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Histomorphometric changes in the ovaries of thymectomized guinea pigs

Murali Punniakotti[1,*], V.Nithya[2], James Villanueva[1]

[1] Bridgetown Internal university, School of Medicine, Barbados
[2] SRM Medical College Hospital and Research Centre, Faculty of Medicine and Health Sciences, SRM Institute of Science and Technology, SRM Nagar, Kattankulathur, 603203, Kanchipuram, Chennai, Tamil Nādu, India

Abstract

Hypothalamic-pituitary-gonadal axis function is necessary for maintaining proper female reproductive cycle. This study aimed to evaluate the ovarian histomorphometric and histoarchitectural changes in neonatal, prepubertal and pubertal thymectomized female guinea pigs. A total of 30 female guinea pigs, sham-operated (n-5) and thymectomized (n-5) were studied in each group. The diameter and number of ovarian follicles among the thymectomized and sham operated female guinea pigs during estrus phase of estrous cycle was compared. Gonadal and accessory reproductive organs weights and microscopic features were studied in the sham operated guinea pigs and thymectomized. There were statistically significant changes in the number and diameter of follicles in the ovary in neonatal thymectomized female guinea pigs, but no significant changes were observed in prepubertal and pubertal female guinea pigs. Neonatal thymectomized female guinea pigs showed significant changes in their weight as well as changes in the microscopic features including reduced thickness of myometrium of uterus and less mucosal folding in the fallopian tube compared to the sham-operated group. But prepubertal and pubertal thymectomy did not affect the weight and microscopic features of gonads and accessory reproductive organs. Depending on the time of thymectomy, these results indicate morphological changes in the ovaries after thymectomy in females.

Keywords

thymus; ovary; estrous cycle; morphology; histoarchitecture; thymectomy.

Introduction

Thymectomy performed in Tx3 inbred mice showed increased frequency of independent autoimmune diseases that target the ovaries, stomach, thyroid, lacrimal gland, prostate, and testis, and in the production of the respective organ-specific auto antibodies and pathogenic T cell responses (Kojima et al., 1976; Taguchi et al., 1981; Nishizuka et al., 1980; A. Kojima et al., 1985; Tung et al., 1987; Kosiewicz MM et al., 1990). The production of circulating auto antibodies against the ooplasm of oocytesin in Tx3 mice was an important autoimmune etiology for ovarian failure. The antioocyte antibodies, lymphocytes infiltration was detectable until 25 days of age. The disruption of the hypothalamic-pituitary–ovarian–thymic axis is caused by thymectomy, which resulted in autoimmune ovarian dysgenesis (Plant TM, 2015). The role of thymus during early life is essential for the normal develop-

* Corresponding author. E-mail: pmurali.pt@gmail.com

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ment of immune system and as well as maturation of the hypothalamic – pituitary-gonadal axis. The Hypothalamic- Pituitary- Gonadal axis begins in the hypothalamus which secretes gonadotropin releasing hormone (GRH) that acts on the anterior pituitary and stimulates the synthesis and secretion of follicular stimulating hormone (FSH) and luteinizing hormone (LH). Follicular maturation and estrogen synthesis are promoted by FSH and LH which act on the ovarian follicle to promote ovulation and corpus luteum development (Calzolari. A, 1898). There has been a heightened enthusiasm in the previous decades on the connection between thymic and reproductive gonads. Studies show thymic enlargement after gonadectomy on rabbits and guinea pigs in both sexes (Dougherty SM, 2006). Androgen and estrogen induce the destruction of the thymocytes when administered to thymic bearing animals (Nishizuka Y, 1969). Nishizuka and Sakakura, 1969, reported a correlation between thymus and reproduction after they found that neonatal thymectomy in mice 72 hours after birth caused ovarian dysgenesis and created wasting sickness that occurred in 2- to 3-month-old female mice. These defects in female guinea pigs were due to immune suppression affecting the reproductive system. Its effects on the ovaries of thymectomized mice were extremely small, absence of follicle and corpus lutea, but no significant effect was seen in male mice. Besedovsky and Sorkin, 1974, and Listern – Moore and Norbaek Sorensen, 1976, noticed the development of ovarian formative disturbance after neonatal thymectomy in mice, Flanagan, 1966, was the first to observe decreased fertility, delayed vaginal opening and follicular atresia in athymic female nude mice. Reber et al., 1981a, reported congenitally athymic mice showed decreased level of pituitary gonadotrophins as well as circulating gonadotrophins in both sexes (i.e., puberty) and decreased level of estrogen in adult athymic mice showed. Previous results that showed enlargement of thymus while other studies showed atrophy of the thymus upon hypophysectomy gland were contradictory (Farookhie R, 1988). So far functional correlation between the thymic hormones and gonadal hormones has been sufficiently studied. However, histomorphometric and histoarchitecture changes of the gonads in female after thymectomy have not been explored.

