

Exogenous Melatonin Causes Gonadal Regression in Scotorefractory Japanese quail (*Coturnixcoturnix japonica*): Involvement of Pineal Photoreceptors

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ABSTRACT: Japanese quail (*Coturnixcoturnix japonica*) an interesting photoperiodic species, exhibits annual gonadal cycle under natural day length condition, and continuously developed gonad under constant long daylength condition. Under constant short day-length condition, initially regressed gonad (scotosensitivity) attains reproductively active condition (Scotorefractoriness) after prolonged exposure (13 weeks) to short day-length. Light perceived by retinal and extra retinal photoreceptors are conveyed to brain in terms of electrical impulses, via cascade of RHT where pineal gland is responsible for secretion of melatonin dependent upon duration of dark period, photoperception and initiation of photo transduction pathway in avian species. The aim of present study was to observe the effect of exogenous melatonin (25µg/100g body weight/day over a period of 21 days) upon developing testis of short day birds as well as on the Pineal photoreceptors Rhodopsin & Transducin under scotorefractory (SR) condition.

Present results indicate melatonin induced suppression of cloacal gland, testicular volume and spermatogenesis as well as decreased immunosignaling of pineal photoreceptors compared to SR quail. These findings led us to conclude that, exogenous administration of melatonin causes testicular regression as well as down regulation of immunoreactive Rhodopsin and Transducin in the pineal of short day quail even under scotorefractory condition. It is suggested that melatonin had significant impact on HPG axis by suppressing the scotorefractoriness in quail, possibly via decreasing the expression of Pineal photoreceptors. These evidences are possibly the first report confirming that melatonin may not only suppress the gonadostimulatory effect of long day-length but also that of gonado-stimulatory short day-length.

KEYWORDS: Melatonin, photoreceptors, scotorefractoriness, rhodopsin, transducin

I. INTRODUCTION

In general like other photoperiodic species, in Japanese quail (*Coturnixcoturnix japonica*), long day-length (LD) is gonado-stimulatory and short day-length (SD) is gonado-inhibitory. When maintained under natural day length (NDL) condition, Japanese quail exhibits annual gonadal cycle similar to any seasonally breeding photoperiodic species [1]. However, this species has other characteristic features- (i) if maintained under constant long day-length condition its gonad remain stimulated like any continuous breeder, (ii) if maintained under constant short day-length condition, as expected, initially gonad regress (scotosensitivity) but after a certain period gonadal stimulation occurs even under SD condition (scotorefractoriness) [2, 3,4,5, 6] . In Avian gonads, there is a potential to respond to plasma melatonin, and in galliform birds (chicken, duck, quail), the melatonin- binding site in gonads appears to be coupled to a guanine nucleotide-binding protein second messenger system [7]. On the other hand, eyes are the most essential part that are responsible for photoperception, photoperiodic response system and regulation of daily and seasonal physiology in mammals.

Unlike birds, blind mammals fail to respond to external clues such as day length and are not able to synchronize their circadian rhythms with the light-dark (LD) cycles [8, 9, 10].

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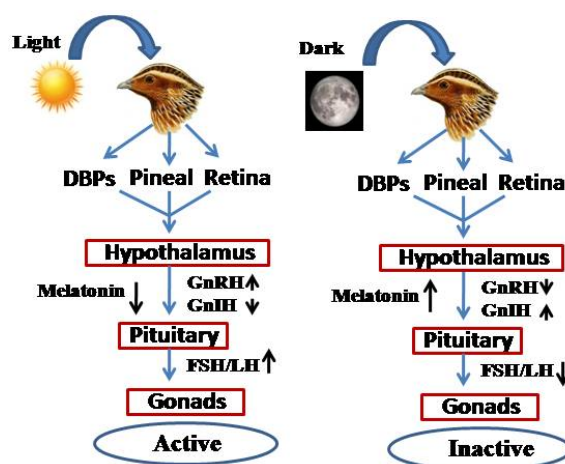


Fig. A. Schematic diagram showing the perception of light through retinal and extra retinal organs and modulation endocrine hormones and melatonin level during different photoperiod in Japanese quail.

