*, 2014). It was revealed recently that supplementation of different feed additives in post molt period improved semen quantity and quality in molted broiler breeders and resulted in better reproductive performance in male birds (Khan *et al.*, 2013b). Although there has been ample research on semen evaluation in layer breeders, there has been no investigation of male fertility in layer breeders after molting. Therefore, this study was aimed to investigate the effect of different feed supplementation on semen quality and immunohistochemistry of pituitary gland of molted layer breeders.

MATERIALS AND METHODS

Experimental birds and their treatments: A total of 270 White Leghorn breeder males (Bovans®) at the age of 59 weeks were used. After 7 days of acclimatization period, they were subjected to forced molt through dietary inclusion of ZnO (3g/kg feed) with moderate decline in lighting schedule from 16 hours to 12 hours (Khan *et al*., 2012a). The phase of molting continued for two weeks. After molting, birds were divided randomly into six groups with 45 birds in each group. One group was kept as control, four groups were fed diet supplemented with protein (12%), probiotics (Protexin®; 50 mg/kg feed), vitamin C (500 mg/kg) and vitamin E (90 mg/kg), respectively, while the 6th group was fed the combination of above mentioned supplementations for a period of 5 weeks. Semen production started after 5 weeks of molting and semen samples were collected weekly for the next 5 weeks.

Semen collection and evaluation: Semen was collected from five birds per group through abdominal massage method as described by Burrows and Quinn (1937). Semen volume was determined by aspirating the semen into a graduated insulin syringe. The sperm motility was assessed by placing a drop of semen on a clean slide and observing under the microscope. Motility was expressed as the percent of motile spermatozoa with rapid forward movement. Sperm concentration was estimated with Neubauer hemocytometer. Eosin-nigrosin staining was used to perform the assessment of live and dead spermatozoa (Khan *et al*., 2013a).

Immunohistochemistry of pituitary gland: For the collection of pituitary glands, birds (three birds per group) were slaughtered once a week for five weeks. Pituitary glands were removed from birds immediately after slaughter and kept in Bouin's Hollande solution for 24 hours, followed by dipping in 4% formaldehyde. Then pituitary samples were processed for immuno-histochemistry as described by Sandhu *et al*. (2010). Briefly, individual samples were mounted on Poly-L-Lysine slides. After rehydration and dewaxing, mounted sections were treated with hydrogen peroxide block for 10 minutes. Then, prediluted (1:200) primary LH antibody was applied on the section and incubated for 2 hours. A drop of secondary antibody (Biotinylated goat anti-rabbit IgG) solution was poured on the tissue and incubated for 10 min. After washing, streptavidin peroxidase was applied on the sections for 30 min. Then sections were incubated for 10 min with diluted chromogen and DAB substrate (1:50). The same procedure was repeated for other sections and primary antibodies for FSH, GH and ovine prolactin were applied to differentiate between different cell types. The quantitative analysis was performed under compound microscope using Image J Software (Image J 1.44P Wayne Rasband, National Institutes of Health, Bethesda, MD, USA) to measure cell size and area.

Statistical analysis: The data were subjected to two-way ANOVA and Duncan Multiple Range test by using the software package GraphPad Prism 5.04®. Results were accepted as significant at $P<0.05$.

RESULTS

Overall mean semen volume, sperm concentration, motility, and dead sperm percentage are given in Table 1. The results obtained for semen traits revealed that overall mean semen volume and sperm motility significantly increased ($P<0.05$) in vitamin C and E treated groups. Concentration of sperms did not differ among the groups, while percentage of dead sperms reduced significantly ($P<0.05$) in vitamin C and E fed group as compared to control and other treated groups. The results of immunohistochemistry (Table 2 and Table 3) revealed that as a result of vitamin E supplementation, cell size, cell area, nucleus size and nucleus area of FSH and LH gonadotrophs and lactotrophs increased significantly ($P<0.05$). There was non-significant difference in cell size, cell area, nucleus size and nucleus area of somatotrophs. The combined effect of probiotics, protein, vitamin E and C was not as good as individual effect of vitamin E and C.

DISCUSSION

In present study, overall sperm motility and semen volume were increased, while the percentage of dead sperm was reduced, significantly in birds supplemented with vitamin E and C. The volume of cock semen ranges from 0.5 to 1 ml, but the amount below or above this is attained commonly (McGoven, 2002). Previous researchers also concluded that volume of semen, concentration of total sperm, motility and liveability in broiler breeder males were enhanced significantly by dietary inclusion of vitamin E @ 200-300 mg/kg (Lin *et al*., 2005; Cerolini *et al*., 2006; Biswas *et al*., 2009). In sperm cells, mitochondria are present in abundance which supply energy for sperm motility. In damaged mitochondria, production of ROS (reactive oxygen species) increased significantly due to which the function of mitochondria in sperm cells is affected negatively. Vitamin E and C react with free radicals and stable ROOH group is produced. It has been proposed that biological stability to the membrane of spermatozoa is provided by vitamin E (Siegel *et al*., 2001). The scavenging ability of vitamin E and C may be responsible for higher sperm motility and increased semen volume in this study.

