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Pineal-Thymus Interrelation in Maintenance of T-Cell Dependent Immune Responses in a Tropical Seasonal Breeder *Funambulus pennanti*

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Abstract

Crosstalk between the neuro-endocrine axis and immune cells is documented in many laboratory and clinical studies. The pineal gland and its hormone melatonin play a central role in this network by positively regulating immune cell proliferation and differentiation via influencing the synthesis of immunomodulatory molecules. However, the pineal-thymus interaction in modulating their bi-directional communication remains elusive. In the present study we investigated the effect of pineal-thymus interaction on the structure and functional status of lymphoid tissues (i.e., spleen and lymph nodes) in a tropical seasonal breeder, *F. pennanti*. We observed that pinealectomy severely compromised the immune status of the squirrels. Besides pinealectomy, simultaneous ablation of pineal and thymus gland, further resulted in atrophy of the lymphoid tissues along with reduced total leucocyte and lymphocyte count. Exogenous melatonin administration improved the total leucocyte and lymphocyte count and restored T cell dependent immune responses and lymphoid tissue architecture in pinealectomized (Px) group. Our observations suggest that suppression of endogenous melatonin in Px group decreased the efficiency of the immune system probably by modulating the production of thymic factors, which becomes even severe with simultaneous ablation of the thymus and pineal gland, resulting in declined immune responsiveness. Thus, it can be inferred that the pineal melatonin and its interaction with thymus plays an important role in regulation of immune status of the squirrels.

Keywords: *F. pennanti*, Immunity, Lymphoid Tissues, Pinealectomy, Thymectomy

1. Introduction

Studies suggest the existence of complex neural-endocrine-immune interactions that regulate season dependent changes in the physiology and behaviour of the vertebrates[40]. This neuroendocrine modulation of immunity is of great interest to the ecologists because of its distinctive set of mechanisms that modulate immunocompetence and influence the overall fitness of an organism. In this regard, a major component that has been the subject of intense research is the pineal gland and its hormone melatonin. Melatonin is known to influence humoral and cell-mediated immunity[1,5,9] and significantly enhance the T-cell dependent immune functions under normal and immune compromised conditions[6]. The effect of melatonin on thymus and thymus derived T-cells was evident from the earlier reports[33] demonstrating atrophy of thymo-lymphatic system under continuous light. Reports further revealed of thymic involution under constant photoperiodic exposure and thymic hypertrophy under darkness[25].

The importance of thymus in the development of immune machinery and the relationship between thymus involution and senescence led to the hypothesis that this gland is the "clock for immunity aging"[27]. It is responsible for differentiation and subsequent maturation of thymus-derived lymphocytes (T-cell). The various peptide hormones secreted by thymus help in T-cell differentiation[51]. The hormone thymopoietin (TP) has been shown to regulate thymic differentiation and lymphocyte activation[14,30]. It has been reported that the loss of immune responsiveness in neonatally

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thymectomized rat can be largely ameliorated by either transplantation of thymic tissue or administration of thymic extracts[26,42].

Likewise, pineal extracts and melatonin administration have also been shown to have a stimulatory effect on thymic growth. The administration of pineal extract induced the hyperplasia of the thymus gland while, pinealectomy resulted in thymic atrophy[8,22] and reduced the blood level of the thymic factor. The most investigated pineal factor, melatonin, and thymosin from thymus are concomitantly high during the night time suggesting a possible interrelationship between the two. Melatonin mediated effects on thymic functions are triggered by the synthesis and/or release of opioid peptides from activated CD4+T lymphocytes[35,36] also known as Melatonin Induced Immune Opioids (MIIO).

Reports till date reveal the immunomodulatory role of thymus and pineal melatonin either in clinical studies or in experimental animals. However, only few reports exist elucidating the immunomodulatory role of thymus in relation to the pineal gland in seasonal breeders; therefore, the present study has been planned to get better insight into the interrelationship between thymus and pineal gland in immunomodulation of a seasonally breeding rodent, Indian palm squirrel *F. pennanti*. To investigate the pineal-thymus interrelationship in the maintenance of T-cell dependent immunocompetence, we simultaneously pinealectomized and thymectomized the squirrels and examined the effect of exogeneous melatonin administration on T-cell dependent immune parameters. A synergistic effect of intact thymus and pineal gland was noted on the T-cell mediated immune responses. The observations suggest for the existence of a functional unity and mutual complementarity that help in maintenance of neuroendocrine-immune interactions in seasonally breeding rodents. We pinealectomized and thymectomized squirrels and supplemented melatonin (25μg/squirrel/day) for 40 days after which they were evaluated for various parameters of immunity.

