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P Senthamil Selvan
Department of Veterinary Anatomy & Histology, RIVER, Puducherry, India

K Rajalakshmi
Department of Veterinary Anatomy & Histology, RIVER, Puducherry, India

E Raja Ravi Teja
Department of Veterinary Anatomy & Histology, BHU, Mirzapur, Uttar Pradesh, India

S Ushakumary
Department of Veterinary Anatomy and Histology, MVC, Chennai, Tamil Nadu, India

M Sivakumar
Department of Veterinary Anatomy & Histology, RIVER, Puducherry, India

Corresponding Author:
P Senthamil Selvan
Department of Veterinary Anatomy & Histology, RIVER, Puducherry, India

Uterotrophic and histomorphological changes in the postpubertal uterus of bilateral ovariectomized BALB/c mice

P Senthamil Selvan, K Rajalakshmi, E Raja Ravi Teja, S Ushakumary and M Sivakumar

Abstract
The uterus undergoes periodic changes in its morphology during every estrous cycle and is controlled by the ovarian hormones after puberty. In the absence of ovarian hormones, the weight of the uterus reduces. This reduction is drastic in the first week and is approximately 72% of its original weight (at estrous) with an additional 7% in the end of the second week post surgery. Histological changes revealed a reduction in the surface epithelial cell height from tall columnar epithelium in control animals to cuboidal/short columnar epithelial cells with limited cytoplasm in OVX mice. The proprial glands are fewer and smaller without any secretion inside their lumen. Their reduction is more than 22% and 33% against control group after 1st and 2nd week respectively. The stromal cellular density is normal but with few blood vessels. The stromal cells are polymorphic, basophilic with limited cytoplasm. The thickness of the endometrium reduced 63% and 66% after 1st and 2nd week of ovariectomy. Its % relative surface area is 33.5% and 32.7% in these animals. The myometrial thickness reduced to 30% and 32% post ovariectomy at first and second week respectively. The myometrial smooth muscle cells showed a distinct, elongated and central basophilic nucleus with narrow, peripheral eosinophilic cytoplasm. Their percentage relative surface area is comparatively more and is 54.4% and 54.7% due to an atrophied endometrium in these animals.

Keywords: Uterus, bilateral ovariectomy, histology, surface area, thickness

Introduction
Laboratory mice are common animal models for biomedical research. They are similar to humans in terms of anatomy and physiology and are often the preferred animal model for studies of human disease. Numerous mouse models are developed till date to understand various biological process *in vivo*. These models serve as powerful tools for advancing our knowledge in understanding the disease processes across species (Bryda, 2013) [4]. Ovariectomised mice models (OVX) are one among them whose ovaries are surgically excised for understanding physiological, metabolic and immunological alterations in various organs and systems due to estrogen deficiency during menopause (Souza, 2018) [19]. OVX model for loss of ovarian function have been employed in various studies like menopause related changes in muscle contractility and myosin function (Moran *et al*., 2007) [12] metabolic pathologies such as insulin resistance, obesity and metabolic dysregulation (Rogers *et al*., 2009) [16], Osteoporosis (Inada *et al*., 2011) [7], life span (Benedusi *et al*., 2015) [3], Hepatic steatosis (Quinn *et al*., 2018) [14], memory (Tao *et al*., 2020) [23], FAO and Oxidative stress (Oliveiria *et al*., 2018) [13] etc.,
Nevertheless, this model is also commonly used in studying the effects of hormones especially estrogen (Modder *et al*., 2004) [11], progesterone (Davoudi *et al*., 2015) [5] and other pharmacologically active phytoestrogens such as soy tempeh flour extract (Utami *et al*., 2017) [24] Plumeria acuminata ait stem extract (Taid *et al*., 2016) [22] etc., on their exogenous administration. However, detailed reports on the trophic and micromorphological changes on the female reproductive organs due to the deprivation of ovarian hormones in this mouse model is still scarce. Thus, we aim to report on the gross, microanatomical and cellular changes in the uterus of this OVX mice which may help other contemporary researchers to evaluate the uterine changes effectively in these animal models.

Materials and Methods

Mice for the present study were from the institutional laboratory animal unit, Konkuk University, South Korea. All the procedures were conducted in strict accordance to the Animal Care and Use Committee, Seoul, South Korea. Adult cycling, healthy postpupertal 3 months old virgin female mice that weighed 20±3.2 gms were selected for the present study. Four sets of 10 number of normal and adult healthy female mice were used. The control set (2 sets of 10 mice each) were sham operated and estrous synchronized. They were sacrificed at the proestrous stage at 13 weeks and 14 weeks of age. While the third set and fourth set were removed of both their ovaries under Avertin anesthesia. They were sacrificed at 1 week and 2 weeks after bilateral ovariectomy and proper postoperative care.

