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Research Article

EFFECT OF L-ASCORBIC ACID AND ALPHA-TOCOPHEROL ON OVARIAN REGRESSION, HORMONAL CHANGES AND GENE EXPRESSION IN JAPANESE QUAIL DURING STRESS

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ABSTRACT: L-ascorbic acid (L-AA) and á-tocopherol (α-TP) facilitate the first line of defence and regulate neuro-endocrine mechanism to optimize performance during stress but how molecular mechanism controls ovarian functions in birds are still unclear. In view of this fact, the study was aimed to appraise the effect of natural antioxidants on ovarian functions of Japanese quail during stress. One hundred and forty four Japanese quails (10weeks) were equally divided into four groups i.e. Gr I (control), Gr II (positive control), Gr III and IV (feed withdrawal). Birds from Gr II and IV received the L-AA and α-TP@250ppm each through drinking water and studied for a period of 10 days. Six birds were sacrificed each on 1, 2, 4, 6, 8 and 10 days and morphological changes were evaluated. Serum concentration of estrogen and progesterone were estimated using RIA protocol. The expression study of luteinizing hormone receptor (LHR) and progesterone hormone receptor (PHR) gene were carried out in ovary and follicles (F_1, F_2, F_3) by Quantitative RT-PCR. The significant reduction in body weight and reproductive tracts' weight were observed in Gr III and IV though low severity was recorded in the later group. The concentration of both estrogen and progesterone hormones were significantly *(P<0.05)* lowered with the study period. The expression study revealed a significant *(P<0.05)* down regulation of LHR and PHR gene in hierarchial follicles and the magnitude of fold expression was moderate in Gr IV. This study concludes that supplementation of L-AA and á-TP may counteract the negative impact of stress in ovarian functions and long term treatment would synchronize better neuro-endocrine and molecular mechanism in Japanese quail.

Key words: Ovarian regression, Hormones, Gene expression, Induced stress, Japanese quail.

INTRODUCTION

Stress induced corticosterone level through the activation of HPA axis causes reduction in feed intake and subsequently lowers the plasma level of LH, estradiol and progesterone (Sundareson *et al.* 2007, Agarwal *et al.* 2013) in dose dependent manner (Moudgal *et al.* 1991). It is also established that vitamin C and E in plasma and minerals are declined which subsequently increases oxidative damage and imbalance the antioxidant status (Klasing 1998, Sahin *et al.* 2002). Many efforts have been made with L-ascorbic acid (L-AA) and á-tocopherol in-order to improve reproductive performance upon alleviation of stress impact in domestic chicken.

Literally, L-ascorbic acid (L-AA) is a 6-carbon lactone is synthesised from glucose in the kidney of birds and reptiles and liver in some mammals. It plays an important role in the biosynthesis of corticosterone and serves as co-factor for the bio-conversion of vitamin D_3 to 1,25-$(OH)_2D_3$ to compensate homeostasis mechanism during stress. However, this biological process becomes insufficient under stress at temperature and relative humidity fluctuation, high productive rate and parasite infestation (Gursu 2004). Vitamin C has evidently suppressed the adrenalin mediating alpha-receptors induced follicular atresia in chicken during in-vitro controlled study (Moudgal *et al.*1985). Since poultry

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cannot synthesise vitamin E (α-TP), it must be met from dietary source to stabilize the cellular function during stress. It acts as a natural antioxidant and facilitates the first line of defence against per-oxidation of unsaturated fatty acids and free radicals in the cell membrane. Dietary supplementation of vitamin E improves general performance, immunity and reproductive efficiency in various poultry species including Japanese quail (Biswas 2003). Biological trials of α-tocopherol @ 250ppm and in combination with L-ascorbic acid (L-AA) are synergistically beneficial in combating heat stress in pullets (Sinkalu and Ayo 2008) and in Japanese quails (Ciftci *et al.* 2005).

Considering the anatomy and physiological homology to chicken, Japanese quail (*Coturnix coturnix japonica*) has now been introduced as a model animal in heavy expensive bio-medical research. Similar to chicken, they up-hold a uniform reproductive sequence, ovarian follicular growth and clutch pattern based on the interactions between endogenous and exogenous rhythm controlled by granulosa and theca cell layers of the follicles (Onagbesan and Peddie 1988). Induced stress by feed withdrawal (FW) in White Leghorn hens (Agarwal *et al.* 2013) and Japanese quail (Shit *et al.* 2016) has documented significant negative impact on tissue regression, expression of luteinizing hormone receptor (LHR) and progesterone hormone receptor (PHR) mRNA. Sahin and Kucuk (2001) reported vitamin C is effective in increasing feed efficiency and ameliorating detrimental effects of stress on reproduction in Japanese quail. In our previous study, dietary L-ascorbic acid (L-AA) showed positive effect in ameliorating cold stress on production performance and fertility percentage inlaying Japanese quail (Shit *et al.* 2012). Most studies related to the adverse effect of stress and its amelioration by dietary supplementation of L-AA and α-TP have been carried out in domestic chicken. However, the role of these anti-stressors on ovarian function and subsequent cellular changes during feed withdrawal in Japanese quail is still unknown.