2. Material and Methods

All the experiments were conducted in accordance with Institutional practice and within the framework of experimental animals (Scientific Procedure) Act 2007, of the Committee for the Purpose of Supervision and Control on Experiments on Animals (CPSCEA), Government of India, on animal welfare.

2.1 Animals and Maintenance

Young male squirrels having average weight, 80±10 g and same age (6-8 months old) as determined by body length, incisor length and cranium diameter[22] were collected from the vicinity of Varanasi, India (Lat. 25°18' N: Long. 83°01' E) during the month of September, and were maintained in open-air wire net enclosures, fully exposed to ambient conditions of temperature, day length and humidity. Squirrels were fed with soaked gram seeds (*Cicer arientium*), nuts, seasonal fruits/vegetables and provided with water *ad libitum*. Prior to the commencement of experiments during the reproductively inactive phase (i.e., November-December), the squirrels were acclimatized to the laboratory condition for two weeks.

2.2 Experimental Protocol

For the study, squirrels underwent surgical procedures. The operations of thymectomy (Tmx) and pinealectomy (Px) were performed in the month of November. After 14 days of post-surgery the operated and sham-operated animals were randomized in 7 groups of 12 animals each and melatonin treatment was continued till the end of the experiment. First group comprised of simultaneously sham operated squirrels (n = 6) in which following cranial and thoracic incision, the pineal and thymus were left intact. Second group comprised of Px while, third group consisted of Tmx squirrels. Fourth group comprised of Tmx + Px squirrels while, the fifth group of Px squirrels were treated with melatonin. Sixth group consisted of Tmx+Px squirrels treated with melatonin while, the sham operated squirrels with melatonin treatment formed the seventh experimental group.

Melatonin was administered at the dose of 25 μg/squirrel/day for 40 consecutive days during the evening hours (17:30 to 18:00 h IST). After 24 hours of the last injection, 6 squirrels from each group were subjected to the Delayed Type Hypersensitivity (DTH) response assay to oxazolone while, remaining other 6 animals from each group were subject for TLC, LC, and blastogenic response of lymphoid cells (splenocytes, LN cells) following the method[24]. Spleen, thymus and mesenteric lymph nodes were weighed on a microelectrical balance (Sartorias, Germany). The histo-architecture of the spleen and mesenteric lymph nodes was also observed under higher magnification light microscope (Leitz MPV3) and documented.

2.3 Surgical Procedures

2.3.1 Thymectomy

Thymectomy was performed following the method[8]. Briefly, the squirrels were anesthetised using Nembutal (4mg/0.2ml saline/100g.B.Wt). The thoracic region was shaved and an incision was made on the skin along the midline from the third rib to a point about 2 mm above on the sternum. The wedge of the sternum from sternal notch to the third rib was cut to expose thymus. Thymic capsule was held out by forceps and suction was applied through a fine glass Pasture pipette to gently suck both the thymic lobes. Following removal of the thymic lobes, the wound was stitched and sprayed with erythromycin ethyl sulfonamide.

2.3.2 Pinealectomy

Pinealectomy was performed following the method[20]. Briefly, following anaesthesia, the cranial region was cleaned with 90% alcohol and a small incision was made on the skin. Local anaesthesia was applied on the parietal bone region and a 1.5 mm wide hole was made by a specially designed dental drilling pin on the cranium bone in between the temporalis muscles. The bone piece was lifted away without damaging the dura till the pineal was superficially visible in the confluence sinus. The pineal gland with its stalk was removed by single puncture in the confluence sinus with the help of watchmaker's forceps without disturbing any other part. The bone piece was replaced to its original position and 5.0 mm square piece of 'spongostan' (Ferrosan, Denmark), a coagulation activator strip, was placed over the wound to check the bleeding and to hasten the coagulation process. The skin was stitched by 2-3 discontinuous stitches and sulphadiazine powder was applied over the wound. Animals took 1-2 hours to recover from anaesthesia and the mortality rate following surgical procedures was minimal (1-4%).

