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Reproductive Responses of Japanese quail (*Coturnix coturnix japonica)* under different photoperiodic conditions

S. Upadhyay, S. Banerjee, R. Soni and C.M. Chaturvedi*
Department of Zoology, Institute of Science, Banaras Hindu University, Varanasi 221005, India
Corresponding author at: Department of Zoology, Institute of Science, Banaras Hindu University, Varanasi 221005, India . Email: upadhyaybhu01@gmail.com

Abstract

Photoperiod plays important role in the regulation of reproductive performances of avian species. It acts through the neuroendocrine axis to execute the photosexual responses in photoperiodic avian species including Japanese quail and is mediated through photoreceptor system. Photoperiod is considered as a main temporal factor for seasonal reproduction in many tropical and subtropical avian species including Japanese quail and artificial photoperiod (long or shortday length) also regulates reproduction under laboratory conditions. Hence present study was design to understand the basis of reproductive performance in long and short daylength quail. Our experimental study demonstrates increased testicular and accessory sex gland activities including the expression of androgen receptor (AR). Contrarily in short day (SD) quail all the studied parameters (Cloacal gland volume, Gonadosomatic index, Testosterone level, Histology of Testes, Epididymis and cloacal gland as well as AR) were suppressed compared to longday (LD) quail. Based on these observation it is concluded that changes in gonadal functions in response to photoperiod mediated through the hypothalamo-hypophyseal gonadal (HPG) axis. The photoperiodic information of daylength is translated in photosexual responses via the deep brain photoperception system and subsequently modulates the hypothalamo-hypophyseal-gonadal axis.

Keywords: Photoperiod; Cloacal gland; Plumage; sexually dimorphic; shortday quail; Longday quail; Natural day length

Introduction

Japanese quail (*Coturnix coturnix japonica*) is a domesticated and easily reproducible avian species. This poultry species breeds continuously when kept under longdaylength conditions, but if maintained under natural daylength (NDL) conditions it display seasonal gonadal cycle. Japanese quail matures sexually in about 6 weeks and life expectancy is only 2 to 2½ years. Similar to any tropical photoperiodic species the plumage of the Japanese quail is sexually dimorphic, allowing for differing sexes to be distinguished from one another (Adkins, E. 1977; Balthazart et al., 1983). Although as chicks, both male and female individuals exhibit the same kind of plumage and coloring. In general, both male and female adults exhibit predominantly brown plumage. However, markings up to the age of 3-4 weeks on the throat and breast, as

well as the particular shade of brown of the plumage, can vary quite a bit. The breast feathers of females are littered with dark spots among generally pale feathers. Contrastingly, male breast feathers show off a uniform dark reddish-brown color that is devoid of any dark spots. This reddish brown coloration also appears in the male cheek, while female cheek feathers are more cream colored. Some males also exhibit the formation of a white collar, whereas this does not occur in any female members of the species (Hubrecht and Kirkwood 2010). Males also have a cloacal gland, a bulbous structure on the dorsal lip of the cloacal vent that secretes a white, foamy material containing sperms. This androgen dependent sex accessory, and unique gland is a reliable indicator of gonadal activity of the males (Massa et al., 1980). In general, long daylength or long photoperiod is gonado-stimulatory whereas short-daylength or short photoperiod is gonado-suppressive. Increasing daylength of spring and summer i.e., long daylength stimulate the onset of seasonal reproductive activities in photoperiodic avian species and these birds are said to be photosensitive. Similarly decreased daylength or post summer markedly initiate post reproductive gonadal regression and these photoperiodic species become photorefractory. Seasonal gonadal growth in short day breeders is initiated with decreasing daylength and breeding occurs during short days of winter. Like long day breeders these species also undergo the phase of scotosensitivity and scotorefractoriness (Follett et al., 1978, 1990; Chaturvedi et al., 1992, 1993).