It has been reported that with increasing age, there was decline in fertility of male birds (Romero-Sanchez *et al*., 2008). Reduced vitamin E level in testes has been linked with this age related drop in fertility of cockerels which can be restored with supplementation of vitamin E at the rate of 200 mg/kg of feed (Surai *et al*., 2000). It was also observed in experimental quails that fertility decreased in the absence of vitamin E in diet and was restored when vitamin E was supplemented (Biswas *et al*., 2007). Conventionally, vitamin E is known as anti-sterility vitamin (Khan, 2011) and is an important lipid soluble antioxidant (Panda and Cherian, 2014). In the membrane of sperm cells, a major chain breaking antioxidant is vitamin E. All three types of free radical, namely, H_2O_2, hydroxyl radical and superoxide are scavenged by vitamin E (Makker *et al*., 2009). It was reported that lipid peroxidation in biological membrane is inhibited by vitamin E which acts as scavenger of alkoxyl [LO-] and lipid peroxyl [LOO-] radicals (Lin *et al*., 2005; Zaniboni *et al*., 2006).

Table 1: Mean semen volume, sperm motility, dead sperm percentage and sperm concentration in different groups of post molt male layer breeders

Groups	Semen volume (mL)	Sperm motility (%)	Dead sperm percentage (%)	Sperm count (×10^9/ml)
Control	0.17±0.01b	61.08±0.57b	14.36±0.39a	1.17±0.03
12% CP	0.17±0.01b	60.48±0.84b	13.77±0.22a	1.23±0.01
Probiotics	0.17±0.01b	61.52±0.83b	13.69±0.21a	1.21±0.01
Vitamin C	0.22±0.02a	67.40±0.41a	12.15±0.09b	1.17±0.03
Vitamin E	0.23±0.02a	68.44±0.68a	12.50±0.24b	1.18±0.02
Combination	0.16±0.01b	60.96±0.84b	14.01±0.34a	1.20±0.02

Values (mean±SE) within a column bearing different alphabets differ significantly (P<0.05). White Leghorn breeder males in groups 2-5 were fed diet supplemented with crude protein (12%), probiotics (Protexin®; 50 mg/kg feed), vitamin C (500 mg/kg) and vitamin E (90 mg/kg), respectively, while the 6th group was fed the combination of above mentioned supplementations for a period of 5 weeks.

Table 2: Mean cell size (μm±SE) and mean cell area (μm^2±SE) of FSH gonadotrophs, LH gonadotrophs, somatotrophs and lactotrophs in different trial groups of post molt male layer breeders

Groups	FSH gonadotrophs size	FSH gonadotrophs area	LH gonadotrophs size	LH gonadotrophs area	Somatotrophs size	Somatotrophs area	Lactotrophs size	Lactotrophs Area
Control	6.49±0.14c	134.8±5.45c	5.63±0.05c	100.6±1.9c	6.35±0.08	127.70±3.15	6.31±0.07c	126.34±2.99c
12% CP	6.22±0.25c	123.7±9.90c	5.67±0.09c	102.1±3.36c	6.61±0.02	140.20±1.89	6.28±0.08c	125.00±3.03c
Probiotics	6.25±0.19c	124.5±7.48c	5.61±0.09c	99.9±3.42c	6.32±0.06	128.2±2.89	6.26±0.15c	124.32±5.79c
Vitamin C	8.00±0.24b	203.0±11.8b	6.73±0.15b	143.5±6.3b	6.38±0.02	130.61±2.10	7.50±0.07b	177.64±0.07b
Vitamin E	9.56±0.23a	291.3±14.3a	7.98±0.18a	202.6±9.02a	6.82±0.20	148.42±9.75	8.13±0.11a	209.11±5.90a
Combination	6.28±0.12c	114.0±4.53c	5.56±0.12c	98.14±4.03c	6.47±0.03	134.20±0.51	6.34±0.09c	127.00±3.71c

Values (mean±SE) within a column bearing different alphabets differ significantly (P<0.05).