Materials and Methods

Experimental Animal

Experiments were conducted in the neonatal (1st week animals, Average weight 90 to 100gm), pre-pubertal (5th week animals, Average weight 200 to 250gm) and pubertal (7th week animals, Average weight 280 to 300gm) female guinea pigs. A total of 30 female guinea pigs were studied. The animals were procured from the Institute of Experimental Animals in Karnataka. All guinea pigs were housed at the SRM Central Animal House; room temperature was maintained at 25±2^{o}C and adequate dark and light cycle for 12 h/ day. All the experimental protocols and procedure were approved by the Institutional Animal Ethical Committee of SRM Institute of Science and Technology Tamilnadu, in accordance with the guidelines of CPCSEA (16111/835re-S-04/IAEC2016).

Experimental design

The present study used 30 female guinea pigs divided into 2 main groups. In Group I (n-15), female guinea pigs underwent a surgical procedure without extirpation of thymus gland which was considered as sham operated group. In Group II (n-15), female guinea pigs with surgically removed thymus gland was considered as thymectomy group. In both groups 5 guinea pigs were each assigned to neonatal, prepubertal, and pubertal subgroups.

Transcervical Thymectomy

Thymectomy was performed according to the procedure described by Adams, 1977, as shown on Figure 1. In the Sham-operated group of guinea pigs (Group I), all the steps in the surgical procedures were followed except for the removal of the thymus gland. Post-surgical procedures included recovery under heat lamp, a special cage with soft corn cob bedding material with adherence to standardized aseptic methods.

Assessment of estrous cycle

The estrous cycle of guinea pigs consists of 4 stages: Proestrus, Estrus, Metestrus and Diestrus with a mean length of ovarian cycle of 16-18 days. Vaginal smear findings were used to assess the stages of estrous cycle. A moist cotton bud with normal saline was slowly inserted into the vagina at a depth of approximately 8-10 mm. Swab was gently turned (clock and counter-clockwise) against the vaginal wall and then removed. Immediately after withdrawal, the tip of the cotton bud was rolled along the whole length of a glass microscopic slide and immediately fixed in absolute alcohol for staining purpose (Jadarmkunti UC, 1999). The slide was then air

