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Effect of Feed Withdrawal Stress on Reproductive Tissue, Sex Steroids and mRNA Expression of IGF-1, Survivin, Caspase 2 and HSP 70 Gene in the Ovarian Follicles of Japanese Quail

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ABSTRACT

Background: Japanese quail, similar to domestic chicken, is equally sensitive and susceptible to stress but limited literatures are available so far. Therefore, this study was aimed to investigate the effect of feed withdrawal stress on the changes in reproductive tissues, steroid hormones and gene expression in Japanese quail.

Methods: Forty two quail hens (10weeks) were individually caged and subjected to feed withdrawal for a period of 10 days without water restriction. Six birds each were sacrificed on 0, 1, 2, 4, 6, 8 and 10 day. The reduction in oviduct weight and serum concentration of steroid hormones was evaluated. The quantitative expression of IGF-1, Caspase-2, Survivin and HSP-70 genes were performed in ovarian follicles using real-time PCR.

Result: No hierarchial follicles were detected after day 6. The ovary and oviduct weight was significantly reduced on day 2 and 4 respectively. The serum estrogen and progesterone were declined significantly when corticosterone was increased from day 1. The IGF-1 gene expression was significantly ($P<0.05$) down regulated in yellow and atretic follicles. The gene expression of Survivin and caspase-2 was up-regulated in F_3 follicle. It concludes feed withdrawal brings noticeable change in reproductive tissues, steroid hormones and associated gene expression in Japanese quail.

Key words: Gene expression, Hormones, Japanese quail, Ovarian follicle, Stress.

INTRODUCTION

Stress leads to the activation of the hypothalamo-pituitary-adrenal (HPA) axis which accelerates glucocorticoid secretion (Brandt *et al.,* 2007) and 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). Further, such events accentuate metabolic changes like hepatic glycogenolysis, protein catabolism andgluconeogensis to mobilize energy reserves to assist an individual toevade the stressors (Razdan, 2003). The regression of ovary and oviduct and follicular atresia is well evident in White Leghorn hens during moulting by feed withdrawal (Agarwal *et al.,* 2013). The increased expression of cytokines in the ovary causes significant reduction of estrogen and progesterone and subsequently increased serum corticosterone in single comb White Leghorn hens subjected to feed withdrawal (Sundaresan *et al.,* 2007). Interestingly, Japanese quail farming now-a-days is gaining popularity as potential alternative to chicken dominated poultry industry because of its small size, less floor space requirement, short generation interval, rapid growth, early sexual maturity and disease resistance (Amrutkar *et al.,* 2013). Their maturation is directly associated with body weight gain and attained highest body weight by 9th weeks of age (Shit *et al.,* 2014).

However, this smallest domesticated avian species is also highly sensitive to stress and encounters negative impact on reproductive functions (Shit *et al.,* 2014). Shit *et al.* (2016) concluded that the ovary and oviduct weight in Japanese quail were reduced by 82.60% and 53.54% respectively during immobilization stress. The regression of the reproductive tract induced by feed withdrawal is a non-inflammatory event mediated by apoptosis (Sundaresan *et al.,* 2007). Stress associated changes in the expression of IGF-1 gene in ovarian follicles confirms that it plays crucial role in avian reproduction as they hasten dose dependent gonadal steroid hormone synthesis, cell proliferation, selection and inhibition of follicular apoptosis by inhibiting oligonucleosome formation (Lovell *et al.,*2002, Tosca *et al.,* 2008). Survivin gene controls cell proliferation and cell death

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during cell division (Wheatley and McNeish, 2005). It is evident that the over expression of survivin gene is only reported in embryonic and foetal tissues though it remains undetected in many normal adult tissues (Ambrosini *et al.,* 1997). The relative expression of IGF-1 and survivin gene in the hierarchial follicles of Japanese quail is changed in reverse manner during evaluation of the immobilization stress (Shit *et al.,* 2014). Caspases-1 and -2 are differentially up regulated in the ovary of the single comb White Leghorn hens subjected to feed withdrawal (Anish *et al.,* 2008). The cell with increased Heat Shock Protein (HSP) exhibits better survival against the additional stress hence they are often called as stress markers (Figueiredo *et al.,* 2007). The expression pattern of HSP-70 gene represented an inconsistent variation in the hierarchial follicles of Japanese quail (Shit *et al.,* 2014). Though, feed withdrawal stress induced physiological and cellular change in domestic chicken is well established but scanty literatures are available so far in Japanese quail.