3. Results

3.1 Weight of Lymphoid Organs and Lymph Node

Removal of the pineal (Px) significantly ($p<0.01$) reduced the spleen weight however, no significant effect of pinelectomy was noted on Mesenteric Lymph Nodes (MLN) compared with sham control group. Melatonin treatment of pinealectomized squirrels showed significant ($p<0.01$) increase in spleen weight while no effect was observed on MLN weight when compared to Px group. In addition, melatonin treatment of intact squirrels significantly increased ($p<0.01$) the thymus and spleen weights compared with sham-control group while, no effect of melatonin administration was observed on the weight of MLN (Fig. 1a). On the other hand, thymic ablation (Tmx) caused a significant ($p<0.01$) decrease in spleen and MLN weights compared to the sham control group. Simultaneous removal of thymus and pineal gland also significantly ($p<0.01$) decreased the spleen and MLN weights compared to the thymectomized group. Further, melatonin treatment during evening hours to Tmx plus Px squirrels showed a significant ($p<0.01$) increase in the spleen and MLN compared to Tmx plus Px squirrels (Figure 1(a)).

3.2 Total Leucocyte and Lymphocyte Counts (TLC & LC)

Pinealectomy significantly reduced the TLC and LC when compared with sham-control group. Treatment of melatonin to the Px squirrels significantly enhanced the TLC and LC when compared with Px group. However, melatonin treatment to the intact sham control animals significantly enhanced the lymphocyte count, whereas no significant change was noted in TLC, when compared with sham-control group (Figure 2(a)). Thymectomy significantly ($p<0.01$) decreased the total leucocyte and lymphocyte counts when compared with sham–operated control squirrels. Removal of thymus and pineal gland together significantly ($p<0.01$) decreased the TLC and LC when compared with the thymectomized squirrels. However, melatonin administration to Tmx plus Px squirrels significantly ($p<0.01$) increased the total leucocyte and lymphocyte counts when compared with the Tmx plus Px group (Figure 2(a)).

3.3 Blastogenic Response of Thymocytes, Splenocytes, and Mesenteric Lymph Node Cells

Px significantly ($p<0.01$) reduced the blastogenic response of splenocytes (Figure 3(a)), thymocytes (Figure 3(b)) and MLN cells (Figure 3(c)) when compared with sham control group. Melatonin treatment to the intact and Px squirrels significantly ($p<0.01$) enhanced the blastogenic response of splenocytes (Figure 3(a)), thymocytes (Figure

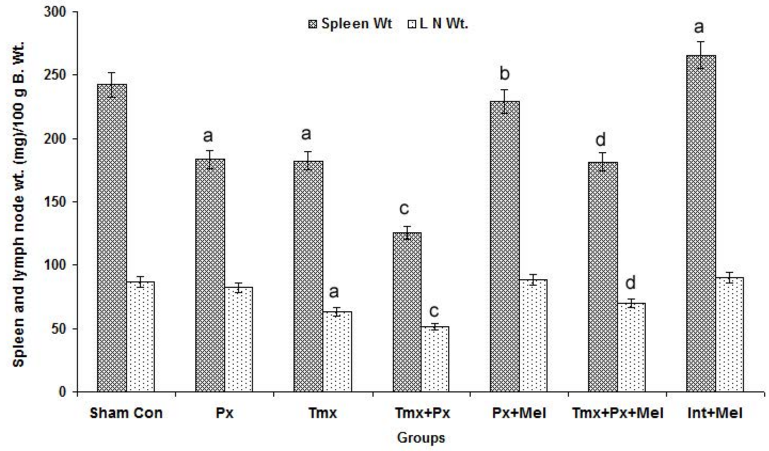

Figure 1. Effect of thymectomy (Tmx), pinealectomy (Px) and melatonin treatment on spleen and mesenteric lymph node weight in seasonally breeding rodent, Indian plam squirrel *F. pennanti* during inactive phase (Nov-Dec). Histogram represents mean ±SE (n=6). Significance of difference p<0.01; a = Sham Con. vs. Px; Int+Mel; Tmx; b = Px vs. Px vs. Px+Mel; c = Tmx vs. Tmx+Px; d = Tmx+Px vs. TmxPx+Mel.