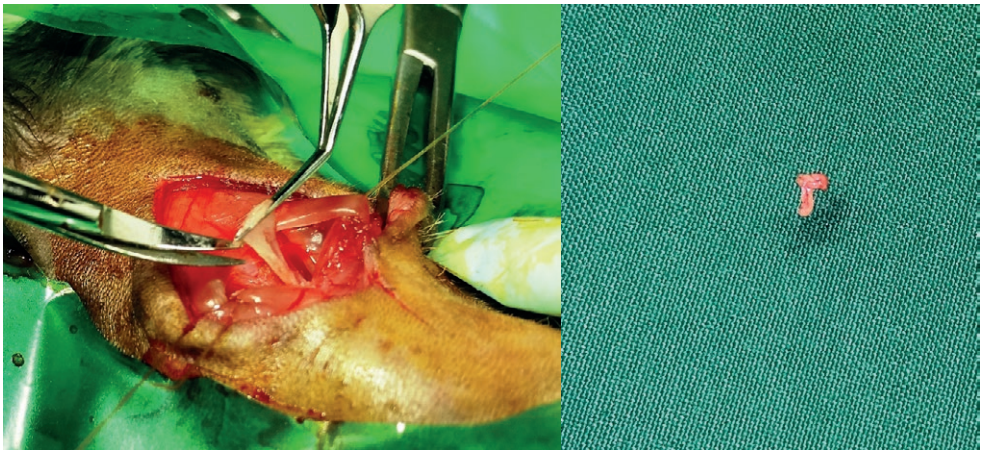

Figure 1. Surgical removal of the thymus gland in guinea pigs by transcervical approach.

dried and stained with Papinocolaou staining (PAP). In the proestrous phase, smears-stained nucleated cells with an intense pink colored cytoplasm; aggregated nucleated cells stained blue to purple with granulated nuclei. In the estrus phase –squamous epithelial cells were cornified. These cells were arranged in sheets, clustered and were uniformly stained orange and pink without nucleus. In the metestrus phase, the nucleated cells-stained pale blue and dark blue with polymorphic nuclei. In the diestrus phase, densely packed leukocytes in groups with presence of nucleated cells (Lilley KG, 1997).

Morphometric assessment of ovarian follicles

Both sham operated and thymectomized guinea pigs in each group were sacrificed in the estrus phase of the first estrous cycle after the procedure. The guinea pigs were euthanized using carbon dioxide for the removal and examination of their ovaries and accessory reproductive organs. Ovaries and accessory reproductive organs were fixed in Bouin's aqueous fixative. In the case of the ovaries every 20^{th} section was mounted and stained with hematoxylin-eosin and evaluated by confocal fluorescence microscopy at a 20X magnification. In the present study, follicles were categorized according to the layers of the granulosa cells (GC) surrounding oocyte; primary follicle (oocyte surrounded by a single layer of cuboidal GC), small secondary follicle (if at least oocyte surrounded by a two layers of GC), medium secondary follicle (oocyte surrounded by a three layers of GC), large secondary follicle (oocyte surrounded by four layers of GC without antrum) and antral follicle (follicle with the presence of an antrum) Karakaş, 2010. The mean diameter of the follicles in the ovary was measured under 40X magnification (Figure 2). The accurate calculation of diameter was taken by using the integrated measuring tools in the ZEN 2010 software Germany after calibration with a stage micrometer [LSM 700 Laser Scanning Confocal Microscope] Griffin J, 2006.

Statistical analysis

Two individual groups were compared and assessed by Student's t-test. Data values were expressed as mean and standard error of means (SEM). Values were considered statistically significant when $p< 0.05$.

Results

Effect of thymectomy on histomorphometry and histoarchitecture of ovarian follicles of thymectomized guinea pigs were compared with sham-operated in neonatal, prepubertal and pubertal female guinea pigs.

In Group I (Sham-operated), the mean values of follicle diameter expressed in mean ± SEM (μm) in the ovary were determined. Table 1 shows the statistical analysis of diameters of primordial follicles in neonatal, prepubertal and pubertal were 80.2±1.80; 87.5±0.97 and 86.8±0.82, respectively. The small secondary follicle diameter in neonatal, prepubertal and pubertal were 181.8±1.42, 189.3±1.52 and 191.8±1.76, respectively. The medium secondary follicle diameter in neonatal, prepubertal and