Each testis consists of a mass of convoluted seminiferous tubules, which in quail, unlike mammals, are anastomotic and not restricted by septa. They are lined by the germinal epithelium consisting of developing germ cells and non-germinal Sertoli cells. During breeding season, stem spermatogonia causes the propagation of numerous germ cells, which mature successively into secondary spermatogonia (type B), primary and secondary spermatocytes, spermatids, and spermatozoa, so that germinal epithelium becomes several layers thick (Marshall and Serventy, 1957).

Maintenance of spermatogenesis depends on intratesticular testosterone, provided in part by the steroidogenic actions of LH on the Leydig cell masses in between the seminiferous tubules and in part, by the production of androgen binding protein (ABP) by the Sertoli cell following the action of FSH (Nicholls et al., 1974). Thus, both gonadotropins act in concert to maintain germ cell production and steroidogenesis. Androgen plays potent role in male reproduction by regulating the sexual behavior and aggression. Androgen is critical for maintenance of the spermatogenesis and male sexual behaviour in quail. It also helps in regulation of the luminal micro environment to guarantee the sperm transport, maturation and storage. Androgen and its derivatives (testosterone and dihydrotestosterone) act through androgen receptor and thus mediate the physiological actions of testosterone and dihydrotestosterone (Brown et al., 1977; Balthazart et al., 1979). Little is known about the expression pattern and action of androgen receptor in quail testes under different photoperiodic conditions. The differential level of testosterone and androgen receptor expression will promote the testicular stimulation/regression in long day and short day

quail respectively. Present study was designed to investigate the initial reproductive responses of Japanese quail to long and short daylength in addition to the expression of androgen binding protein in the testes and epididymis.

Materials and Methods:

Sexually mature 3-4 week old male Japanese quail were purchased from Chuck Gazaria Farm, Sultanpur Road, Lucknow, India and acclimatized under laboratory conditions in a photoperiodically controlled room. These male quail (n=20) were randomly divided into two groups. Quail of one group (LD) maintained under constant long day condition (16L: 8D) while second group (SD) under short day condition (8L: 16D) for 12 weeks and provided commercial poultry feed and water *ad libitum*. Experiment was conducted in accordance with institutional practice and within the framework of the revised animals (Scientific procedures) act of 2002 of the Government of India on Animal welfare. This study is performed under the regulation of "Institutional Animal Ethical Committee, Institute of Science, Banaras Hindu University".

During the experiment, body weight and cloacal gland volume of LD and SD quail (n=10) was measured every week. At the end of the experiment, quail were weighed, and the length and width of the cloacal gland was measured *in situ* by dial calipers. The birds were anesthetized with sodium pentobarbital (3–4 mg/100 g body weight). Blood was collected from the wing vein into a heparinized tube and centrifuged at 5000 rpm for 15 min at 4 °C to separate the plasma for the testosterone assay.

For whole body perfusion, quail were perfused transcardially with phosphate buffered saline (PBS) followed by Zamboni's fixative (4% paraformaldehyde in 0.1 M sodium phosphate buffer; pH 7.4) and testes were fixed in Zamboni's solution for histological studies.

Both the testes were excised, weighed and the Gonado-Somatic Index (GSI) was calculated as

$$\boldsymbol{paired\ testicular\ weight\ (g)/bodyweight(g) \times 100}.$$

The length and width of the left testis was measured *in situ* with dial calipers. The cloacal gland and testicular volume was calculated using Bissonett's formula $\boldsymbol{\frac{4}{3}\pi ab^2}$ (a = half of the long axis; b = half of the short axis) (Chaturvedi et al., 1993).

Testosterone assay

For plasma testosterone levels, enzyme immunoassays (EIA) were performed using a commercial testosterone kit (DSI s.r.l., Italy) following the manufacturer's protocol. The antiserum used in the assay was specific for testosterone. The cross reactivity of the assay was 0.056% with progesterone, 0.004% with cortisol, 0.005% with estradiol, 4.8% with dihydrotestosterone, 3.6% with androstenedione, 0.048%

with androsterone, 0.004% with cortisone, 0.002% with estriol and 0.007% with estrone. The analytical sensitivity of the assay was 0.0576 ng/ml. The intra-assay coefficient of variation (CV) was 5.6% whereas the inter-assay CV was 7.1%. The accuracy for this assay was 99%.