Table 3: Mean nucleus size (μm±SE) and mean nucleus area (μm^2±SE) of FSH gonadotrophs, LH gonadotrophs, somatotrophs and lactotrophs in different trial groups of post molt male layer breeders

Groups	FSH gonadotrophs nucleus size	FSH gonadotrophs nucleus area	LH gonadotrophs nucleus size	LH gonadotrophs nucleus area	Somatotrophs nucleus size	Somatotrophs nucleus area	Lactotrophs nucleus size	Lactotrophs nucleus area
Control	2.14±0.02c	14.55±0.39c	2.29±0.03 c	16.69±0.53 c	2.43±0.03	18.93±0.51	2.36±0.03c	17.58±0.46c
12% CP	2.16±0.01c	14.76±0.16c	2.34±0.03 c	17.41±0.46 c	2.51±0.03	20.37±0.49	2.36±0.01c	17.55±0.23c
Probiotics	2.23±0.03c	15.70±0.46c	2.34±0.02 c	17.45±0.33c	2.48±0.04	19.68±0.70	2.38±0.02c	17.94±0.30c
Vitamin C	2.53±0.05b	20.15±0.81b	2.61±0.05 b	21.56±0.84 b	2.54±0.02	20.71±0.44	2.72±0.03b	23.29±0.57b
Vitamin E	2.88±0.08a	26.25±1.52a	2.95±0.04 a	27.74±0.85 a	2.58±0.03	21.33±0.61	2.97±0.02a	27.85±0.38a
Combination	2.16±0.02c	14.69±0.23c	2.41±0.01 c	18.51±0.27 c	2.51±0.02	20.43±0.45	2.39±0.01c	18.10±0.13c

Values within a column, bearing different alphabets differ significantly (P<0.05).

Similar to the valuable effect of vitamin E, it has been suggested that dietary supplementation of vitamin C improves semen quality in poultry birds. Fertility and semen quality in broiler breeder males were reported to improve by supplementation of vitamin C in diet (Nowaczewski and Kontecka, 2005; Khan *et al*., 2013b).

Pituitary gland is the master gland in the body, as it plays a vital role in many physiological processes including reproduction and growth in addition to various other functions (Anwar *et al.*, 2012). It has been suggested by Sandhu *et al*. (2008) that during and after induced molting, LH gonadotrophs have potency to proliferate. Likewise, it is also reported by Chowdhury and Yoshimura (2002) that during the egg laying resumption process, cell size and area of LH and FSH gonadotrophs increased. In the current study, the cell size and area of LH and FSH gonadotrophs were increased significantly in group supplemented with vitamin E and C. It has been suggested that in pituitary-gonadal axis, an important role is played by vitamin E and C in the production of hormones (Umeda *et al*., 1982).

Lactotrophs and somatotrophs are group of cells which belong to acidophilic classification and have capability to change or modify the hormonal profile of the birds depending upon the physiological state of the birds (Sandhu *et al*., 2010). In the current experiment, there was no significant difference in nucleus size, nucleus area, cell size and cell area of somatotrophs among different groups. In birds, complex and intricate mechanisms control the phenomena of growth. This system includes environment, nutrition and genetics in addition to other intrinsic physiological and endocrine factors.

Prolactin hormone is released from lactotrophs which takes part in the synthesis of sperm cells and stimulates the testicular growth (Erdost, 2005). The production of LH and FSH is influenced by higher levels of prolactin (Khan *et al*., 2013a). In the present study, the higher cell size and area of lactotrophs in vitamin E and C supplemented groups is associated with better semen quality of birds in these groups.

In the present study, additive effect of vitamin C and E was not observed when they were given in combination. This may be due to antagonism and biological variability among different dietary ingredients (Khan *et al*., 2012b). A negative interaction between antioxidant vitamins in the gastrointestinal tract of broiler birds was reported by Aburto and Britton (1998). Similarly, low protein and addition of probiotics in diet did not show any beneficial effect on semen quality and pituitary gland's cell parameters. These findings may be directly linked to variations in the protein metabolism rate which may lead to increased cellular stress (Iftikhar *et al*., 2015) and hence, results in decreased semen quality.

Conclusions: In this study, we attempted to recycle layer breeder male birds through zinc-induced molting and supplemented them with different feed additives, the beneficial effects of which are well documented. We did not find any significant effect of protein and probiotics in this study. In conclusion, these data suggested that vitamin E and C are beneficial in improving the reproductive performance of White Leghorn breeder males after zinc-induced molting.

Acknowledgments: This research work was conducted under Research Project PSF/NSLP/P-AU (245). Authors are highly thankful to Pakistan Science Foundation, Islamabad for financial support.

Author's contribution: The work is a product of the intellectual environment of the whole team; and all the members have contributed in various degrees in designing the study, developing the methodology, performing the analysis and writing the manuscript.

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