The uterus from all the three sets were collected carefully, trimmed free from its adhering fat and peritoneal connections. The uterus were washed with saline and weighed for their wet weight. To reduce differences in uterine weight due to body weight, the relative weights of the uterus were calculated as follows: % Relative weight of the uterus = [(wet weight of uterus /body weight at sacrifice) x 100] (Lee *et al*, 2021) [8].

The uterine tube were cut at the middle of its length and tissue pieces from it were collected, washed with saline and fixed in suitable fixatives viz., 10% neutral buffered formalin, bouin's fluid and Zenkers fluid. The tissues were then processed for paraffin embedding. Paraffin sections of 4-5 micrometer thickness were cut using a microtome and were utilized for standard hematoxylin and eosin staining technique, (Bancroft and Stevens, 1996) [2] Masson's trichrome method for collagen and Gomori's method for reticulum (Luna 1968) [10]. Micro morphological and Quantitative histological observations on these sections were performed using a stereo microscope aided with Axio Vision 4.6.3.0 software.

Data are presented as mean±standard deviation (SD) or as indicated in figure legends. Significance was determined with the one-way Anova/Tukey test in case values were considered to be normally distributed. Differences were considered statistically significant with $p \leq 0.05$ and highly significant with $p \leq 0.001$.

Results and Discussion

Effect of bilateral ovariectomy on uterine weight in OVX mice

The wet weight of the uterus varied during different stages of the estrous cycle. It was heavier during proestrous when estrogen level was highest and lighter during diestrus (Balmain *et al*., 1956) [1]. In our present study we recorded that the uterine weight during proestrous stage in both 13 week old and 14 week old mice were 132.6±23.42 mgs and 133.5±22.01 mgs respectively. Its % relative weight accounted 0.62±0.10 and 0.61±0.09 in control groups. Post-ovariectomy the uterus appeared grossly thinner and their weight after 1 week and 2 weeks were 36.88±11.98 mgs and 24.31±0.68 mgs respectively. This sharp reduction in weight accounted 72.2% and 81.8% against the controls in proestrous stage (Chart 1). Their % relative weight was 0.16±0.053 and 0.115±0.032 that accounted 74.2% and 81.2% lesser against the controls (Table 1). Similar observations though have been reported by other researchers like (Modder *et al*, 2004) [11] in ovariectomised C57BL/6 mice and (Taid *et al*, 2016) [22] in ovariectomised adult female albino mice (C3H strain). The controls that were sham operated for comparison were not specified of their stage of estrous cycle at the time of tissue collection. Our results against the synchronized controls especially at their proestrous stage was more appropriate and a better platform for the evaluation criteria of this assay in OVX mice. The rate of reduction was rapid at the 1st week postsurgery similar to the findings of Lemini *et al* 2015 [9] in mice and rats who reported that the decrease in uterine weight was marked from day 1 to day 9 post surgery and it was gradual thereafter.

This decrease in uterine weight was due to the lack of ovarian hormones especially the estrogen (Stevenson *et al*, 1993 and Lemini *et al*, 2015) [20, 9] in wistar rats and CD1 mice. Nevertheless, exogenous administration of estrogen improved the uterine wet weights in these OVX animal models (Taid *et al*., 2016) [22]

Effect of bilateral ovariectomy on micromorphology of the uterus in OVX mice

The uterine wall of both the control and OVX mice showed all the histological layers. The total uterine diameter of the control groups were the greatest and measured 1071.3±72.9 µm and 1109.6±97.4 µm in 13th and 14th week respectively. Whereas, in OVX mice it measured 584.7±56.8 µm and 567.3±51.8 µm after 1st and 2nd week of surgery (Table 2). This accounted for 45.4% and 49.8% reduction in these OVX mice. In general, the diameter of the uterus varied along the dorsolateral-medial (mesometrial - free border) and dosal-ventral axis. The dorsolateral-medial diameter (mesometrial - free border) was more and therefore the uterus was wider along this axis while, the dorso- ventral diameter was comparatively less (Fig: 1). For uniformity we recoded and compared the total diameter only along the dorsal-ventral axis in these mice.