Therefore, in the present study the expression pattern of Luteinizing hormone receptor (LHR) and Progesterone hormone receptor (PHR) gene in the ovary and hierarchial follicles were investigated using real-time PCR. The reproductive tissue regression and hormonal changes were also evaluated to correlate the cellular changes during feed withdrawal and subsequent amelioration considering the synergistic effect of L-AA and α-TP, the natural anti-oxidants.

MATERIALS AND METHODS

Birds and experimental design

The protocols involving the care and use of animals for these experiments were in accordance with the rules of the 'Animal Ethics Monitoring Committee of the Central Avian Research Institute, Izatnagar. A total of one hundred and forty four (144) Japanese quail hens (10 weeks) from the same hatch were randomly selected from the institute quail farm and used for this study. They were equally divided into four groups i.e. Group I (Gr I), Group II (Gr II), Group III (Gr III) and Group IV (Gr IV) with an account of thirty six birds /group. All were caged individually (20x20x20 cm^3) under 14:10h light:dark cycle.

Induction of stress

Birds from Gr I served as un-treated control while Gr II was considered as positive control who received quail layer ration (ME-2716 Kcal/kg, CP-20.03 %, Ca-3.06%, P-0.33%, Lysin-1.09 and Methionine-0.45%). There was a complete feed withdrawal (FW) for those birds from Gr III and Gr IV for a period of 10days according to Bell (2003). However, L-ascorbic acid (L-AA) and α-tokopherol (α-TP) @ 250ppm each were supplemented to Gr-II and IV through drinking water for this study. Birds from control group were supplied *ad-libitum* feed and water throughout the study period.

Sample collection and processing

Six birds from each group (I-IV) were sacrificed by cervical dislocation on day 1, 2, 4, 6, 8 and 10 of experiment. Immediately after slaughter, reproductive organs of individual bird were accounted (0.01g specificity) to specify the percent reduction. Ovarian follicles were separated from the ovary immediately and weighed in order to categories the largest (F_1), second-largest (F_2) and third-largest (F_3) follicles. Follicles were cut open transversely along the stigma to drain the yolk material completely. The follicular membrane was washed repeatedly with ice-cold sterile saline to devoid of yolk material. Follicles were collected without separating granulosa and theca layers. All tissue samples were incubated separately in RNA stabilization solution (RNAlater, Ambion Inc., USA) at 4 ^oC overnight. Then, samples were removed from RNAlater and stored at -80 ^oC for 1–2 weeks as per the manufacturer's instructions, till the RNA isolation was performed.

Hormones assay

Serum was extracted following standard protocol and

subjected to estimation of estradiol and progesterone concentration. The sex steroids were assessed using commercial RIA kit (Immunotech, SAS) following the manufacturer's instructions. Inter- and intra-assay coefficients of variation were 8.5% and 7.5%, 6.8% and 4.2%, and 6.8% and 4.4%, respectively.

Expression study by Quantitative RT-PCR

Total RNA was purified from follicular tissues, homogenised in Trizol reagent according to manufacturer's protocol (Invitrogen, USA). The integrity of the RNA samples was verified at A_{260} vs A_{280} by Nano-Drop system (Thermo, 2000). Each RNA sample (5mg) was treated with 5U of RNase-free DNase (Biogene, USA) at 37 °C till 1h to make free from genomic DNA contamination and subsequently inactivated by incubation at 65 °C for 10min. Complementary DNA (cDNA) for the Quantitative RT-PCR reactions were generated from 1ìg total RNA from all samples using 'Revert Aid First strand cDNA synthesis kit' (MBI Fermentas, USA) according to the manufacturer's instructions. The resultant cDNA was stored at -20 °C for further used.

The expressions of individual gene targets were analysed using Syber Green master mix in IQ5 cycler real-time PCR system (Bio-Rad, USA). The specific primer pairs were designed from the coding region of chicken luteinizing hormone and progesterone hormone receptors mRNA sequences available in Gene Bank considering the close relationship between chicken and quail in phylogeny and sequences. The sequence of forward and reverse primer for Luteinizing hormone receptor (LHR), Progesterone Hormone Receptor (PHR) and â-actin (control gene) are shown in Table 1.