Histological preparations

Testes removed after whole body perfusion was post-fixed in fresh Zamboni's fixative. Twenty four hours after fixation, all the testes were dehydrated in a graded series of alcohol, treated with xylene and embedded in paraffin wax. 6 µm thick sections were cut by Lieca RM2125 RT rotatory microtome (Leica Biosystems Nussloch GmbH, Nussloch, Germany) and processed for the haematoxylin-eosin staining and immunofluorescence of androgen receptor (AR) as follows using polyclonal antibody.

Androgen receptor Immunofluorescent Study:

Immunofluorescence for androgen receptor (AR) was performed in a two-step procedure as described by Banerjee et al (2016). In the first step, testis and epididymis section slides were processed for initial deparafinization in xylene and rehydration in graded series of alcohol. Antigen retrieval was performed using 1 M citrate buffer (pH 6) in a microwave oven at 1000 W (3-4 min). Tissue sections were then treated with blocking solution for 2 hr at room temperature (5% HIGS- Heat Inactivated Goat serum) and then sections were incubated with rabbit anti-AR polyclonal antibody (1:100) (Generous Gift from Prof. Frank Claessens, Molecular Endocrinology Laboratory, Department of Cellular and Molecular Medicine, University of Leuven, Leuven, Belgium) for 24 h in humid chamber. In second step, sections were washed with TBS (3 X 5 min.) and then incubated with donkey anti-rabbit IgG H&L (1:200) (conjugated with FITC sc-2090, USA) for 3 hrs at RT in dark. After incubation, sections were again washed with TBST (3 X 5 min.) and two drops of the fluorescent media (0.5% N-propyl gallate + 20mM Tris in 90% glycerol + DAPI counterstained) was applied on the sections. Coverslips were applied and then sealed with nail polish after ensuring the spread of the mounting media over all the sections without any bubble formation). A fluorescent microscope (Axioskop 2 plus; Carl zeiss, Oberkochen, Germany) was used to observe the sections and the images were captured with an Axioskop HR cam camera. Nuclei were counterstained with DAPI (1µg/10 ml PBS).

Statistical Analysis

All the data were presented as the mean ± SEM. For statistical analysis, Student's t-test was performed for the comparison of group means. A p-value of less than 0.05 was considered significant. All the statistical analyses were performed using SPSS Statistics software version 17.0, Chicago, USA.

Results:

Body weight: No significant difference was observed in between the LD and SD quail (Fig: 1).

Cloacal gland volume (CGV): Significantly increased CGV was observed in LD quail while SD quail maintained regressed CG compared to LD quail (Fig: 2).

Gonado-Somatic Index (GSI): Gonado-somatic index was significantly low in SD quail compare to LD. (Fig: 3).

Testosterone Assay: Plasma testosterone was found to be significantly decreased in SD quail as compared to LD quail. The increased plasma testosterone clearly suggests increased steroidogenesis in LD quail (Fig: 4)

Histology

Histologically, testis of LD quail exhibited full breeding condition with enlarged seminiferous tubules having all the stages of spermatogenesis and bunches of spermatozoa in the lumen. Contrarily, testis of SD quail has small sized seminiferous tubules containing only single layer of inactive spermatogonial cells. Inter-tubular spaces exhibit many undifferentiated interstitial cells and lumen of the seminiferous tubule contained cellular debrises (Fig: 5).

Epididymis of the LD quail showed enlarged lumen of ductus epididymidis, extensively filled with spermatozoa. The mucosa of the ductuli efferentes was highly folded. The connecting ducts were much smaller in diameter. Ductus epididymis and connecting ducts were lined by non-ciliated pseudostratified epithelium. The ductus epididymis was larger in diameter than that of the connecting ducts, and its lumen was filled with sperm. Contrarily, SD quail showed significantly reduced ductus epididymidis lumen diameter, scanty number of spermatozoa and cellular debris as well. The SD quail ductus epididymidis epithelium lining formed relatively few microvilli, and height of the cells was less than that of epithelium lining the ductus epididymis of LD quail (Fig:5).