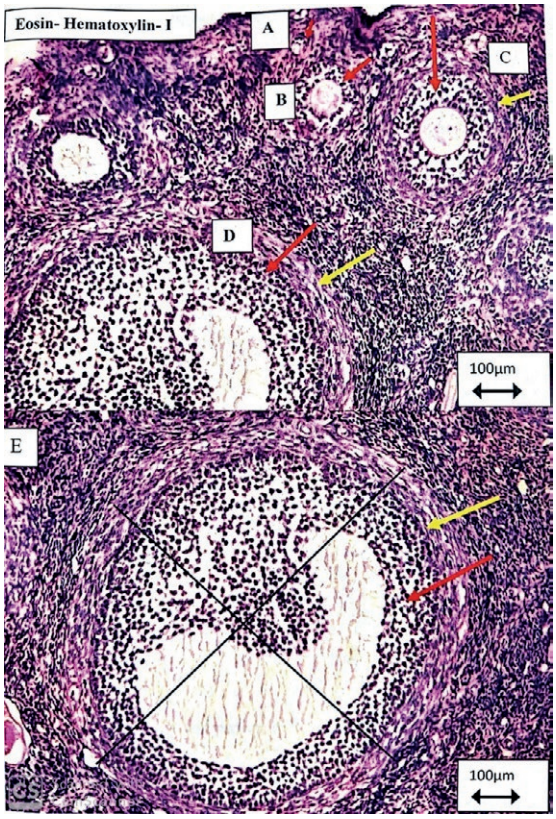

Figure 2. Hematoxylin-eosin staining of guinea pig ovary shows ovarian follicles at different stages. Yellow arrow – theca layer Red Arrow - Granular cells A. primary follicle with single layer of granular cells B. Small secondary follicle with two layers of granular cells C. Medium secondary follicle more than four layers of granular cells with formation of theca layer. D. Antral follicle E. Oocyte is surrounded by granular cells with theca layer and continuous line for measurement of diameter.

pubertal were 202±2.14, 213±1.16 and 223.5±2.4, respectively. The large secondary follicle diameter in neonatal, prepubertal and pubertal were 552±2.69; 621±1.74 and 628±1.25, respectively and the antral follicle diameter in neonatal, prepubertal and pubertal were 915±1.20, 934±2.60, 941±1.94, respectively.

In Group II (thymectomized), the same measurements were done as in Group I. The values are summarized in Table 1 that showed primordial follicle diameter in neonatal, prepubertal and pubertal were 62.5±0.8, 82.5±1.0 and 86.5±1.1, respectively. Small secondary follicle diameter in neonatal, prepubertal and pubertal were 141.7±1.0, 185±1.38 and 195±1.5, respectively. Medium secondary follicle diameter in neonatal, prepubertal and pubertal were 168±1.83, 195.3±1.53, 204.3±1.39, respectively. Large secondary follicle diameter in neonatal, prepubertal and pubertal were 364±1.67, 595±1.42 and 612±1.36, respectively and antral follicle diameter in neonatal, prepubertal and pubertal were 655±1.37, 893±1.16 and 930±1.71, respectively.

Table 1. Mean ± SEM for Different Follicle Diameter of Thymectomized and Sham-operated Group (*p < 0.05 significant).

Age	Primordial (μm)		Small secondary(μm)		Medium secondary(μm)		Large secondary(μm)		Antral(μm)	
	Shax	Tx	Shax	Tx	Shax	Tx	Shax	Tx	Shax	Tx
1st week	80.2±1.80	62.5±0.8	181.8±1.42	141.7±1.0	202±2.14	168±1.83	552±2.69	364±1.67	915±1.20	655±1.37
5th week	87.5±0.97	82.5±1.0	189.3±1.52	185±1.38	213±1.16	195.3±1.53	621±1.74	595±1.42	934±2.60	893±1.16
7th week	86.8±0.82	86.5±1.1	191.8±1.76	195±1.51	223.5±2.41	204.3±1.39	628±1.25	612±1.36	941±1.94	930±1.71
P value	1st week-0.001*		1st week-0.044*		1st week-0.003*		1st week-0.001*		1st week-0.041*	
	5th week -0.091		5th week –0.131		5th week -0.376		5th week –0.466		5th week –0.437	
	7th week-0.346		7th week-0.177		7th week-0.406		7th week-0.532		7th week-0.210	

Table 2 Number of Ovarian Follicles in Neonatal, Pre-pubertal and Pubertal in Thymectomized Guinea Pigs When Compared with Sham-operated Guinea Pigs. (*p< 0.05 significant).