The amplification was carried out in 25µl volume (triplicate) containing 1x QuantiTect SYBR Green PCR master mix (QIAGEN GmBH), a 0.2-mM concentration of each gene-specific primer and 1µl of cDNA template. The three-step real-time PCR program included an enzyme activation step set to 10min at 95 °C (first segment, one cycle), 10s at 95 °C and 30s at T_m of a specific primer pair (second segment, 40 cycles) followed by 10s at 95 °C and 72 °C for 45s (dissociation curve segment). Controls lacking cDNA template were included to determine the specificity of target cDNA amplification. The melting curve generated for each sample upon completion of amplification was used to determine the specificity of polymerase chain reaction. To generate gene-specific standard curves, plasmids containing each of the different genes were serially diluted from 10^{-1} to 10^{-5}. Each RT-qPCR experiment contained triplicates of test samples, one no-template control (NTC) and a $\log_{10}$ dilution series.

Statistical analysis

The data collected on physical parameters were analyzed using two-way analysis of variance and means compared using Duncan's multiple range tests (Duncan 1955). The data received on serum hormones was analyzed using Graph pad prism, version 4.0 (Graph pad, La Jolla, USA) software. The relative mRNA expression level was normalized against ß-actin and the fold expression of the gene of interest was calculated according to Pfaffl *et al.* (2002).

RESULTS AND DISCUSSION

The percent body weight reduction was found 42.68 and 35.49 in Gr III and Gr IV respectively (Figure 1). Though a similar trend of reduction was noticed in both FW treatments but the drastic change was recorded in the former group. The birds in Gr II gained 2.47 percent more body weight compared to those of control birds (Gr I).

In spite of the same treatment, presence of anti-oxidant vitamins could be the cause of stress alleviation resulting Gr IV confirmed less severe change in their body weight. The result agreed with Sahin and Kucuk (2001) who stated that dietary ascorbic acid (250ppm) significantly stimulate growth and improved feed efficiency in stressed chicken and quail respectively. Ascorbic acid (250mg/kg) has relieved the birds from the negative effects of

Table 1. Primer pair sequences with their amplicon size and annealing temperature used for Quantitative RT-PCR.

Target gene	Primer sequence* (5'-3' order)		Amplicon size (bp)	Annealing temp. (°C)
	Forward	Reverse		
LHR	ATTGTGCTCCTCGTCCTC	GTCTATGGCGTGGTTGTAG	162	56
PHR	GGAAGGGCAGCACAACTATT	GACACGCTGGACAGTTCTTC	83	56
B-actin	GGAAGTTACTCGCCTCTG	AAAGACACTTGTTGGGTTAC	127	58

LHR, luteinizing hormone receptor, PHR, progesterone hormone receptor

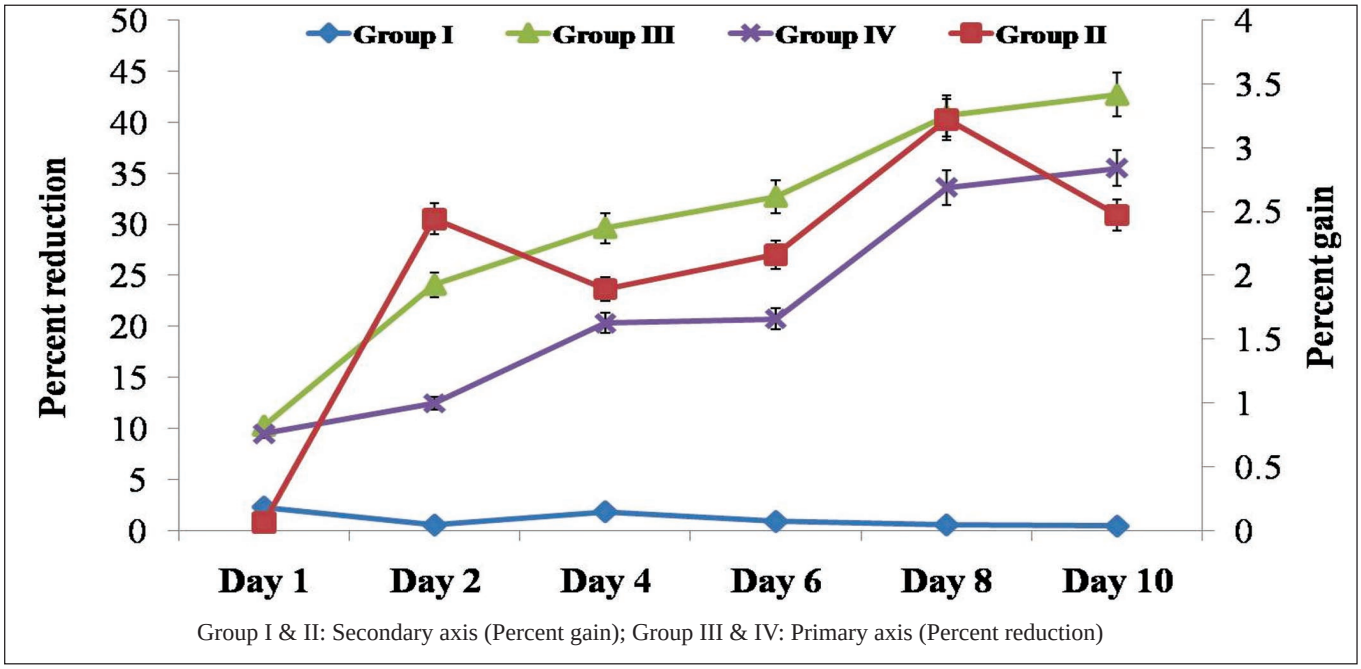


Fig. 1. Percent body weight change in the different treatment groups of laying Japanese quail during induced stress. Graphics represent the mean value of body weight under different days of experiment (mean ± SE; N=6).