In view of the above facts, the present study aims to investigate the effect of feed withdrawal as stress factor on the reproductive tissues and to correlate the expression profile of IGF-1, survivin, caspase-2 and HSP-70 genes with the serum level of sex steroids in Japanese quail.

MATERIALS AND METHODS

The code of behaviours for the care and use of animals considered for this study were in accordance with the rules of the 'Animal Ethics Monitoring Committee of the Central Avian Research Institute, Izatnagar, Uttar Pradesh.

Experimental birds

The experiment was conducted in the Department of Physiology and Reproduction, Central Avian Research Institute, Izatnagar, Uttar Pradesh, India. Forty two healthy Japanese quail hens at 10 weeks of age from the same hatch were randomly selected from the institutional quail breeding farm. Following uniform husbandry conditions, birds were housed in individual cages (20x20x20 cm^3) with 14:10h photo-schedule. Stress was induced following complete feed withdrawal (FW) method as described by Bell (2003) for a period of 10 days. Drinking water was *ad-libitum* throughout the study. Six birds each were sacrificed by cervical dislocation on days 0, 1, 2, 4, 6, 8 and 10 when day 0 served as the untreated control.

Samples collection

Ovary with largest follicles and oviduct were aseptically dissected out and weighted up to two decimal points. Serum was extracted following standard protocols (Melissa *et al.,* 2009) and stored at -20°C until use for the biochemical analysis. The hierarchial follicles (HFs) and atretic follicles (AFs) were separated carefully and placed immediately on ice. Follicles were weighed in order to identify the largest preovulatory follicle (F_1) and classify their position and numbers for subsequent follicles. Both HFs and AFs were cut open transversely along the stigma to drain the yolk material. The follicular membranes were rinsedsufficiently with ice-cold sterile normal saline (0.9%) tillthere was no adhering of yolk material. The tissue samples from both HFs and AFs after overnight incubation (4°C) in the RNA stabilization solution (RNAlater, Ambion Inc., USA), were stored at -80°C for 1-2 weeks as per the manufacturer's instructions, till the RNA isolation was performed.

RNA isolation and reverse transcription

The total RNA was extracted from individual HFs (F_1, F_2 and F_3) and AFs using Trizol reagent (Invitrogen, Carlsbad, CA, USA) as per the manufacturer's instructions. The concentration and purity of RNA preparations were determined at A_{260}versusA_{280} by Nano-Drop system (Thermo 2000) and the purity was confirmed upon the ratio ≤2.0 for all the samples. Each RNA sample (5mg) was treated with 5U of RNase-free DNase (Biogene, Cambridge, UK) at 37°C for 1h to be free from the contamination of genomic DNA and subsequently inactivated by incubation at 65°C for 10min. With suitable negative and positive control, DNase-treated total RNA sample (5mg) was reverse transcribed using 'RevertAid First strand cDNA synthesis kit' (MBI Fermentas, Hanover, MD, USA) according to the manufacturer's instructions. The resultant cDNA was stored at -20°C till used.

Quantification of IGF-1, Caspase 2, Survivin and HSP 70 by RT-qPCR

The real-time PCR was performed to quantify the transcripts of the IGF-1, Caspase 2, Survivin and HSP 70 gene using

Table 1: Oligo-nucleotide primer sequences used in the real-time polymerase chain reaction (RT-PCR) in ovarian follicles (F_1, F_2, F_3 and atretic follicle) of Japanese quail hens.