Retina is the most important part of eye which perceives light and allows and mediates the effect of light on growth and behavior [11]. The avian pineal gland or epiphysis cerebri of birds, common with other vertebrates, is derived from the posterior roof of the diencephalon, consists of modified paraneuronal cells known as pinealocytes and few supporting cells, i.e., ependymal and glial cells [12]. The retina and the pineal gland have capacity to synthesize an indolamine, melatonin (N-acetyl-5-methoxytryptamine) by using the environmental light impulses rhythmically (day/night) and synthesis of melatonin increases at night [13,14]. In birds, mainly three melatonin receptor genes have been identified: mel-1a and mel-1b are the homologues of mammalian MT1 and MT2 respectively, while Mel-1c receptor is very specific and found only in birds [15].

In mammals, melatonin influences reproductive physiology and activity in short day breeders and inhibits gonadal activity in long-day breeders. In photoperiodic birds, the relationship between melatonin signal and reproductive timing is more complex than in photoperiodic mammals [16]. In avian gonads and brain, other hormones such as GnIH and its receptor (GnIHR) are also expressed along with expression of melatonin receptor, and for birds it is predicted that melatonin could directly inhibit secretion of GnIH and interfere in gonadal development in long day breeding birds. [17]. Many photoreceptors are located in retina, and some extraretinal photoreceptors are located in the pineal gland [18] and the brain. Deep brain photoreceptors are thought to be responsible for avian photoperiodism [19].

Avian Pineal, Retina and Deep Brain Photoreceptors (DBPs), have many photopigments (viz: Rhodopsin, Transducin, Iodopsin, Melanopsin etc.) involved in diverse function including image formation etc [20]. The outer segments of the rods and cones are having a region filled with membrane-bound discs, which contain proteins bound to the chromophore 11-*cis*-retinal. Isomerization of chromophore occurs when it absorbs any visible light [21]. Rhodopsin is the main pigment of rod cells present in retina. It is a light sensitive protein which can sense a very dim beam of light hence adapted for vision in night or dark. It shows its maximum sensitivity at 500 nm (in the visible green part of the spectrum) [22]. After the absorption of light, rhodopsin gets converted into metarhodopsin II which is an important intermediate for vision. This intermediate causes activation of another phototransduction protein Transducin, which is responsible for starting the impressive signal transduction cascade which results into production of a nerve impulse to the brain [23]. After the perception of visible light, changes take place in molecular arrangement i.e., all 11-*cis*-retinal converts into all-*trans*-retinal. As the changes occur in the conformation of protein, initiation of biochemical reaction

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cascade occurs resulting in the closing of Na⁺ channels in the cell membrane. When the Na⁺ channels are closed, a large potential difference builds up across the plasma membrane and membrane becomes hyperpolarized. Due to this event, an electrical impulse is generated at the synaptic terminal, and visual information is sent to the brain [24]. In view of the above cascade of information in photoperception and, previous studies from our laboratory related to photoperiodism in Japanese quail, present study was designed to study the response of developed gonad of scotorefractory quail to external melatonin in addition to the modulation in the expression pattern of extra retinal photoreceptors.

II. MATERIALS AND METHODS

Sexually immature 3-week-old male Japanese quail (*Coturnixcoturnix japonica*) were purchased from State poultry farm, Chakganjaria, Jahangirabad, Barabanki (U.P) India. Quails were weighed and maintained continuously in short day-length (SD- 8L: 16D).

After 13 weeks of age, when cloacal gland started developing in SD condition, birds were weighed and randomly divided into 2 groups (n=10 in each group) and continued to be maintained under the same photoperiodic condition. Both the groups were provided commercial food and water *ad libitum*. One group served as scotorefractory control (SR) and received normal saline subcutaneously whereas another group received Melatonin (SR+Mel.) (Sigma, St. Louis, USA), at a concentration of 25µg/100g body weight/day (dissolved in few drops of ethanol and diluted with normal saline), subcutaneously for 21 days [12]. Melatonin was injected between 16:30 and 17:00 h. Twenty four hours after the last injection birds were anesthetized and whole body perfusion was done. [25, 26]

The 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, Faculty of Science, Banaras Hindu University.

Tissues Fixation and Measurement

Quails were anesthetized with sodium pentobarbital (3–4 mg/100 g body weight), followed by whole body perfusion. Quail were perfused transcardially with phosphate buffered saline (PBS) followed by 10% neutral buffer formalin. Pineal were dissected out, and then post-fixed in fresh 10% neutral buffer formalin fixative. Twenty four hours after fixation, tissues were dehydrated in a graded series of ethanol, treated with xylene and embedded in paraffin wax. Ten µm thick sections were cut using Leica RM2125 RTS rotatory microtome (Leica Biosystems, Nussloch, Germany) and processed for immunohistochemistry of photoreceptors (Rhodopsin and Transducin).