environmental stress and increased feed intake, protein digestibility, dressing percentage and FCR (Attia *et al.* 2009). The present findings may correspond to Ciftci *et al.* (2005) who concluded that ascorbic acid (200mg/kg) in combination with vitamin E (125mg/kg) significantly improved feed efficiency and body weight gain in laying hens exposed to heat stress.

Birds from Gr II did not show any noticeable change in their body weight compared to control birds which may be correlated with the established fact that ascorbic acid is not essential nutrient for avian species as they possess gulonolactone oxidase enzyme which is required for the biosynthesis of this vitamin in kidney (Khan *et al.* 2012).

The current study showed a significant reduction in ovary and oviduct weight in birds from both Gr-III (92.70% and 80.44%) and IV (82.63% and 50.07%) and co-related with the gross morphological changes of the reproductive tract (Fig.2).

Obviously, the regression rate was less severe in those birds (Gr-IV) received L-AA and α-TP (Table 2). The oviductal regression noticed in this study might be the cause of less feed intake which is in accord to McCormick and Cunningham (1984). Similar to domestic fowl, the gonadal regression in male Japanese quail evidently reported a direct effect of food deprivation on reproductive organs (Kobayashi *et al.* 2004). The ovary and oviduct was significantly regressed in laying hens and Japanese quail subjected to feed withdrawal (Anish *et al.* 2008) and immobilization (Shit *et al.* 2016), respectively. Substantial reduction in ovary and oviduct weights indicated that there was a high level of gross reproductive regression in moulted birds. It may be postulated that anti-stressors i.e. L-AA and α-TP may induce gonadotropic support from the pituitary to compromise atresia and ovarian regression that may be a possible basis of present findings. No remarkable effect of L-AA and α-TP was noticed in the morphological change among control and supplementary control groups.

The concentration of serum estrogen was decreased significantly ($P<0.05$) from 2nd day of treatment (DOT) in both Gr III and Gr IV (Fig.3A). Hence, the level was reduced gradually in advance to the study and became harsh in the former group (46.58pg/ml). Similarly, a significant ($P<0.05$) down grade progesterone level was recorded in both Gr III and Gr IV (Fig.3B).

The concentration of both the hormones was found lowest on day 6 which were valued below threshold in rest of the study period. The mean values of steroid hormones did not differ among Gr I and Gr II throughout the study. The present findings coincide with the reports documented in Japanese quail (Shit *et al.* 2017), White Leghorn (Anish *et al.* 2008) and laying hens (Sundareson *et al.* 2007).

Stress brings alteration in metabolic pathways through HPA activation which reduces feed intake (Etches *et al.*