Immunofluorescence of Androgen Receptor (AR) in Testis and Epididymis:

Immunofluorescence study revealed that the LD quail testes showed strong/intense immunoreactivity for AR unlike SD quail testis. Significantly increased *ir*-AR positive cells were exhibited in all the testicular cell types, viz., Leydig cells, Sertoli cells, peritubular myoid cells and germ cells (spermatogonia, spermatocyte etc.). On the other hand, significantly weak immunoreactivity for AR was observed in the Leydig cells, Sertoli cells, peritubular myoid cells and spermatogonia of SD quail Testis (Fig: 6A).

The LD quail epididymis epithelial cells showed very strong/intense immunostaining for androgen receptor (AR). Contrarily epididymal epithelia of SD quail showed weak AR immunoreactivity (*ir*-AR positive cells) (Fig: 6B)

Discussion:

From our study, it is evident that long day quail showed significantly increased cloacal gland volume, testicular weight, gonado-somatic index and plasma testosterone level (Fig: 2, 3 and 4), but no significant change in body weight (Fig: 1), was observed compared to short day quail. Plasma testosterone level was also significantly increased in long day (LD) quail compared to short day (SD) quail. The gonado-somatic index clearly indicates that testicular and somatic growth runs parallel in the LD quail. But in spite of somatic growth i.e., increased in the body weight, gonad remain in regressed condition even in adult quail maintain in SD condition.

Increased plasma testosterone in LD quail is responsible for the sexual/copulatory and aggressive behavior not evident in SD quail (Fig: 4). Injections of testosterone and androstenedione restored aggressive behavior in castrated male quail (Beach and Inrnan 1965).

The aggressive behavior in adult male Japanese quail, as well as their sexual behavior, is induced by testosterone as well as estrogen converted by aromatase in the brain from testosterone (Howard and Bermant 1967). In males, testosterone can act at its target tissues by binding to testosterone receptors, or first being converted to a more potent androgen, dihydrotestosterone, by the enzyme 5α-reductase, or by being converted to estrogen by the enzyme aromatase and acting by binding to estrogen receptors (Sun et al., 2001).

The histological studies of LD quail testis showed significantly increased seminiferous tubule diameter and tiers of germ cells inside the tubules. It was also observed that increased area/volume of Leydig cell masses in the decreased interstitial spaces clearly indicate increased activity of Leydig cells i.e., increased steroidogenesis in LD quail. Increased plasma testosterone in LD quail further supports our finding. Significantly increased number of spermatozoa/bunches of spermatozoa was also seen in the lumen of the tubules of LD quail. Hence, it is evidently enough the LD quail testis is not only sexually active but also showed testicular hypertrophy. On the other hand, in SD quail, testis showed significantly decreased seminiferous tubule diameter and single layer of spermatogonia inside the tubules indicating the arrest/ suppression of spermatogenesis. It was also observed that decreased area/volume of Leydig cell masses in between the tubules and decreased interstitial spaces with vacuolization/gaps clearly indicate decreased activity of Leydig cells i.e., decreased steroidogenesis in SD quail. Significantly decreased plasma testosterone in these quail further supports our finding. Scanty/no spermatozoa were observed in the lumen of the tubules of SD quail. Hence, it is clear enough the SD quail testis is not only sexually inactive/quiescent but also showed testicular atrophy (Fig: 5).

In addition to LD quail testes, the epididymis also showed enlarged lumen of ductus epididymidis, extensively filled with spermatozoa. The ductuli efferentes mucosa was also highly folded suggesting developed epididymis. Contrarily SD quail showed significantly reduced ductus epididymidis lumen diameter with cellular debris

and no spermatozoa. The SD quail ductus epididymidis epithelium lining formed relatively few microvilli, and height of the cells was less than that of epithelium lining the ductus epididymis of LD quail (Fig: 5).