Group	1st week		5th week		7th week	
	Shax	Tx	Shax	Tx	Shax	Tx
Primordial	605	395*	554	520	420	412
Small secondary	354	250*	329	330	295	296
Medium secondary	613	400*	552	510	432	421
Large secondary	400	258*	382	372	350	348
Antral follicle	432	291*	392	388	366	358

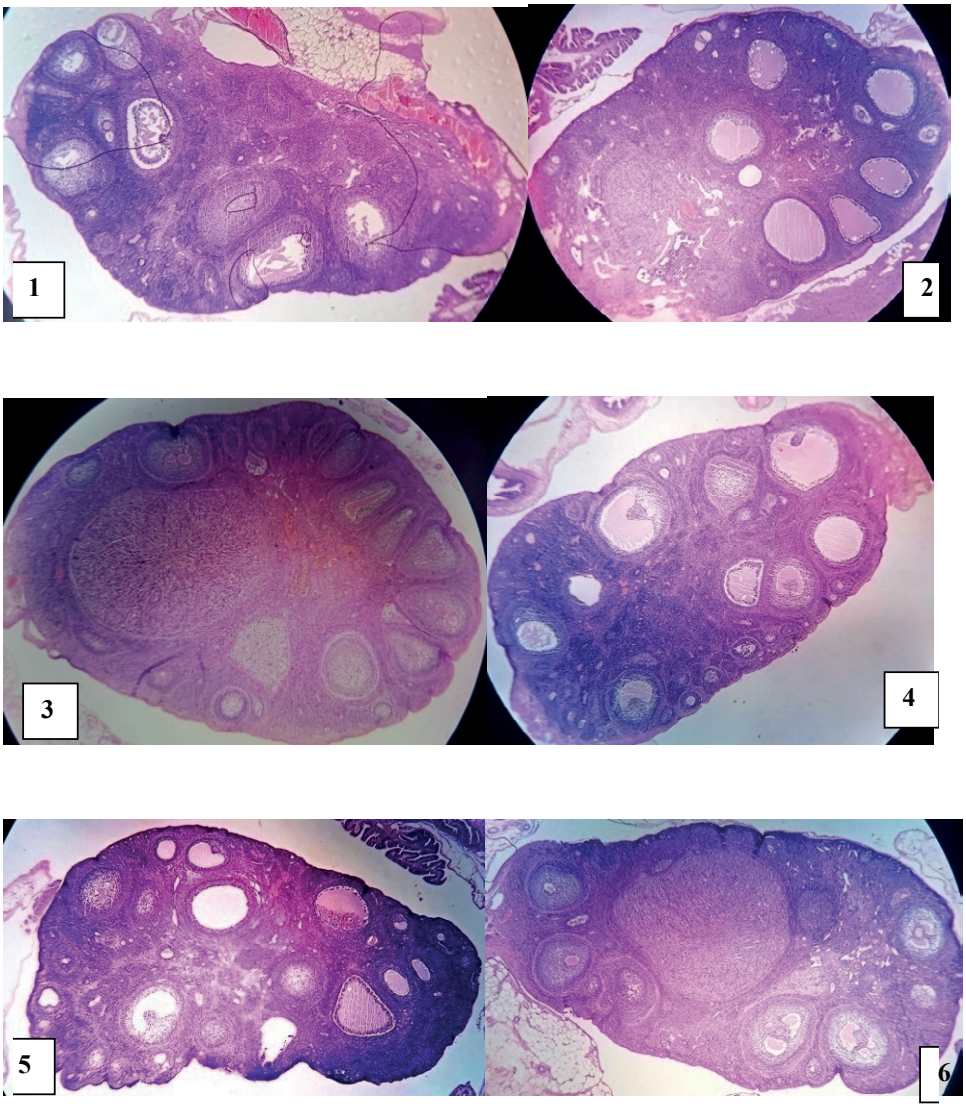

Figure 3. Female guinea pigs ovary Comparison between sham-operated and thymectamized.1. Neonatal Sham-operated. 2. Neonatal Thymectomized 3. Pre-pubertal Sham operated 4.pre-pubertal Thymectomized 5. Pubertal sham-operated 6. Pubertal Thymectomized.