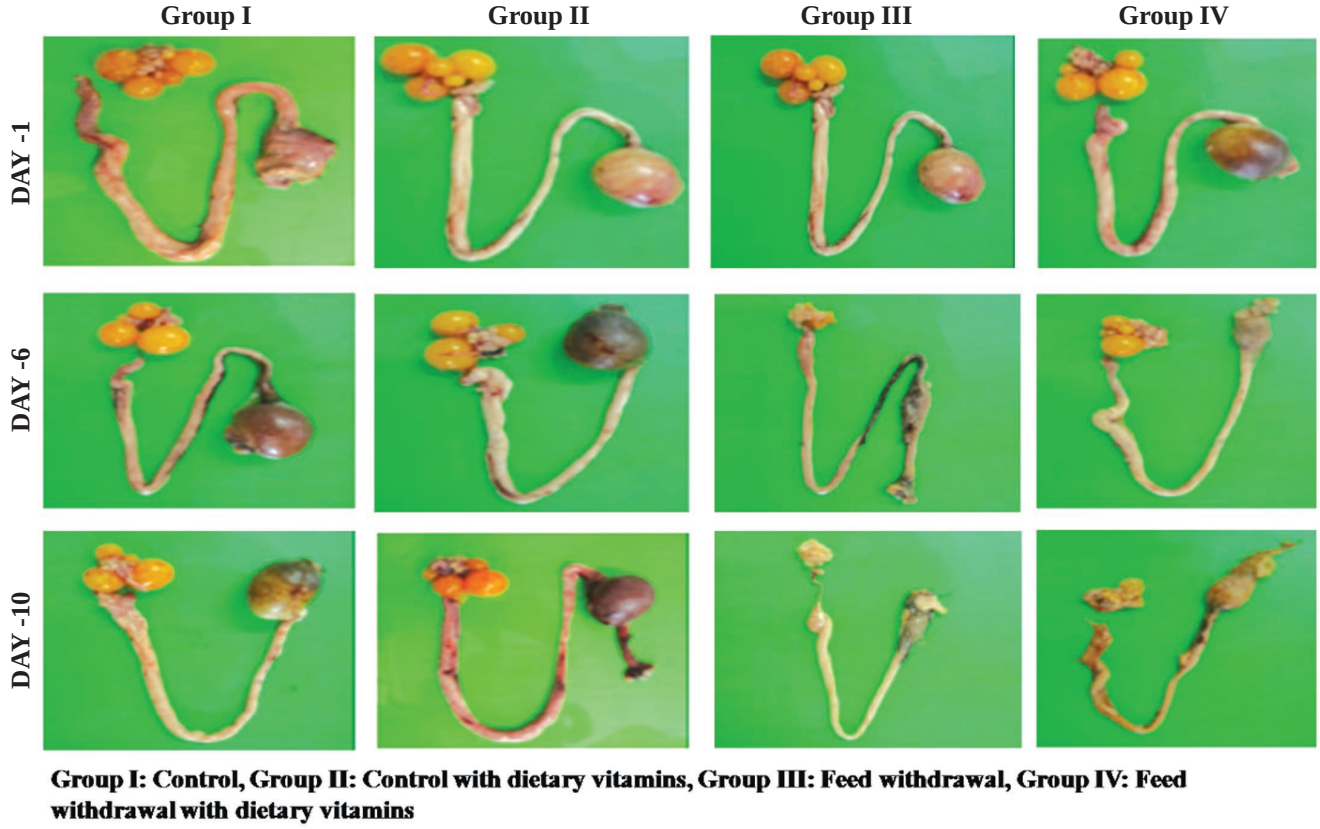


Fig.2. Changes in the gross morphology of reproductive system under different experimental groups of Japanese quail during induced stress.

1984) and subsequently lowers the plasma level of sex steroids through the ovarian regression in dose dependent manner (Moudgal *et al.* 1991). The tocoferol is the major chain-breaking antioxidant in lipid phases such as cellular membranes or low density lipoproteins (LDL), and the oxidising free radical chain reactions are terminated in aqueous compartments with ascorbic acid as terminal reductant and their synergistic effect could be the result of stress alleviation in Gr IV. Supplementation of natural anti-oxidant did not exhibit any role on steroid hormones as observed in control groups.

The expression profile of LHR and PHR genes supposed to be involved in the physiological and molecular events of ovarian functions during induced stress by feed withdrawal. As there were no hierarchical follicles detected after 6thday of the experiment, expression study was carried out in the whole ovary. However, gene expression analysis during the rest of the days was performed in three larger hierarchical follicles (F_1, F_2 and F_3).

The luteinizing hormone receptor (LHR) has been classically described as a receptor present in gonads (ovary and testis) controlling various reproductive processes. However, recent evidences suggest the presence of LHR in the extragonadal tissue, particularly in the female reproductive tract of human, bovine, porcine, rat, mouse, rabbit and turkey (You *et al.* 2000, Mukherjee *et al.* 1994). The LHR gene expression was significantly ($P<0.05$) down regulated in the follicles from Gr-III and Gr-IV on day 4 and 6 respectively of this study (Fig.4). However, the magnitude of expression was more rigorous in GR-III at any point of this study compared to its counterparts. This finding is in agreement to Agarwal *et al.* (2013) and Anish *et al.* (2008) who hypothesized that the pituitary response to luteinizing hormone–releasing hormone (LHRH) for the secretion of LH during moulting is reduced which may result in inhibition of gonadotrophin-sensitive steroidogenesis in hens. No significant improvement was recorded in the relative fold expression of LHR gene in Gr-IV which implied longer dietary inclusion of L-AA and α-TP may optimize anti-stressors effect to assuage the negative blow of stress.

Activation of the G-protein coupled LH-R on mural granulosa cells in response to the LH surge stimulates adenylate cyclase, increases intracellular cAMP, thereby activating protein kinase A and inducing expression of

Table 2. Changes in the ovary weight (A) and oviduct weight (B) of control and different treatment groups of Japanese quail during induced stress (mean ± SE; N=6).