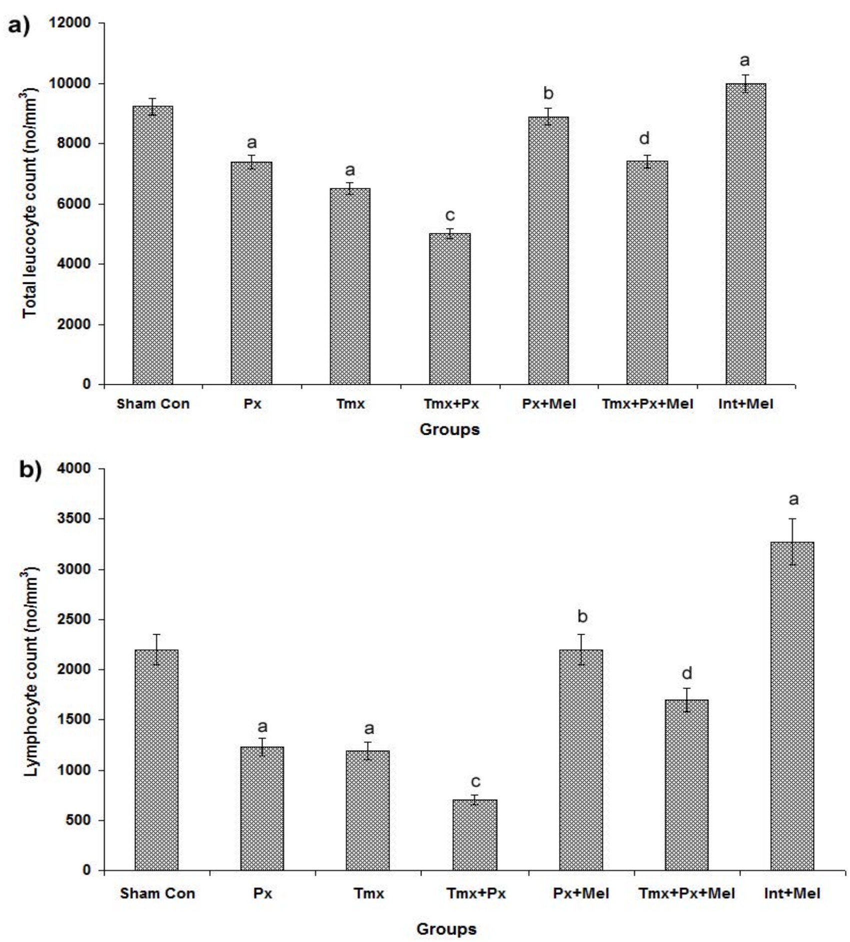

Figure 2. Effect of thymectomy (Tmx), pinealectomy (Px) and melatonin treatment on. **(a)** Total leukocyte. **(b)** Lymphocyte count in seasonally breeding rodent, Indian plam squirrel *F. pennanti* during inactive phase (Nov-Dec). Histogram represents mean ±SE (n=6). Significance of difference p<0.01; a= Sham Con. vs. Px; Int+Mel; Tmx; b= Px vs. Px vs. Px+Mel; c= Tmx vs. Tmx+Px; d= Tmx+Px vs. TmxPx+Mel.

3(b)) and MLN cells (Figure 3(c)) compared to the Px squirrels.

A significant (p<0.01) reduction in the blastogenic response of splenocytes (Figure 3(a)) and MLN cells (Figure 3(c)) was observed following Tmx. Simultaneous Tmx and Px in squirrels showed further significant (p<0.01) depression in the blastogenic response of splenocytes (Figure 3(a)) and MLN cells (Figure 3(c)) when compared with Tmx group. Melatonin treatment of Tmx + Px squirrels however, improved the blastogenic response of splenocytes (Figure 3(a)) and MLN cells (Figure 3(c)) compared to simultaneous Tmx and Px group of squirrels.

3.4 Delayed Type Hypersensitivity (DTH) Response to Oxazolone Treatment

Px squirrels showed a significant (p<0.01) decrease in the DTH response when compared with sham control squirrels. Melatonin treatment of Px as well as intact squirrels however, significantly (p<0.01) increased the DTH response compared to Px and sham control squirrels respectively. Thymic ablation caused a significant (p<0.01) decrease in DTH response to oxazolone when compared with sham-control group. Simultaneous removal of thymus and pineal gland showed further significant (p<0.01) decrease in DTH response compared to the Tmx squirrels. Melatonin treatment of Tmx and Px squirrels significantly (p<0.01) enhanced the DTH response to oxazolone when compared with simultaneous Tmx plus Px squirrels (Figure 4).

3.5 Plasma Melatonin Concentration

Pinealectomy resulted in significant decrease (p<0.01) in the plasma melatonin concentration compared to the sham-control animals. Exogenous melatonin treatment of Px squirrels significantly (p<0.01) increased the plasma melatonin concentration compared to Px group. Melatonin treatment of intact squirrels further significantly (p<0.01) increased the melatonin concentration compared to sham-control group.