Gene	Primer sequences	Annealing Temp. (°C)	Product Size (bp)	Reference/Accession No
IGF-1	F-5′ TGTACTGTGCTCCAATAAAGC	58	127	Guernec *et al.,* 2003
	R-5′ CTGTTTCCTGTGTTCCCTCTACTTG			
Survivin	F-5′ TCGAAGATGTAGCCAAGG	56	98	HM588003
	R-5′ CAGCGTGGCAGTGTC			
Caspase-2	F-5′ TTGATTGTGGAACATTCTTTGG	56	163	HM587999
	R-5′ CACTGCTGAAATGGATATTGC			
HSP-70	F-5′ GGCACCATCACTGGGCTT	56	74	HM587997
	R-5′ TCCAAGCCATAGGCAATAGCA			
Beta-actin	F-5′ GGAAGTTACTCGCCTCTG	58	114	GU230784
	R-5′ AAAGACACTTGTTGGGTTAC			

IQ5 Cycler system (Bio-Rad, Hercules, CA, USA). Based on the suitability of the SYBR chemistry, specific primer pairs including reference gene (Beta-actin) were designed using Beacon designer software (Premier Biosoft International, Palo Alto, CA, USA) and commercially synthesized (Operon Biotechnologies, Cologne, Germany)for the quantification study. The recombinant plasmids (InsTD Aclone; MBI Fermentas) for target genes including Beta-actin were generated and confirmed by sequencing. Recombinant plasmids with an insert for target genes were used as a positive control and construction of slope. The amplification was carried out in 20ml volume containing qPCR master mix with SYBR Green 1 dye Fluorescein passive reference dye (DyNAmoTM HS; Finnzymes, Woburn, MA, USA), HotStart Taq DNA polymerase and dNTPs with dUTP in optimized buffer components (New England Biolabs, Ipswich, MA, USA), a 0.2-µM concentration of 32 and 52 gene-specific primer (Table 1) and 1µl of cDNA template. PCR cycling conditions for 40 cycles were as follows: initial denaturation of 95°C for 15min, subsequent denaturation at 95°C for 30s; gene specific annealing temperature for 30s and extension 72°C for 30s. For each sample, a dissociation curve was generated after completion of amplification and results were expressed in terms of the threshold cycle value (C_t), the cycle at which the change in the reporter dye (DRn) passes the significance threshold.

Estimation of serum hormones

The concentration of sex steroids (estradiol and progesterone) and corticosterone were estimated using radio immunoassay (Immunotech, France) and enzyme immunoassay (United Biotech Inc.) kits respectively following manufacturer'sguideline. The intra assay is expressed by intra assay coefficient of variation (CV) to monitor the deviation within the same assay. Each sample is measured several times and then %CV is calculated for each sample by dividing the standard deviation of a set of measurements by the set mean and multiplying by 100. Finally, the average of the individual CVs is denoted as intra assay CV. The inter assay variation describes the variation of results obtained from repeated experiments and expressed by inter assay coefficient of variation to monitor the precision of results between different assays.

Statistical analysis

Data obtained during the course of the present study, were analyzed using two-way ANOVA. Graph pad prism software version 4.00 (Graph pad, USA) was used to analyses the data obtained from sex steroid hormones. The relative expression of the genes of interest was examined according to Pfaffl *et al.* (2002).

RESULTS AND DISCUSSION

Gross morphology

The mean ovary and oviduct weight reduced significantly ($P<0.05$) during stress in Japanese quail (Fig 1). The significant reduction of the ovary and oviduct weight was recorded on day 2 and 4 respectively and reached highest by 93% and 80.5% on day 10 compared to the value on day 0. However, the reduction in weight was more severe in the ovary compare to the oviduct. Substantial reduction in the weight of the reproductive organs ensured a high level of gross reproductive regression in quail hens. Berry (2003)

Table 2: Correlation among the six characters considered in the present study.

	Ovary weight	Oviduct weight	Yellow follicles	Estrogen	Progesterone	Corticosterone
Ovary weight	1					
Oviduct weight	0.952489**	1				
Yellow follicle	0.973846**	0.897698*	1			
Estrogen	0.974526**	0.88336*	0.992469**	1		
Progesterone	0.986985**	0.944059**	0.982075**	0.974588**	1	
Corticosterone	-0.39316	-0.27429	-0.41979	-0.4394	-0.28742	1

*Significant at P<0.05; **Significant at P<0.01.