The morphometric analysis showed a significantly higher average range of mean value of diameter of all types of follicles in the ovary in neonatal thymectomized animals when compared to the sham operated animals. In contrast, there was no significant difference in the mean diameter of different stages of ovarian follicles in the ovary in pre-pubertal and pubertal age thymectomized animals when compared to the sham-operated animals.

Statistically there was a significant difference in all kinds of follicles in the ovary between sham-operated and experimental animals in neonatal group animals with a $p < 0.001$, but there were no significant changes of different ovarian follicles ovary in pre-pubertal and pubertal age thymectomized animals when compared to the sham-operated animals (Table 2, Figure 3). Female neonatal thymectomized guinea pig results showed significant changes in their weight as well as changes in the microscopic features including reduced thickness of myometrium of the uterus and less mucosal folding in the fallopian tube compared to the sham-operated group. There were no significant changes in the accessory reproductive organs in pre-pubertal and pubertal thymectomized guinea pigs compared with the sham operated guinea pigs.

Discussion

To our knowledge, this is the first study to examine the effect of thymectomy on morphometric and histoarchitectural changes of the gonads during the estrus phase of the estrous cycle in neonatal, prepubertal and pubertal age female guinea pigs. The study proved that complete thymectomy in five to seven-day old female guinea pigs resulted in changes in the follicular morphology and a reduced number of follicles. Morphological changes in the follicles included the absence or advanced atresia of large size follicle, medium size follicle, primordial follicle, degenerated corpora lutea and proliferation of hypertrophied interstitial cell elements.

Earlier studies by Deschaux et al, 1979, reported that thymectomized guinea pigs showed development of a wasting disease with a loss of body weight and dysfunction of hormonal balance. In mammals, the immune system is essential for the optimal function of the reproductive system. This clearly shows that the immune system affects reproductive function linked to the hypothalamus Garcia L et al, 2000, pituitary gland (S. S. Walusimbi et al,2013, Meinhardt et al,2011 and gonads Greenstein BD et al, 1986, Walsh S et al, 2005. Thymic epithelial cells secrete hormones such as thymulin and thymosin alpha-1. The production of thymic hormones is inhibited by conditions like sex hormone treatment and ovariectomy. This in turn increases the weight of thymus in the mice Greenstein BD et al, 1987, Heng TS et al, 2005, Windmill KF et al, 1993,1998, Miller, E.M et al, 1967, Hardy, B, 1974. The factors which regulate the primordial follicle in the mammalian ovary are poorly understood.

The surgical removal of the thymus gland and ovaries during non-breeding and breeding phase of the animals, respectively results in marked changes in the ovaries of thymectomized and the thymus of ovariectomized animals. The role of thymic hormones in the development of the ovaries is further highlighted in a study that showed retardation of ovarian follicle development in a girl with congenital absence of the thymus Miller ME et al, 1967. Nishizuka and Sakakura 1969, 1971 have revealed that there was a functional connection between the thymus and the ovaries and noted that after neonatal thymectomy, ovarian dysgenesis is replaced by the invasion of lymphocytes into the ovary, a sudden loss of oocytes, an expansion in follicular atresia, a reduced or absence of corpora lutea, a lower weight of the ovaries and tumors developing in the grown-up stage. Besedovsky et al., 1979, have shown that neonatal thymectomized female mice demonstrated delayed primary ovulation and the presence of contracted ovarian follicles with the delay occurring in the

beginning of pubescence in thymectomized mice as well as in athymic nude mice. In the two cases, follicular morphology might be restored by exogenous gonadotropin Jones, E.C et al.,1961a.