A. Ovary weight (g)

Day	Group I	Group II	Group III	Group IV	Significance
Day 1	6.44±0.41	7.39±1.18	6.08±0.99^{p}	6.45±0.77^{p}	NS
Day 2	6.66±0.87^{a}	6.16±0.91^{a}	3.82±0.79^{bq}	4.11±0.80^{bq}	*
Day 4	6.37±0.94^{a}	6.75±1.16^{a}	3.58±1.25^{bq}	3.90±0.33^{bq}	*
Day 6	7.36±0.56^{a}	6.93±0.43^{a}	0.76±0.06^{cr}	3.37±0.22^{bq}	**
Day 8	7.13±0.99^{a}	7.41±0.68^{a}	0.56±0.18^{cr}	1.68±0.26^{cr}	**
Day 10	6.66±0.26^{a}	7.05±0.92^{a}	0.40±0.05^{cr}	1.12±0.02^{cr}	**
Significance	NS	NS	**	**	

Group I: Control, Group II: Control with dietary vitamins, Group III: Feed withdrawal, Group IV: Feed withdrawal with vitamins. Means bearing different superscript in column ^{pqrs} and row ^{abc} differ significantly.
* *P<0.05*; ** *P<0.01,* NS non-significant.

B. Oviduct weight (g)

Day	Group I	Group II	Group III	Group IV	Significance
Day 1	8.16±0.60	9.10±1.02	7.29±0.51^{p}	8.18±0.82^{p}	NS
Day 2	8.28±0.33^{a}	8.17±0.26^{a}	5.87±0.96^{bq}	7.28±0.71^{abp}	*
Day 4	7.46±0.43^{a}	7.85±0.85^{a}	5.71±0.94^{bq}	6.28±1.56^{abpq}	*
Day 6	7.83±0.33^{a}	8.37±1.28^{a}	2.81±0.23^{cr}	5.70±0.79^{bq}	**
Day 8	8.46±0.35^{a}	7.08±0.80^{a}	2.26±0.26^{crs}	5.08±1.52^{bq}	**
Day 10	7.54±0.26^{a}	8.73±1.22^{a}	1.23±0.30^{cs}	3.92±1.53^{br}	**
Significance	NS	NS	**	*	

Group I: Control, Group II: Control with dietary vitamins, Group III: Feed withdrawal, Group IV: Feed withdrawal with vitamins. Means bearing different superscript in column ^{pqrs} and row ^{abc} differ significantly.
* *P<0.05*; ** *P<0.01,* NS non-significant.

PHR (Rebecca *et al.* 2009). Subsequent to selection, follicle undergoes a transition from largely FSH dependence to LH-dependence which elicits progesterone production in preovulatory follicles (Johnson and Bridgham 2001). The expression profile of PHR gene in the follicles and ovarian tissue is shown in Fig.5. The mean expression level of PHR was down-regulated in the yellow follicles from Gr-III on 4th day of the experiment. The sensitivity of ovarian tissues towards FW stress was evidently more and confirmed significant (P<0.05) down regulation throughout this study. The expression pattern in Gr-IV varied inconsistently throughout the study. No statistical disparity was observed in the mRNA expression of PHR gene among control groups. This result could be co-related with serum concentration of progesterone as declined in progress of this study. Though the severity of fold expression was less in Gr-IV but did not vary significantly and could be the effect of L-AA and α-TP to induce gonadotrophin-sensitive steroidogenesis. The expression level of PHR was found to be up-regulated in the hierarchial follicles on sexual maturation in Japanese quail (Shit *et al.* 2014) but no literature is available so far to correlate these present findings. However, further study of longer duration is required to correlate their effects in mitigating the detrimental consequence of stress on the expression pattern of PHR.

CONCLUSION

The results of the study suggested that Japanese quail is as sensitive as domestic chicken to stress induced by FW. The gross morphological regression and profile of steroid hormones were found well correlated with down regulated mRNA expression of IGF-1, LHR and PHR. It appears that supplementation of vitamin C and E may alleviates negative effect of induced stress and improves