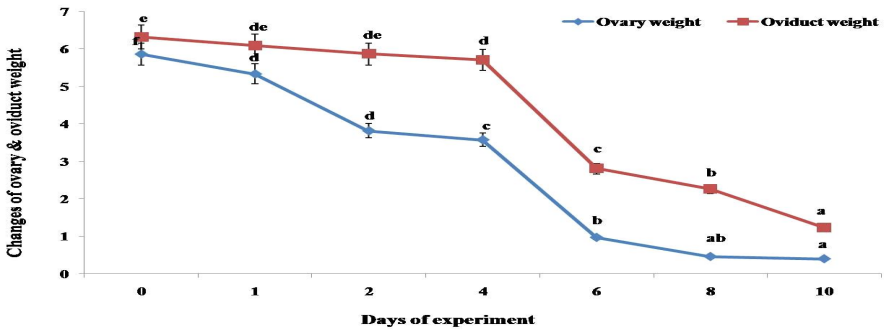

Fig 1: Percent reduction in the weights of ovary and oviduct of the Japanese quail. Graphics represent the mean weights of organs at different days of treatment (mean ± SEM, N=6). Different superscripts indicate a significant difference (*p<0.05*).

argued that lack of gonadotropic support from the pituitary during moulting by feed withdrawal in White leghorn hens has been shown to cause atresia and resorption of yolk material and ovarian regression. The present observation could be due to low level gonadotropic support during feed withdrawal as reported in the domestic chickens. Similar to domestic chicken, the gonadal regression in male Japanese quail has been reported as a result of food deprivation on reproductive organs (Kobayashi *et al.,* 2004). We are in agreement to the findings of Anish *et al.* (2008) who concluded that moulting by feed withdrawal leads to a significant reduction in reproductive organs weight of White leghorn hens.

The present observation revealed an abrupt decrease in the number of hierarchial follicles after day 2 and significant (P<0.05) increase of atretic follicles subject to feed withdrawal stress (Fig 2). There were no hierarchial follicles detected after day 6 though atretic follicles were evident as early as day 2 of the experiment. A significant (P<0.05) positive correlation was observed between the oviduct weight and the number of yellow follicles (Table 2). Similar to the oviduct, we presume that owing to the lack of gonadotropic support from the pituitary, hierarchial follicles undergo into atresia. It is suggested that the small ovarian follicles are more susceptible to atresia during stress (Moudgal *et al.,* 1991). Epinephrine secreted in response to stress induces follicle atresia in vitro and in vivo in hens (Moudgal *et al.,* 1990). It is reported that induction of immobilization stress leads to noticeable follicular regression in Japanese quail (Shit *et al.,* 2016).

Sex steroids and corticosterone

The mean concentrations of serum estrogen (pg/ml) and progesterone (ng/ml) were declined significantly (P<0.05) on day 2 onwards, till the end of the study (Fig 3A and B). However, the concentration goes below threshold value after 6th day due to of severe reduction of the hormones. This result is found similar to Sundaresan *et al.* (2007) and Anish *et al.* (2008) who found down regulation of estrogen and progesterone in the single comb White Leghorn hens. It is suggested that the under nutrition state inhibits gonadotrophin secretion (Robinson *et al.,* 1999). Stress