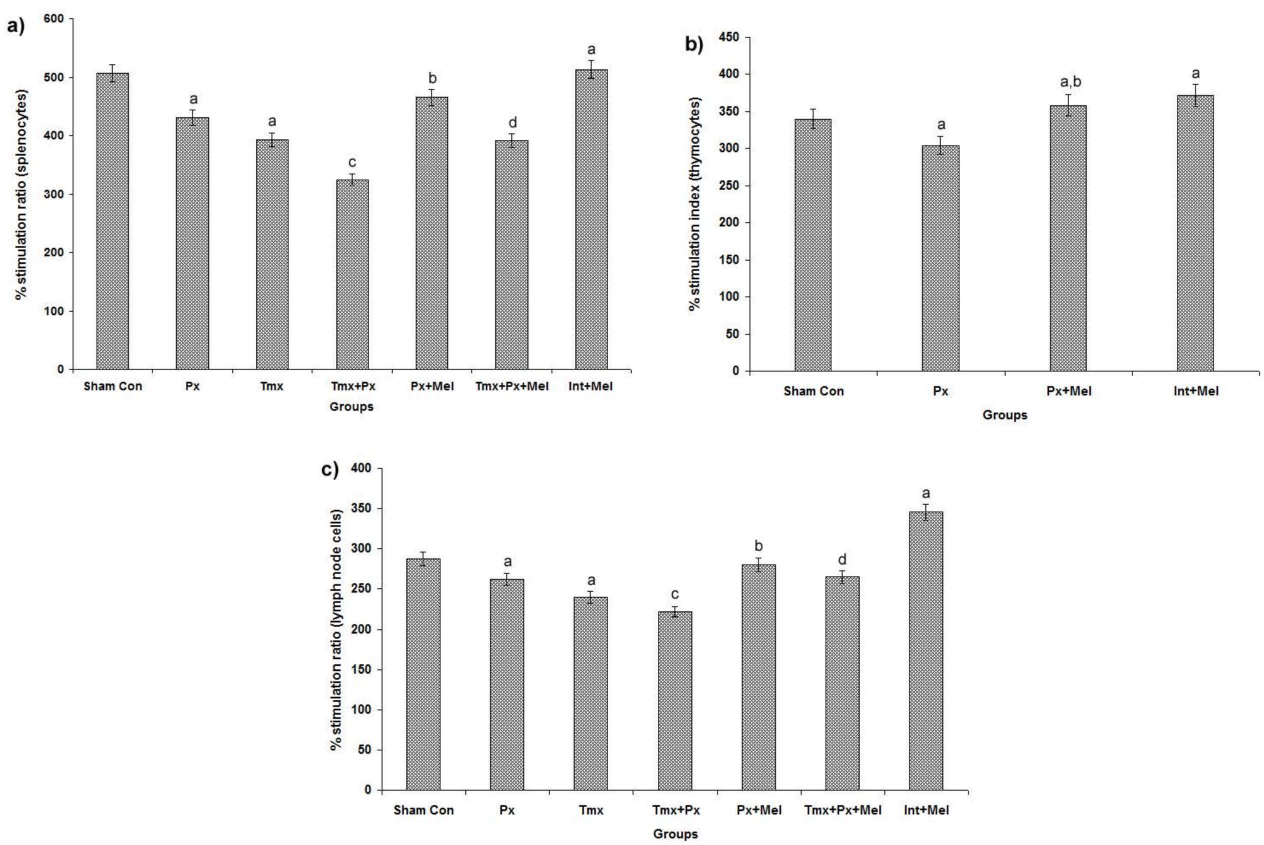

Figure 3. Effect of thymectomy (Tmx), pinealectomy (Px) and melatonin treatment on blastogenic response of. **(a)** Splenocytes. **(b)** Thymocytes. **(c)** Mesenteric lymph node cells in seasonally breeding rodent, Indian plam squirrel *F. pennanti* during inactive phase (Nov-Dec). Histogram represents mean ±SE (n = 6). Significance of difference p<0.01; a = Sham Con. vs. Px; Int+Mel; Tmx; b = Px vs. Px vs. Px+Mel; c = Tmx vs. Tmx+Px; d = Tmx+Px vs. TmxPx+Mel.