Immunohistochemistry

Immunohistochemistry of photoreceptors Rhodopsin and Transducin was performed using the antiserum raised in Chicken and Avidin-Biotin complex. 8µ thick pineal sections were deparaffinized, rehydrated in graded series of ethanol and rinsed in PBS and then immersed in 0.3% hydrogen peroxide for 20 min at room temperature. They were incubated in 10% normal horse serum for 1 h and subsequently incubated in chicken Rhodopsin antibody (1:200) and chicken transducin (1:200) separately for overnight at 4 °C. The second incubation with 2^o antibody (Vector Laboratories, Burlingame, CA, USA) was done for 1 hr, after that sections were rinsed with PBS and then incubated in Avidin-Biotin complex for 1 hr. Sections were finally visualized with diaminobenzidine hydrochloride (Lot# SHBG4549V Sigma-Aldrich, St Louis, MO, USA). The slides were washed with PBS, dehydrated through graded series of ethanol, cleaned in xylene and mounted using DPX. Slides were viewed under Carl Zeiss Axioskop 2 Plus microscope.

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III. EXPERIMENTAL RESULTS

The unmeasurable cloacal gland size and small testis of both the groups observed initially remained suppressed in short day condition until 11-12 weeks of age. Thereafter development was initiated which was apparent by 13 weeks of age. Further after 21 daily injections of melatonin i.e. at the age of 16 weeks, while SR quail receiving normal saline exhibits full cloacal gland development with foam production that of SR+ Mel quail exhibited a decrease compare to SR control (Table1 & Fig.1). Study also showed that during scotorefractory(SR) condition, testis were enlarged and in develop condition while, SR+Mel. Quail has smaller/regressed testicular size (Table 1 & Fig.1).

T.S. of testis of SR quail showed enlarged seminiferous tubules with all the stages of spermatogenesis and bunches of spermatozoa lying in the lumen. On the other hand, in melatonin treated scotorefractory quail (SR+Mel) flattened seminiferous tubules were filled with inactive spermatogonial cells only (Fig.2).

Immunohistochemical localization of both rhodopsin and transducin showed that the expression of these photoreceptors and their signal intensity was down regulated in the pineal of SR+Mel quail compared to SR control (Fig.3&4).

Table.1: Effect of melatonin administration on the cloacal gland and testicular volume of Scotorefractory quail.

***p<.001, Significance of difference from SR.

#p<.05, ##p<.001, Significance of difference from 13 weeks old quail(i.e. before starting the treatment)

Short Day (8L:16D)		Cloacal Gland Volume(CGV) (cm ³)	Testicular Volume(TV) (cm ³)
	3 weeks old	unmeasurable	0.079
	13 weeks old	1.42±0.18	1.36±0.17



Short Day (8L:16D)	16 weeks old	SR		SR+Mel	
		CGV	TV	CGV	TV
		2.27±0.17 [#]	2.06±0.64 ^{##}	0.604±0.03 ^{***##}	0.32±0.07 ^{***##}

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Fig. 1

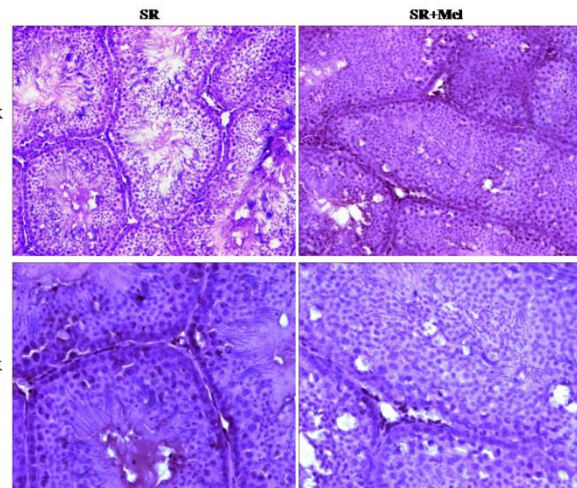


Fig. 2

Fig. 1. In-situ localization of testis in scotorefractory (SR) and scotorefractory+ melatonin (SR+Mel) quail. In scotorefractory quail (left), note well developed cloacal gland with foam along with the enlarged testis. In SR+Mel quail (right) note regressed cloacal gland without foam and small sized testes. **Fig. 2.** Effect of melatonin administration on the histology of testis of SR quail. In scotorefractory quail testis note enlarged seminiferous tubules with active spermatogenesis and lumen filled with spermatozoa. Contrarily, flat seminiferous tubules of SR+Mel quail testis exhibits only inactive spermatogonial cells with some vacuolation/degenerated cells.