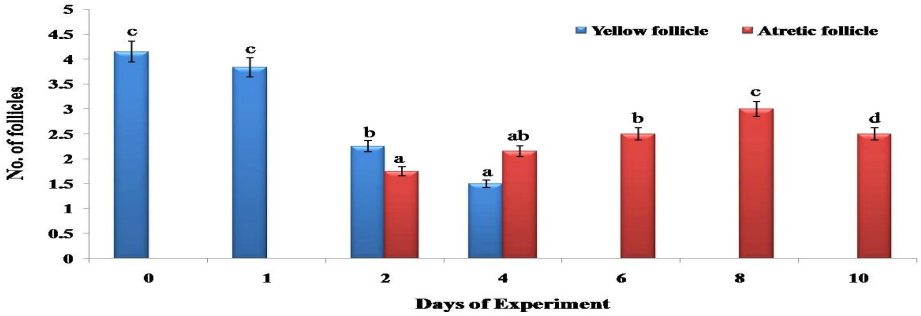

Fig 2: Graphics represent the number of yellow and atretic follicles at different days of treatment (mean ± SE; N=6). Different superscripts indicate a significant difference (*p<0.05*)

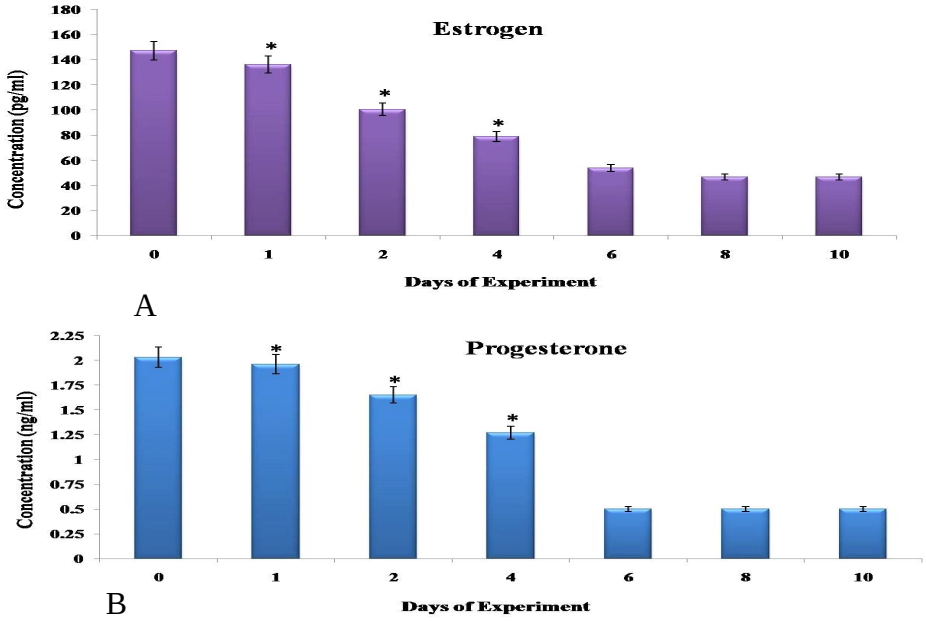

Fig 3: The serum concentrations of estrogen (3A) and progesterone (3B) in Japanese quail during stress (mean ± SE; N=6). * indicates significant difference (*P<0.05*)

induced inhibition of GnRH pulsation affects gonadotrophin secretion from the pituitary and may be the cause of declining sex steroid concentration (Anish *et al.,* 2008). Cytokines may be involved in declining serum estrogen and progesterone concentration in Japanese quail as reported in White leghorn hens (Sundareson *et al.,* 2007).

The corticosterone level (ng/ml) was increased significantly (P<0.05) and became highest on day 2 (4.86±0.53), however, the concentration was recorded then inconsistent throughout the study period (Fig 4). A significant (P<0.01) negative correlation was recorded between the serum corticosterone and ovarian tissue regression (Table 2). Cockrem *et al.* (2004) described that corticosterone increased during stress and extends adjustment to stressors by affecting locomotion, feed intake, fluid balance and energy mobilization. The present observation is in agreement to Harvey *et al.* (1984) who suggested that food deprivation rapidly increases the plasma corticosterone from baseline in laying hens. Similar findings were also reported during stress in turkey (El Halawani *et al.,* 1973), pigeons (Pilo *et al.,* 1985) and Japanese quail (Shit *et al.,* 2016). Stress induced activation of HPA axis and subsequent release of ACTH and other pro-opiomelanocortin (POMC) derived peptides i.e. â-endorphin could be the cause of higher level of corticosterone (Brandt *et al.,* 2007).