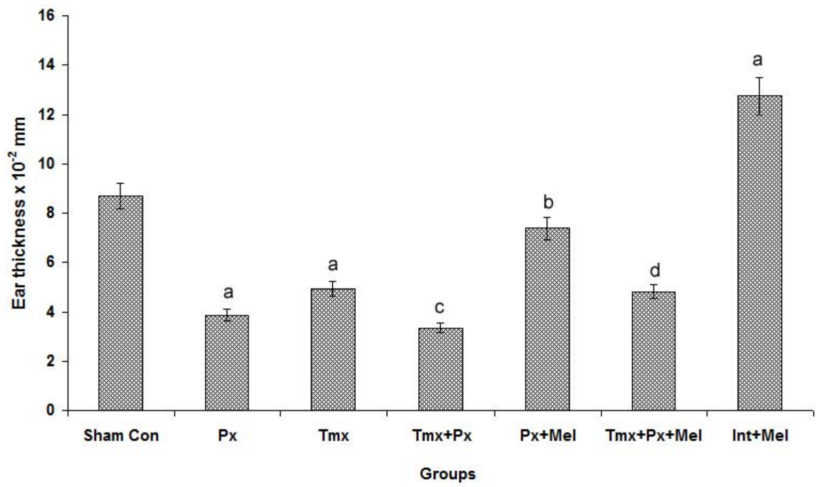

Figure 4. Effect of thymectomy (Tmx), pinealectomy (Px) and melatonin treatment on delayed type hypersensitivity response to oxazolone application in seasonally breeding rodent, Indian plam squirrel *F. pennanti* during inactive phase (Nov-Dec). Histogram represents mean $\pm$SE (n = 6). Significance of difference p<0.01; a = Sham Con. vs. Px; Int+Mel; Tmx; b = Px vs. Px vs. Px+Mel; c = Tmx vs. Tmx+Px; d = Tmx+Px vs. TmxPx+Mel.

A non-significant decrease in the plasma level of melatonin was observed following thymectomy while, simultaneous Tmx plus Px significantly (p<0.01) reduced the plasma melatonin concentration compared to Tmx squirrels. Melatonin treatment of Tmx and Px squirrels significantly (p<0.01) enhanced the plasma melatonin concentration when compared with the simultaneous Tmx plus Px squirrels (Figure 5).

3.6 Histological Changes in the Spleen of Tmx Squirrels

The splenic white pulp contains thymus dependent and independent zones. The periarterial zone of spleen is thymus dependent while, the external lymphoid sheath and follicles are thymus independent zones. Removal of the thymus of adult male squirrel caused depletion of lymphocytes whereas sham–control squirrel showed

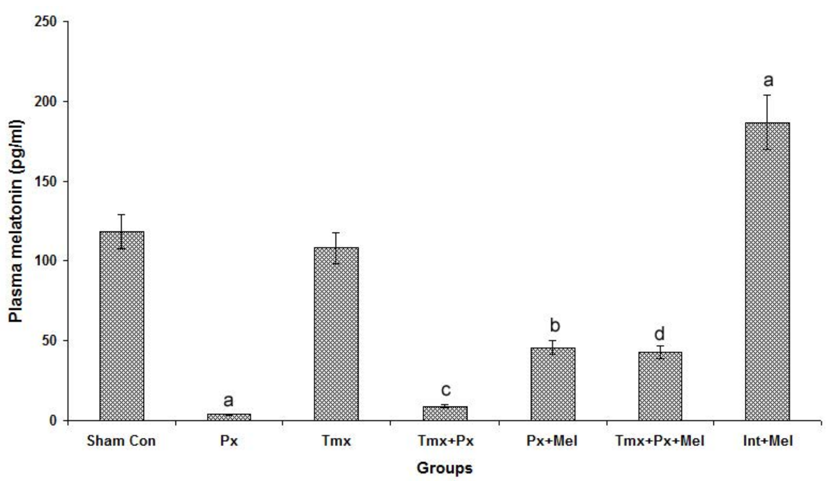

Figure 5. Effect of thymectomy (Tmx), pinealectomy (Px) and melatonin treatment on plasma melatonin concentration in seasonally breeding rodent, Indian plam squirrel *F. pennanti* during inactive phase (Nov-Dec). Histogram represents mean $\pm$SE (n = 6). Significance of difference p<0.01; a = Sham Con. vs. Px; Int+Mel; Tmx; b = Px vs. Px vs. Px+Mel; c = Tmx vs. Tmx+Px; d = Tmx+Px vs. TmxPx+Mel.

densely populated lymphocytes in the periarterial zone of the splenic white pulp. However, Tmx plus Px squirrels presented further depletion of lymphocytes in the periarterial region of the splenic white pulp. However, melatonin treated Tmx plus Px squirrels showed slightly dense population of lymphocytes in the periarterial zone of the splenic white pulp when compared with the Tmx plus Px squirrel (Figure 6).