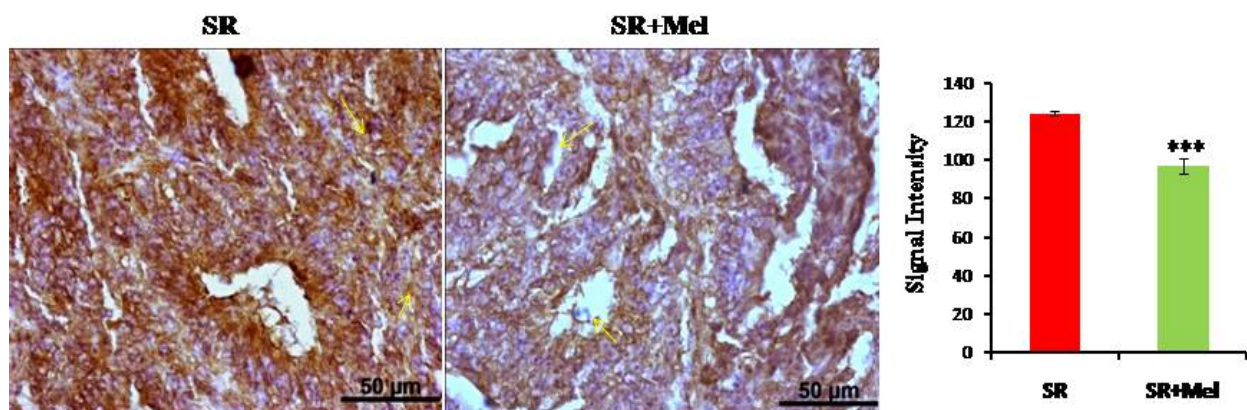


Fig.3. Immunolocalization of Photoreceptors Rhodopsin in the pineal gland of SR and SR+Mel quail. Note decreased immunostaining in SR+Mel quail compared to SR quail.

*** $p < 0.001$, significance of difference from SR.

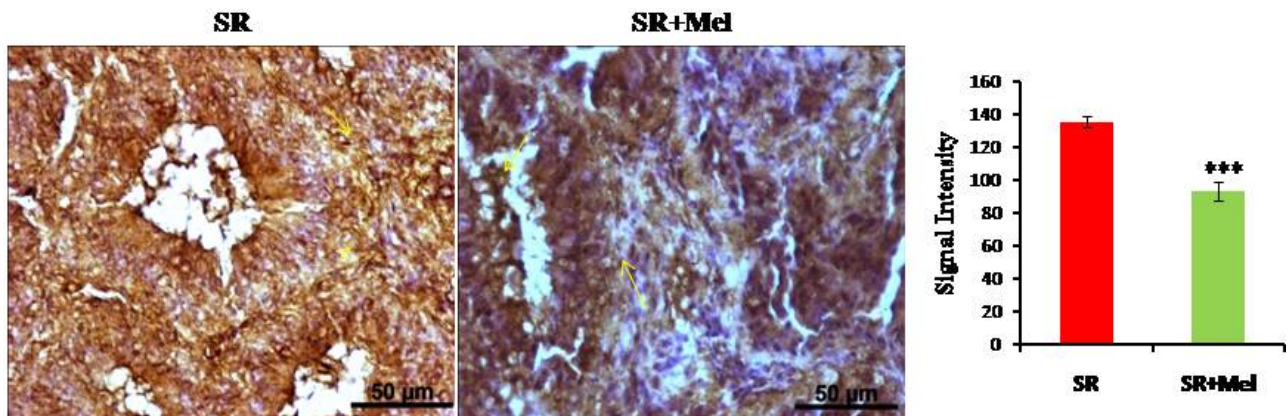


Fig.4. Immunolocalization of photoreceptor Transducin in the pineal gland of SR and SR+Mel quail. Note intense immunoreactivity in SR and low immunoreactivity in SR+Mel quail. *** $p < 0.001$, significance of difference from SR.