Gene expression study

The mRNA expression of IGF-1, Survivne, Caspase 2 and HSP 70 gene was carried out in the yellow follicles (F_1, F_2 and F_3) till day 4. However, the gene expression study was performed in atretic follicles thereafter as yellow follicles were no longer evident.

The insulin-like growth factors (IGFs) maintain growth and differentiation of most cell types through endocrine, autocrine and paracrine control. They play crucial role in avian reproduction as they hasten dose dependent gonadal steroid hormone synthesis, cell proliferation, selection and inhibition of follicular apoptosis by inhibiting oligonucleosome formation (Johnson *et al.,* 2001). During stress we noticed a significant (P<0.05) down regulation in the fold expression of IFG-1in both yellow and atretic follicles (Fig 5). The sensitivity to the stress stimuli was found greater for the largest follicle and the relative change was remarkable in the atretic follicles compared to the hierarchial follicles. Interestingly, both IGF-1 and IGF-2 exerts their action after binding to the same receptor IGF-R (type-1) in avian species. Stress induced decrease in the secretion of ovarian growth factors (IGFs) hastens oligonucleosome formation which initiates apoptosis in the ovarian tissues. Evidently, the mRNA and protein encoding for the IGFs receptor are expressed in immature ovary (Heck *et al.,* 2003), granulosa and theca cells of the developing follicles (Tosca *et al.,* 2008). The present finding is in agreement to Shit *et al.* (2014) who reported a significant down regulation of IGF-1 gene during immobilization stress. However, the molecular mechanism associated with IGF-1gene expression during feed withdrawal in Japanese quail is still unclear.

The current study revealed a significant (P<0.05) up-regulation in the fold expression of survivin gene (Fig 6). The F_2 yellow follicle revealed greater magnitude of

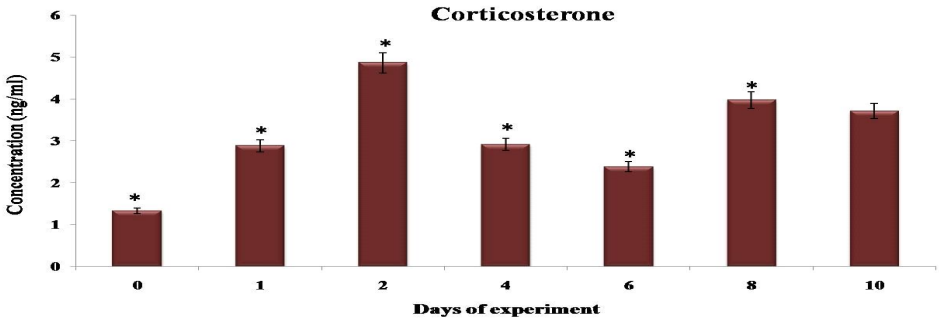

Fig 4: The serum concentrations of corticosterone in Japanese quail during stress (mean ± SE; N=6). * indicates significant difference (*P<0.05*).

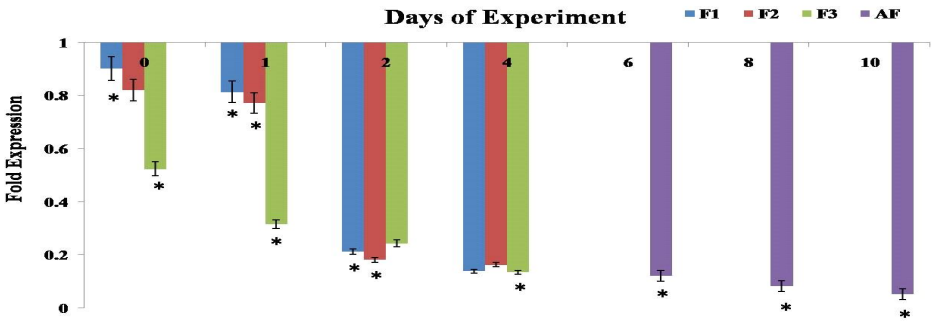

Fig 5: The mRNA expression profile of IGF-1 gene in hierarchial (F_1, $F_2$and F_3) and atretic follicle (AF) of Japanese quail under stress (mean ± SEM, N=6). *Significant at *P<0.05.*