A significant reduction of growing follicles has been reported at 10 days in congenitally athymic nude mice but restoration of multi laminar growth stages upon treatment with gonadotropin Jones, E.C et al., 1961b. The neonatal thymectomy at 2 to 4-day-old female mice results in reduced weight of the ovary, composed of mostly of interstitial- like cells, devoid of follicle and replaced by lymphocyte infiltration in and around the medium and large sized follicles that accompany the dysgenesis. The ovarian dysgenesis occurs between the age of 90'th to 120'th day and further reduction of weight of ovary when compared to the intact animals H.O. Besedovsky, 1979. Neonatal thymectomized female mice at the age of 10, 21 and 30 days did not show statically significant changes in the number of ovarian follicles in different stages and follicular atresia which begins after the 50th day. Complete destruction of follicles occurred at the age of 130th day Eshkol A et al.,1967, These findings were consistent with our present finding of ovarian changes after thymectomy in neonatal female guinea pigs.

Further studies have reported that nude female mice have shown that ovarian dysgenesis begins at approximately 24 days of age and is usually complete by 60 days of age Jones, E.C, et al.,1961. Around the 30'th day of life, the presence of heteropyknotic cells with lymphocytes infiltration and abnormal-looking cells have been reported Pedersen T, 1969a. Michael, 1983, has stated that third day thymectomized mice produce circling autoantibodies against the ooplasma of oocytes, showing an immune system etiology for the ovarian atrophic changes or failure. However, the invasion of lymphocytes and the manifestation of antioocyte antibodies were observable until 25 days of age Tung et al.,2005. Kosiewicz and Michael, 1990, suggested that the thymectomy impact on the ovary may include the interruption of the hypothalamic-pituitary– ovarian– thymic axis, which does not seem to include the immunological factors associated with ovarian dysgenesis. In addition, similar changes indicative of ovarian follicle destruction was recorded in hypophysectomized and mice treated with an antiserum injection to gonadotropins during neonatal period (2-7days) mice and rat Weisz J, 1970, Bagavant H1 et al., 1999, Kleinewietfeld M, 2014.

Neonatal thymectomy on Day 3 after birth showed organ-explicit auto immune system disease influencing various organs including the ovary Fitzpatrick, F. Q et al.,1985. Day three thymectomy after birth instigated oophoritis and ovarian atrophy because of aggravation and raised cytokine expression in the female ovaries, interceded by the proinflammatory Th1 T cells. Th1 cells prevent immunosuppressant and are less in number in female animals when compared to males. Th1cells are capable to maintaining the normal estrous cyclicity, ovarian follicular development, ovulation, and fertility Greenstein, B. D et al., 1986. Above outcomes suggest that loss of various functions of ovaries in autoimmune ovarian disease based upon mechanisms in Th1 cell-intervened oophoritis, and anomalous cytokine creation may produce untimely ovarian failure. Neonatal thymectomized mice initiated immunological disorder results from absence of a one-of-a-kind thymus-determined administrative CD4+T cell subset that constitutively communicates the IL-2 receptor αchain (CD25) Lintern-Moore et al., 1977.

Interestingly Sue Lintern-Moore et al., 1976 examination upon careful evacuation of thymus after 2 days in female Bagg rodent showed no statistical significance on the

number of small, medium and antral ovarian follicles during the initial 12 weeks of life. In this examination, results demonstrated 20% of thymectomized mice over 12 weeks of age created ovarian failure which incorporated the loss of corpora lutea and a general decrease in all parts of the ovarian follicle population. These findings were not consistent with our present findings in neonatal thymectomized female guinea pigs ovaries.

Conclusion

Reduction in the number of ovarian follicles and their decrease in diameter in neonatal thymectomized female guinea pigs strongly indicate that thymus plays a major role in the proper development and function of the hypothalamic –pituitary – gonadal axis in the early age of a guinea pig. But no significant difference was noted in the thymectomized prepubertal and pubertal female guinea pigs when compared with the sham operated group which indicates an age-dependent role of thymus in reproductive development.

List of abbreviations used

LH- luteinizing Hormone
FSH- Follicular Stimulating Hormone
Tx – Thymectomy
Shax – Sham-operated
GC – granulosa cells
ns – Not Significant
s- Significant.

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