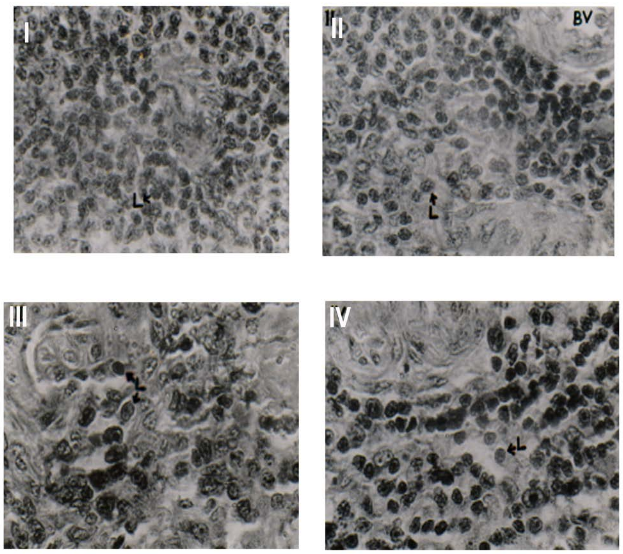

Figure 6. Histology of spleen of (I) Sham control squirrel showing lymphoidal cells (L) in periarterial region of white pulp (II) thymectomized squirrel showing depletion of lymphoidal cells (L) in periaterial region and blood vessels (BV) (III) simultaneously thymectomized plus pinealectomized squirrel showing severe depletion of lymphoidal cells (L) in periarterial region and (IV) melatonin treated thymectomized plus pinealectomized squirrel showing restoration of lymphoidal cells (L) in periarterial region during reproductive inactive phase (Nov-Dec) (x365).

3.7 Histological Changes in the Mesenteric Lymph Nodes of Tmx Squirrels

The mesenteric LN in sham-control squirrels presented distinct cortical and medullary regions. Cortical region had lymph follicles. The primary follicles lack the germinal center whereas, secondary lymphoid follicles show prominent germinal center mainly consisting of B-lymphocytes. The paracortical region was populated mainly by T-lymphocytes. Thymectomy caused severe depletion of lymphocytes in the deep cortex (paracortical region), a thymus dependent zone. Tmx plus Px squirrels further showed a reduction in the volume of deep cortex with decreased lymphocyte count. However, melatonin treatment of Tmx plus Px squirrels showed greater density in the paracortical area of the lymph nodes (Figure 7).

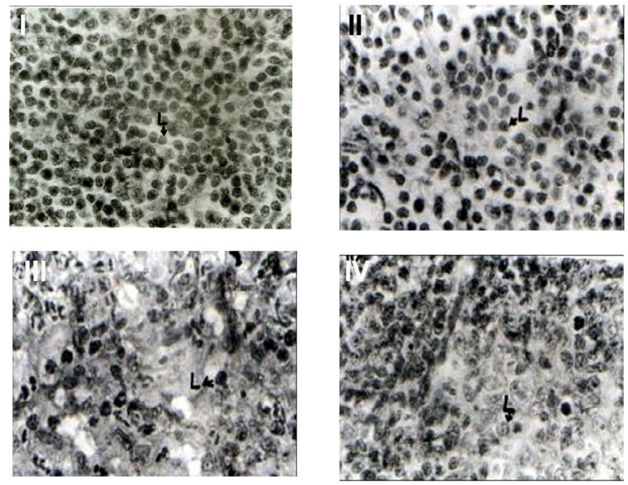

Figure 7. Histology of mesenteric lymph nodes of (I) Sham control squirrel showing paracortical region with lymphoidal cells (L) (II) thymectomized squirrel showing depletion of lymphoidal cells (L) in the paracotical region (III) simultaneously thymectomized plus pinealectomized squirrel showing severe severe depletion of lymphoidal cells (L) in the paracortical region and (IV) melatonin treated thymectomized plus pinealectomized squirrel showing restoration of lymphoidal cells (L) in the paracortical region during reproductive inactive phase (Nov-Dec) (x365).

4. Discussion

The thymus-lymph node-spleen system seems to have a decisive role in T-cell dependent immune responses. The present study on thymus-pineal inter-relationship on maintenance of immunocompetence in a seasonally breeding rodent *F. pennanti* shows that simultaneous Px and Tmx results in drastic reduction in the weight of spleen thymus and lymph node in T-cell dependent immune responses. Melatonin administration significantly improves the cellular architecture of the lymphoid tissues and counteracts pinealectomy and thymectomy induced immune suppression.