expression and reached its peak on day 4 of the experiment. The relative gene expression was evidently down regulated in the atretic follicles in advance to the course of study. Survivin, member of IAPs proteins, has been proposed to control cell proliferation and death (Wheatley and McNeish, 2005) and over expression is reported in embryonic and fetal tissues though detected in many normal adult tissues (Ambrosini *et al.,* 1997). Additionally, it has been found to inhibit apoptosis either by inhibiting the cytochrome-c induced proteolytic events in the cytoplasm or by directly interfering the activities of terminal effector cell death protease i.e. caspases-3, -7 and -9 (Tamm *et al.,* 1998; Shin *et al.,* 2001). According to Johnson and Howerth (2004), survivin regulating F_2 and F_3 follicles may be mitotically more active and are under regular cell cycle (G2/M phase) and this could be correlated with the present observation. No conclusive evidence is available so far, further study is required for clarification.

In the present experiment, we observed significant ($P<0.05$) up-regulation of caspase-2 gene expression in both yellow and atretic follicles (Fig 7). The relative fold expression level was more in the largest yellow follicle (F_3) compared to the rest F_2 and F_3 follicle. However, the magnitude of expression reached to the highest on 4th day of the study. Caspase-2 is a nuclear resident protein and it can trigger apoptosis by release of mitochondrial cytochrome c and other apoptogenic factors into the cell cytoplasm (Paroni *et al.,* 2001; Guo *et al.,* 2002). Evidently, stress induced intra-cellular nitric oxide (NO) accumulation proportionately accelerates the activation of caspase cascade which directly leads to reproductive tissue regression via apoptosis in mammals (Skarzynski *et al.,* 2005). Caspase-2 is known for its pro-apoptotic and anti-apoptotic activities depending on the tissue where it is produced. However, a similar trend of up-regulation was recorded in the ovary and oviductal tissues (Anish *et al.,* 2008) and in the post ovulatory follicles (Sundaresan *et al.,* 2008) in fasted White Leghorn hens. The mechanism beneath the stress induced caspases associated reproductive tissue regression in Japanese quail is yet be explored.

The cells with increased level of Heat Shock Proteins (HSP) exhibit tolerance against the additional stress hence they are often called as stress markers (Figueiredo *et al.,* 2007). Although the mechanism by which HSPs protect cells is known but their expression can be modulated by cell signal transducers, such as changes in intracellular pH, cyclic AMP, Ca^{2+}, Na^{+}, inositol trisphosphate, protein kinase C and protein phosphatase (Kiang and Tsokos, 1998). The amplified fragment sequence of quail HSP-70 is having 98% homology with the chicken and 99% to the helmeted Guinea fowl (Gaviol *et al.,* 2008). The HSP-70 gene expression did not show any noticeable change and varied inconsistently in ovarian follicles throughout the study (Fig 8). This result is in agreement to Shit *et al.* (2014) who concluded an inconsistent variation in HSP-70 gene expression in Japanese quail during immobilization stress. It is demonstrated that heat shock proteins (HSPs) expressed at high level only when cells are exposed to high or low temperature or other stressors. It is also reported that heavy body weight line Japanese quail is inherently more capable