In sexually active LD quail, unlike SD quail the cloacal gland was well-developed and active with abundant foamy secretion. Supporting the fact that cloacal gland is a reliable indicator of testicular activity. LD quail cloacal gland sections demonstrated enlarged lobules (well-developed glandular tissue). Which remained suppress in SD quail. In SD quail, the cloacal gland was significantly regressed and foaming was also not observed. The lobular villi/projections were also regressed significantly. Our findings further support the reproductive quiescence in SD quail.

Immunofluorescence study revealed that the LD quail testes showed significantly increased *ir*-AR positive cells in all the testicular cell types, viz., Leydig cells, Sertoli cells, peritubular myoid cells and germ cells (spermatogonia, spermatocyte etc.). While significantly weak immunoreactivity for ir-AR cells was observed in spermatogonial cells of SD quail. The LD quail epididymal epithelial cells also showed very strong/intense immunostaining for AR. Contrarily, epididymal epithelia of SD quail showed weak AR immunoreactivity. Hence, it is quite clear from our study that in LD quail, the testosterone dependent testicular activity and spermatogenesis is mediated through androgen receptor present in all the testicular cell types. Epididymal sperm maturation, functionality and sperm transport in LD quail is also under the control of testosterone which is mediated via androgen receptor present in epididymal epithelial cells (Kwon et al., 1997; Yoshimura & Kawai 2002). Regressed testis and epididymis in SD quail may result due to the insufficient level of testosterone as well as significantly decreased expression of its receptor in testicular cell types and in epididymal epithelial cells.

Our study clearly suggest that long day and short day response in quail is exhibited through hypothalamic deep brain photoreceptor system as well as hypothalamic and reproductive circuitry (GnRH-I/II-GnIH system) which in turn modulates the hypophyseal-gonadal/testicular axis and the same is reflected in the photosexual responses (Ubuka et al., 2008, 2013; Nakane et al., 2010).

Conclusion:

Findings from our study clearly suggests daylength/duration of the photoperiod dependent testicular maturation and reproductive performance in quail. Long daylength is gonado-stimulatory while short daylength is gonado-inhibitory. Long day (LD) quail are sexually active and their reproductive performance is significantly higher compared to sexually quiescent short day (SD) quail. Obviously, photoperiod induces its effect via higher brain Centre (photoreceptors) and HPG axis ultimately modulates the gonadal activity. The photoperiodic responses are evident not only in the testicular size histology affecting spermatogenesis but also at the level of the sex hormone and sex accessories including AR. It is concluded that increased photoperiod (LD) is gonado stimulatory while decreased photoperiod (SD) is gonado-inhibitory. The photoperiodic

effects are mediated through hypothalamo–hypophyseal complex and finally regulates gonadal activity. Present study clearly provides experimental evidences how the external environmental factor may significantly influences body physiology with special reference to reproduction. The study also manifests the mechanism of seasonal gonadal activity in the avian species which breeds at a longer days of summer and sexual inactivities in shorter days of winter. This report also supports the existence of photoreceptors in the birds which transduce photic stimulation into hormonal response and thus modulate the gonadal activity and reproductive performance of birds.

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Legend to Figures:

Fig: 1. Body weight of LD and SD quail maintain in 16L: 8D and 8L: 16D respectively. No significant difference was found in the body weight of the two groups.

Fig: 2. Cloacal gland volume of LD (16L: 8D) and SD (8L: 16D) quail. Significant increase in CGV was observed in quail, while reveres/opposite condition has been shown by short day quail. Results are expressed in terms of value representing Mean+SEM where n=10 and * $P<0.05$, ** $P<0.01$, * $P<0.001$, (long day group vs. short day group).

Fig: 3. Gonadosomatic index of LD (16L: 8D) and SD (8L: 16D) quail. Significant increase in Gonadosomatic index was observed in LD quail, while reverse/opposite condition has been shown by SD quail. Results are expressed in terms of value representing Mean+SEM where n=10 and * $P<0.05$, ** $P<0.01$, * $P<0.001$, (long day group vs. short day group).