Thymus as a gland is capable of synthesizing a variety of factors/hormones that are reported to show immune regulatory properties[50]. Atrophic changes in the thymus has been associated with a decrease in the number of thymic lymphocytes[39] and other cell types that are otherwise known to synthesize thymic factors necessary for development of adequate response against immune challenges[11]. Moreover, thymus is responsible for the differentiation and subsequent maturation of thymus derived T-cells[41,51] suggesting the importance of thymus in providing immunity to the organism. The functions of thymus gland have been suggested to be modulated by neuroendocrine circuits[47]. Among the many neuroendocrine mediators, the pineal indoleamine melatonin is known to exert profound influence on thymocyte proliferation[18]. Melatonin promotes thymic rejuvenation there by redressing the peripheral components of the immune repertoire under various patho-physiological conditions[12,48]. The present study addresses the interactions between pineal and thymus glands in the maintenance of immune status of *F. Pennanti.* Being a seasonal breeder, *F. Pennanti* exhibits a seasonal cycle of thymic regression and recrudescence which is essentially controlled by the pineal gland and its hormone melatonin[22,23]. We further looked into the effect of simultaneous thymectomy and pinealectomy on the T-cell specific immune responses in this squirrel.

Thymectomy of young adult squirrels results in reduced secondary lymphoid organ weight (spleen and mesenteric lymph nodes) and a suppression of the general immune parameters *viz.* circulating total leukocyte and lymphocyte counts. T-cell specific immune responses like lymphocyte proliferation as well as DTH response also show a significant suppression in the thymectomized squirrels. These observation of ours clearly support an earlier report of immune suppression by the removal of thymus in the rat[46]. Thymic hormones have been reported to reconstitute and augment the response in immune-deficient situations[10]. By inducing surface markers to allow maturation and differentiation of T-cells, thus regulating immune responses[11].

The mechanism of thymectomy induced immune-suppression is not yet clear but, one of the possible reasons might be its lympho-proliferative property. Our experiments clearly suggest an important role for melatonin in immune responses as Px plus Tmx animals show a significantly greater immune suppression over that of Tmx animal. The importance of melatonin in immune-stimulatory functions F. pennanti is very evident from the recorded significant decrement in organ weights, circulating cell counts, cell proliferative responses to the mitogen Con A and DTH responses to oxazolone in Tmx plus Px rodents, and the marked restorative effects exogenous melatonin in these animals. In contrast[46], failed to see any influence of melatonin in immune-competence in rats. This apparent contradiction seems to be due to shorter duration of their study. As against this[15,37], clearly demonstrated the role of pineal and melatonin enhanced humoral and cell mediated immunity in small mammals. Moreover, impaired T-helper cell activity in immune-compromised mice was shown to be restored following melatonin administration[4,6].

Melatonin has been shown to possess a stimulatory effect on mouse CFU-GM[28,49]. Moreover, melatonin has been shown to protect the blood-forming system of mice transplanted with Lewis lung carcinoma and treated with cyclophosphamide or etoposide[32]. Melatonin has been consistently shown to restore the rhythm of CFU-GM and increase the number of Granulocyte-Macrophage Colony Forming Units (CFU-GM) when added to directly bone marrow cultures though only in presence of suboptimal concentrations of CSF[31]. These reports lend support to our present observation on the favourable role of melatonin in the regulation of leucocyte and lymphocyte numbers. Antigen presentation by splenic macrophages to T cells is also enhanced by melatonin; this enhancement was coincident with an increase in Major Histocompatibility (MHC) class II molecules as well as IL-1[43]. However, till date, it is not clear as to how melatonin treatment modulates the immune responses in thymectomized squirrels. It can be suggested that the possible site of action of melatonin might be the lymphoid cells. Since, melatonin receptors have been isolated on circulating lymphocytes in human and other rodents[3,44,45] suggesting a direct effect of melatonin on the regulation of the immune system[15]. On the other hand, the thymic hormone thymopoietin helps regulate thymic differentiation and lymphocyte activation[14] providing support to our data on immune parameters in thymectomized, thymectomized plus pinealectomized and melatonin administered animals. The present study also shows that the immunocompetence of Px animals is better than that of Thx animals indicating the primary role of thymus in this. However, melatonin seems to be potent in restoring the compromised immunocompetence in Thx plus Px animals suggesting the supportive role of pineal in immune functions in mammals.

5. Conclusion

It can be concluded from the present study that a bi-directional communication between pineal and thymus is of importance in immune cell differentiation and immune competency in *F. pennanti*. Moreover, the pineal-thymus axis can be an important target for the development of therapeutic strategies for combating immune-compromising conditions associated with drug induced allergies and inflammations.

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Author's Contribution: All authors have contributed equally.

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