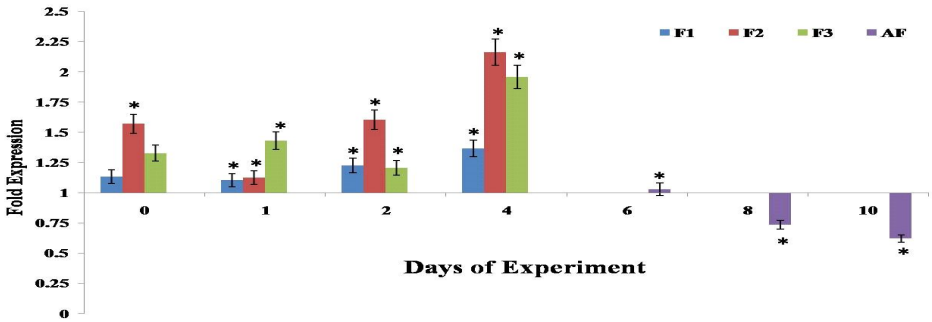

Fig 6: The mRNA expression profile of Survivin gene in hierarchial (F_1, $F_2$and F_3) and atretic follicle (AF) of Japanese quail under stress (mean ± SEM, N=6). *Significant at *P<0.05.*

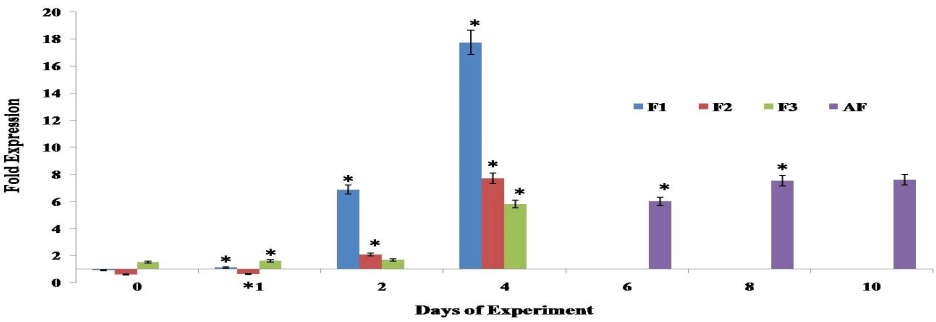

Fig 7: The mRNA expression profile of Caspase-2 gene in hierarchial (F_1, $F_2$and F_3) and atretic follicle (AF) of Japanese quail under stress (mean ± SEM, N=6). *Significant at *P<0.05.*

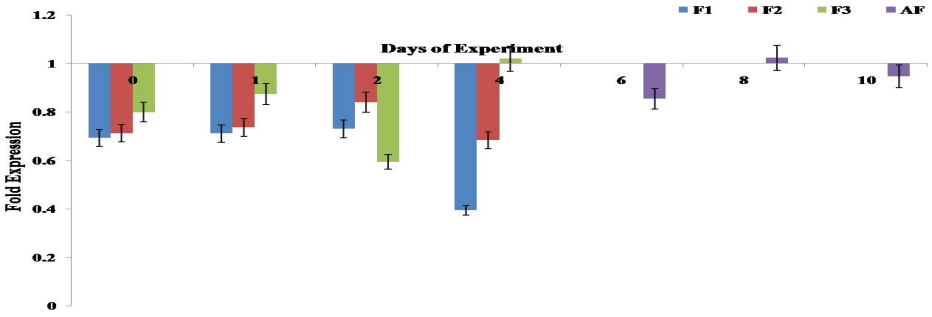

Fig 8: The mRNA expression profile of HSP-70 gene in hierarchial (F_1, $F_2$and F_3) and atretic follicle of Japanese quail under stress (mean ± SEM, N=6). *Significant at *P<0.05.*

in counteracting the negative effect of heat shock on immunocompetence by induction of high intensity of HSP-70 transcription (Faisal *et al.,* 2008). Increased expression of HSP-70 was also detailed in the myocardial tissue of Japanese quail exposed to loud noise, inescapable irritation, cold temperature and isolation in darkness (Hoekstra *et al.,* 1998).

CONCLUSION

The study concludes that stress on feed withdrawal in Japanese quail is enough for reproductive tissue regression. The vibrant changes in the serum level of sex steroids and corticosterone is well correlated with the oviductal regression and follicular atresia. It appears that expression of IGF-1, Survivin, Caspase-2 gene signifies their involvement in the tissue regression via endocrine changes. However, the exact signalling and consequences for reproductive tissue regression in Japanese quail during stress is yet to be established.

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