Fig: 4. Plasma testosterone level of LD (16L: 8D) and SD (8L: 16D) quail. Significant increase in Plasma testosterone level was observed in LD quail, while reverse/opposite condition has been shown by SD quail. Results are expressed in terms of value representing Mean+SEM where n=10 and * $P<0.05$, ** $P<0.01$, * $P<0.001$, (long day group vs. short day group).

Fig: 5. Histology of Testis (A), Epididymis (B) and Cloacal gland (C) in long day and quail. LD quail exhibited full breeding condition with enlarged seminiferous tubules having all the stages of spermatogenesis and bunches of spermatozoa in the lumen, enlarged lumen of ductus epididymidis, and cloacal gland (CG) showed significantly increased folds in the evaginations of the secretory lobules which clearly indicate the hyperactive CG. Which was lacking in SD quail.

Fig: 6. Immunofluorescence of Androgen Receptor in testis (A) and Epididymis (B). LD quail showed significantly increased expression/immunoreactivity for AR in all the testicular cell types where AR located.

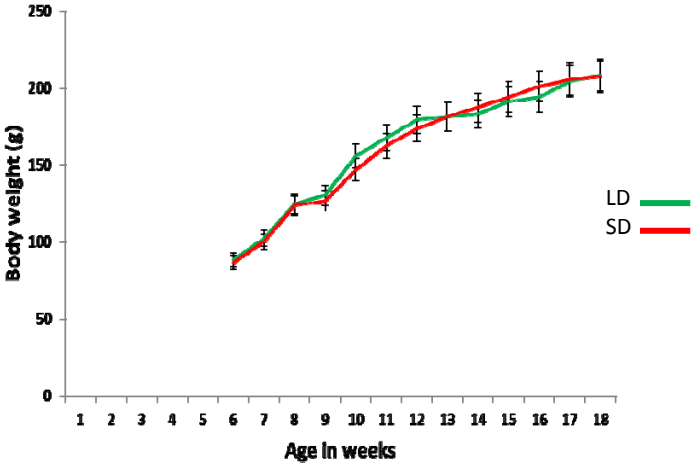

Fig. 1

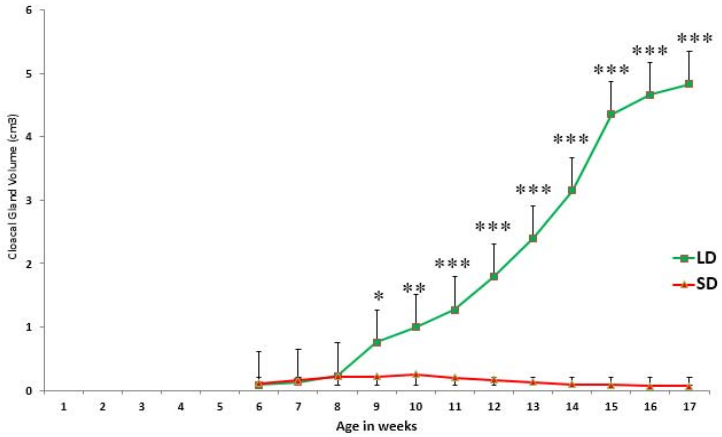

Fig. 2

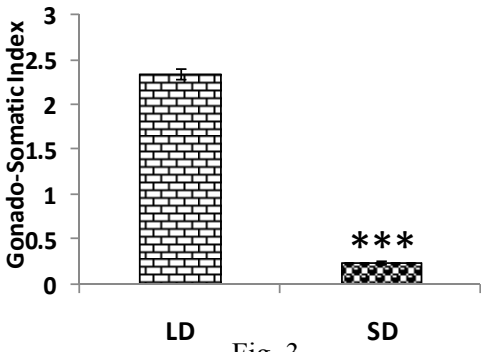

Fig. 3

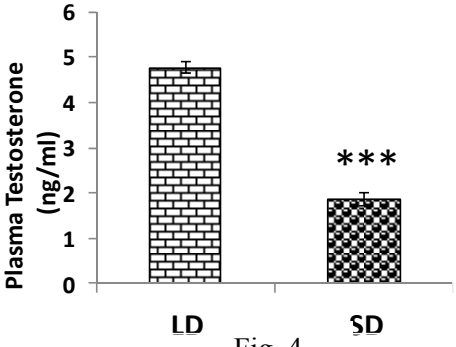

Fig. 4

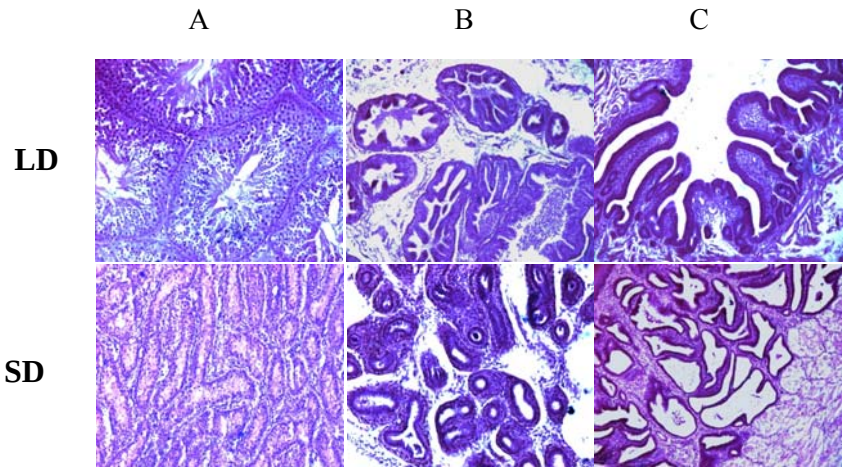

Fig. 5

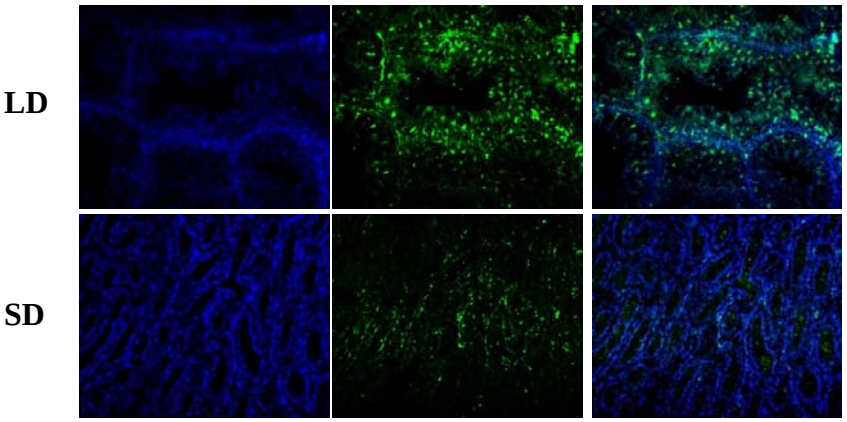

Fig. 6(A)

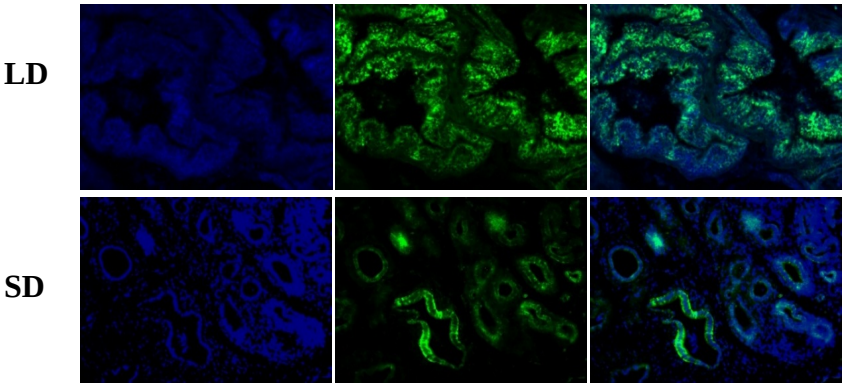

Fig. 6(B)