IV. DISCUSSION

Our findings clearly indicate that under SD condition, Scotosensitive quail (with suppressed gonad) started testicular as well as cloacal gland development at the age of 11-12 weeks. At the stage of 13 weeks, when reproductive development was quite apparent, Quail were continued for another 3 weeks in the same short day-length condition and saline treated SR quail attained full gonadal development indicating that prolonged exposure to constant short day length condition exhibited scotorefractoriness. On the other hand, when exogenous melatonin was administered in these quail for 21 days in scotorefractory quail, gonadal development was suppressed significantly compared to SR quail. Immunohistochemical observations indicate increased expression of Rhodopsin and transducin photoreceptors in the pineal of scotorefractory quail which decreases significantly in melatonin administered SR quail.

Present study suggests that the exogenous administration of melatonin led to the regression of gonads even in the scotorefractory quail (Table 1 & Fig. 1). It has already been reported from our laboratory, that the increased testis size of photostimulated/photosensitive Japanese quail in long day condition was regressed after melatonin treatment [27]. Interestingly our present findings not only supports the gonado-suppressive effect of melatonin but also indicates that it may even overcome the scotorefractoriness leading to the suppression of reproductive development after prolonged exposure to short day length condition unlike its SR counterpart.

In the present study there are only two groups scotorefractory (SR) and scotorefractory +Melatonin (SR+Mel.). Apparently it is expected to have one control group i.e. the control for short day scotorefractory quail (having large active gonad) where short day quail have not attained scotorefractoriness i.e. still maintaining scotosensitivity (having small inactive gonads). But theoretically it is not possible to have both the scotosensitive and scotorefractory condition simultaneously because under constant short days all the sexually inactive scotosensitive quail in a given group of quail will attain scotorefractoriness (sexually active condition) after 9 to 13 weeks. However, for the control of SR+ Mel we have administered normal saline in SR quail which has served as the control (SR) of SR+Mel group.

In birds, melatonin is reported to act directly on GnIH neurons via its receptor to induce hypothalamic GnIH expression and release which in turn inhibits HPG axis. Thus melatonin may act as the regulator of photo-induced reproductive cycle, i.e., via inhibiting the hypothalamic neuropeptide gonadotropin-inhibitory hormone (GnIH) [28]. In mammals also, experimentally manipulating the duration of melatonin secretion causes the change in reproductive physiology of photoperiodic mammals [29]. Intra testicular injection of melatonin decreased androgen receptor and androgen-binding

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protein expression in mammals [12]. Our finding is also supported by the fact that in most seasonally breeding mammalian and avian species, there is an inverse relationship between pineal and gonadal activity [30, 31]. In photoperiodic species (hamster/ Japanese quail etc), short-day condition suppresses gonadotropin secretion by secreting melatonin. [32] Exogenous administration of melatonin either in the form of food or injected directly in the body causes initiation of downstream regulation of melatonin receptors, and it results into desensitization of receptors by endogenous melatonin level. Photoreceptor cells become unresponsive to utilize advantages of melatonin function during night such as adaptation by retina for vision in dark [33].

Many previous studies reported that an increase in melatonin increases the vulnerability of light-induced cell death of photoreceptors in non-pigmented rats, and also suggested that during the dark period, melatonin administration to pigmented male rats may have a toxic effect on retinal cells. [34]. During dark condition, melatonin shows its importance by altering the sensitivity towards light in central visual system [35,36]. These reports also support our present findings that exogenous treatment of melatonin may increase the cell death of photoreceptors during scotorefractory condition resulting into a decrease in the expression of photoreceptors (Rhodopsin and Transducin) compare to non treated group. Although effects of melatonin on the gonad and Pineal photoreceptors are comparable (both following decreasing trend) it is yet to be investigated whether it is cause and effect relationship between the response of gonads and photoreceptors or both are simultaneous but unrelated effects of melatonin after prolonged exposure to short day-length.

V. CONCLUSION

On the basis of present experimental findings it is concluded that melatonin causes gonado-inhibitory effect not only under long day condition but also during the gonado-stimulatory phase of short day condition i.e., in scotorefractory Japanese quail. Further, photoreceptors during gonado-stimulatory condition in SR quail shows increased immunosignaling compared to gonado-inhibitory condition in melatonin administered SR quail. It is suggested that simultaneous effects of short days on gonads as well as pineal photoreceptors to regulate HPG axis differs with the duration of short day-length inducing either scotosensitive or scotorefractory condition. Further, melatonin also plays a crucial role during photoperiodism with special reference to short day effects i.e. it may override the occurrence of scotorefractoriness in Quail.

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