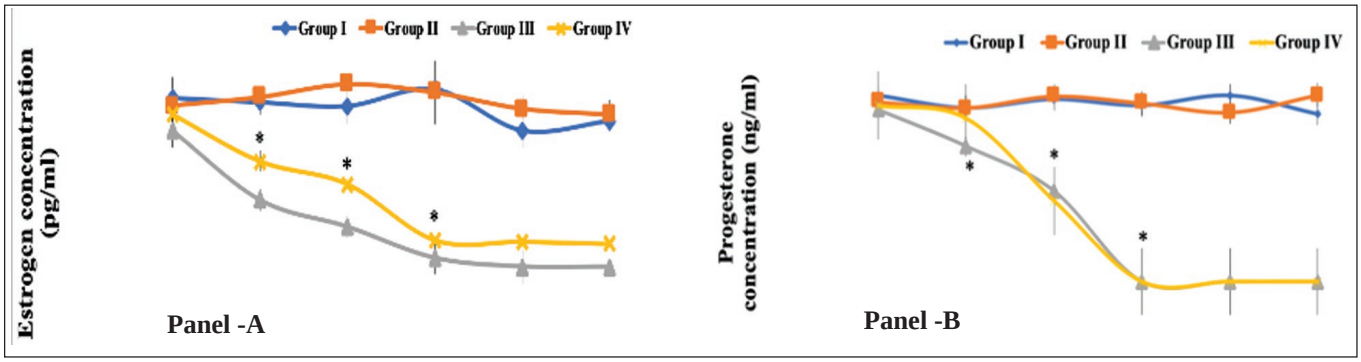
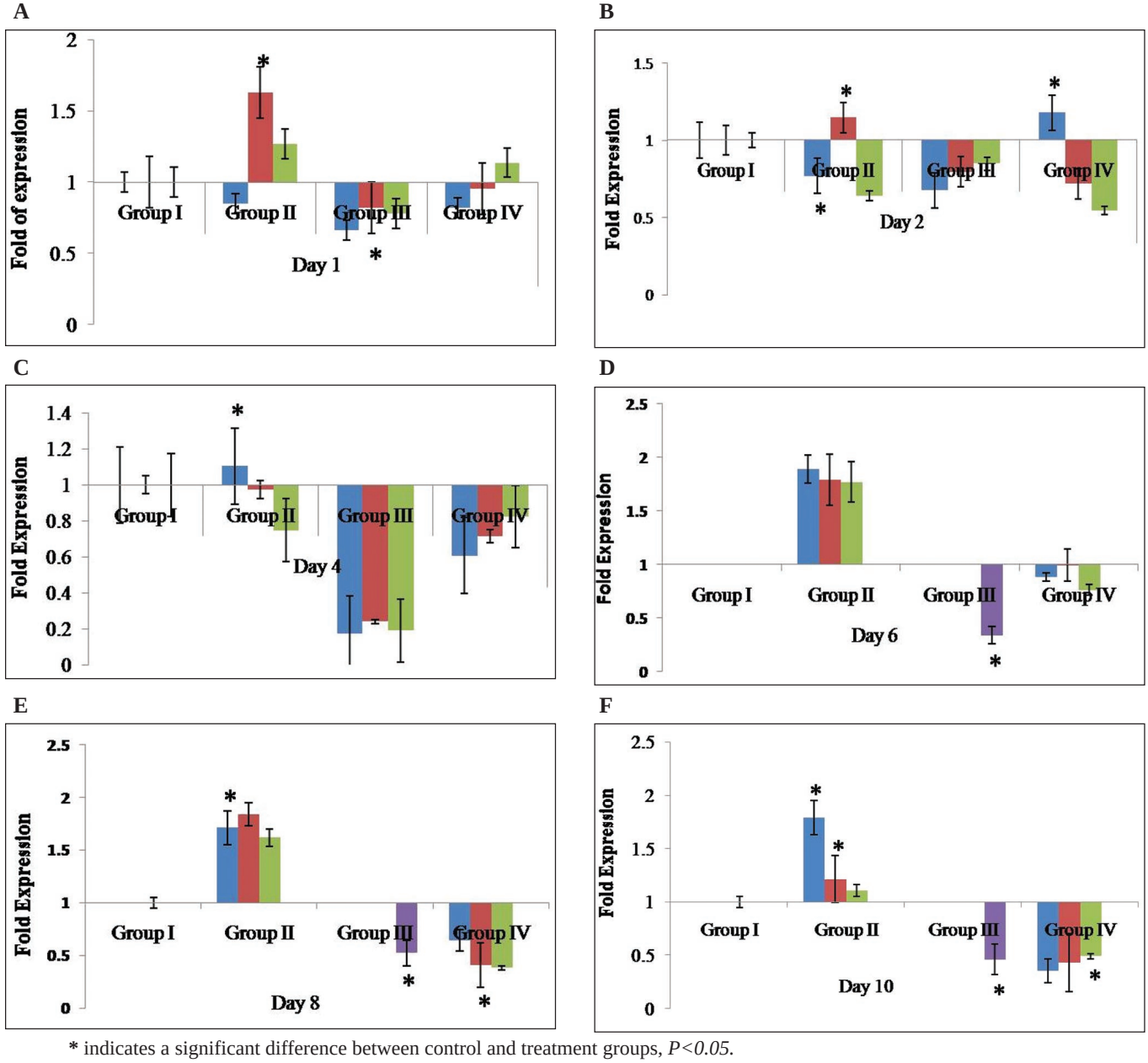
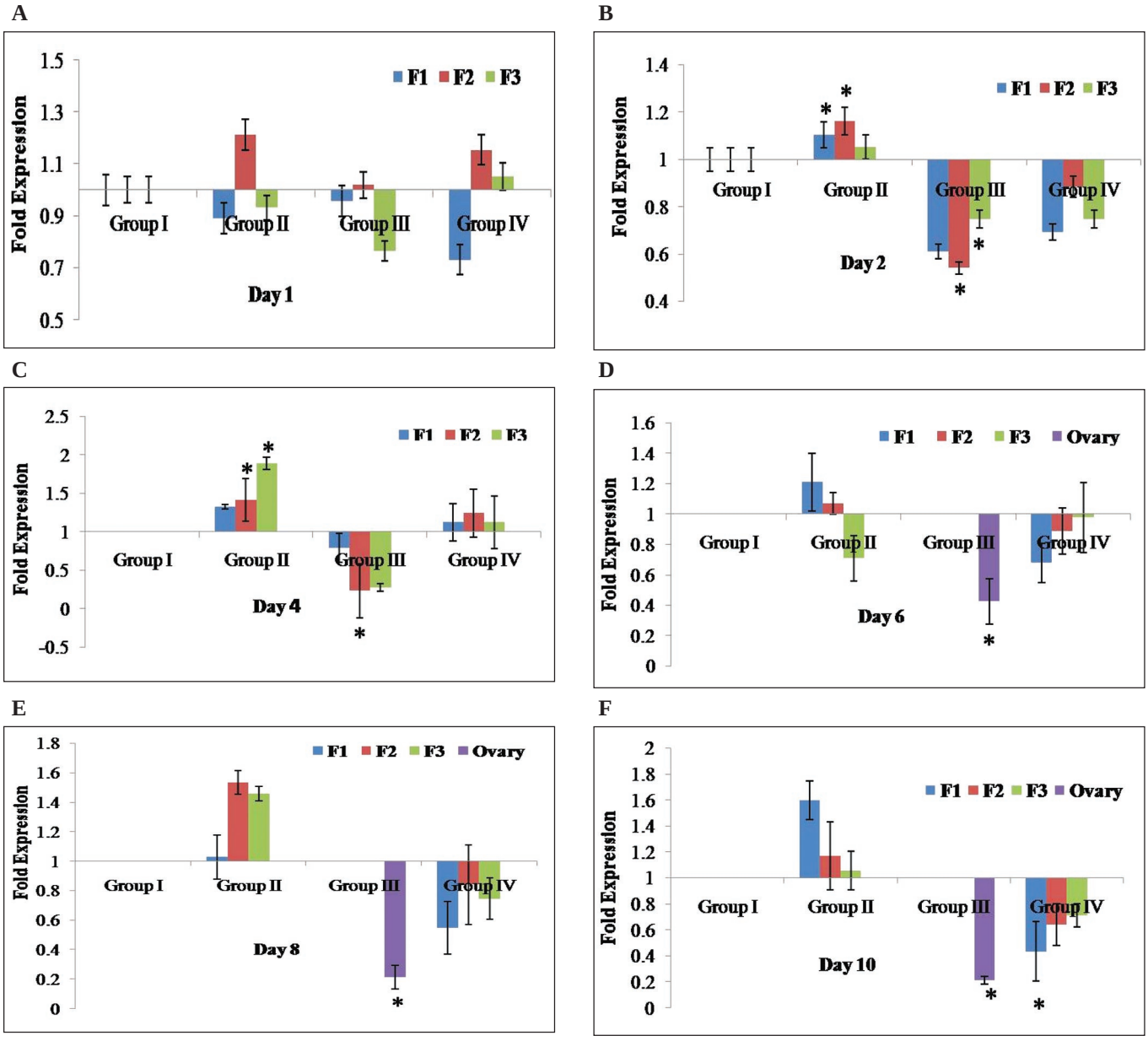


Fig. 3. The serum hormone profile of different treatment groups of Japanese quail during induced stress. Graphics represent the mean level of estrogen (panel A) and progesterone (panel B) at different days of experiment (mean ± SE, N=6).



* indicates a significant difference between control and treatment groups, *P<0.05*.

Fig. 4. mRNA expression of Luteinizing hormone receptor (LHR) in the ovary and ovarian follicles (F_1, F_2 & F_3) from different dietary treatment groups of Japanese quail. The graphics represent the mean ± SE; N=6 and the panel A,B,C,D,E and F denotes days of treatment.



* indicates a significant difference between control and treatment groups, *P<0.05*.

Fig. 5. mRNA expression of Progesterone hormone receptor (PHR) in the ovary and ovarian follicles (F_1, F_2 & F_3) from different dietary treatment groups of laying Japanese quail. The graphics represent the mean ± SE; N=6 and the panel A,B,C,D,E and F denotes days of treatment.

the morphological, bio-chemical and cellular function in Japanese quail. Further it is to mention that vitamin C and E is found dietary non-essential when the species is maintained under standard management without stress. However, the exact mechanism of FW induced reproductive regression and sequence of events in alleviation of stress by vitamin C and E supplementation in Japanese quail is yet to be unravelled.

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