Since, the size of the lumen may have an influence on the total diameter of the uterus, the luminal diameter as well as the % relative area was recorded and analysed between controls and OVX mice. In cross section, the uterine lumen was oriented along the dorsolateral-medial axis/(mesometrial - free border) and its diameter in the present study was recorded only along the dorsal-ventral axis for unformity in both groups. Its diameter and % relative surface area in control group was 45.6±8.8 µm (12.2%) and 43.7±10 (11.7%) µm in 13th and 14th week respectively. Whereas, in OVX mice it measured 57±8.4 µm (12.4%) and 59.5±9.4 µm (13.1%) after 1st and 2nd week of surgery (Table 2). The higher luminal diameter and % relative luminal surface area with lesser total uterine diameter in OVX mice confirmed a reductional/involutional change in other uterine layers/tunics.

The endometrium contained surface epithelium that is formed of cuboidal/ low columnar cells (Fig 2) as reported by Modder *et al*, 2004 [11] in C57BL/6 OVX mice. Their height was 14.3±2.3 µm and 13.5±1.9 µm in OVX mice after 1st and 2nd week of surgery. This was 54% to 58% lower than the high columnar epithelial cells of the control groups. Our findings agrees with Suzuki *et al*, 1996 [21], who reported that the uterine epithelial cell height significantly reduced in 1 to 20 days after ovariectomy when compared to controls at the estrous stage in mice. Very few subepithelial leucocytes where noticed in OVX mice (Fig 2)

The nucleus of these cells are oval, dark, basophilic and basal. The cytoplasm was eosinophilic and restricted to their apical ends. All the cells were intact. The endometrial glands also showed a reduction in size and number as reported by Lee *et al*, 2021 [8] in ovariectomized DDY mice that the uterine glands undergo significant atrophic changes due to estrogen

depletion. In the present study, the number of glands reduced 32% to 43.7% than the control group at the proestrous stage and the glandular epithelial cells were of simple cuboidal with a central, spherical basophilic nucleus and very few exhibited apoptotic changes (Fig 3). The lumen of the glands were narrow/absent in few. The endometrial stroma had connective tissue fibers predominantly reticular that didn't vary between the controls and OVX mice. Subepithelial and stromal blood vessels were fewer and narrower (Fig 2). Stromal cellular density was normal in both catagories. However, (Saruhan *et al*, 2006) [17] reported that the endometrial stroma appeared more loose in the overiectomised rat than controls. The stromal cells in Ovx group were polymorphic, basophilic with limited cytoplasm and resembled dormant fibroblasts. They were tightly packed as reported by Modder *et al*., 2004 [11] in C57BL/6 ovx mice. Further, the absence of infiltrated immune cells and the presence of only few macrophages (Fig 2) confirmed the physiological inflammatory process and thereby, the remodeling of uterus is absent in these mice.

The endometrium of the uterine horn is uneven with very small mucosal folds (Fig 1). It is limited medially (free border) and almost thinner laterally (at the mesometrial border). However, its thickness was higher at the roof/dorsal and floor/ventral regions. For uniformity, the endometrial thickness in this present study was measured only at dorsal/ventral and medial areas in both groups. These results were supplemented with % relative endometrial surface area for better analysis & interpretation without any bias.

Endometrial thickness and its % relative surface area in control group was 259.8±33.6 µm (44%) and 271.7±35.7 (44.5%) µm in 13th and 14th week respectively. Whereas, in OVX mice it measured 95.1±7.4 µm (33.5%) and 90.9±12.8 µm (32.7%) after 1st and 2nd week of surgery (Table 2 & 3). This endometrial reduction accounted 63.4% and 66.5% for thickness and 10.5% and 11.8% for surface area after 1st and 2nd week of surgery in OVX mice. Thus ovariectomy resulted in drastic endometrial atrophy. This could have resulted due to the absence of growth response as reported by (Rockwell *et al*, 2001) [15] and also, due to higher apoptotis as reported by Sato *et al*, 2003 [18] in ovariectomised mouse. However, in our present study, only very few apoptotic cells were noticed only by the end of 2 week after Ovariectomy (Fig 2).

The submucosal layer is absent in both the groups. Tunica muscularis formed the uterine myometrium and possessed an inner circular and outer longitudinal layer in mice. Whereas, it possessed three layers in humans (Escalante, 2017) [6]. The myometrial smooth muscle cells in OVX mice were distinct, elongated and had a central basophilic nucleus. Their cytoplasm is lesser but eosnophilic (Fig 4). The stratum vasculare between the inner circular and outer longitudinal layer was highly reduced with very few and narrow blood vessels (Fig 1). The total myometrial thickness and their % relative surface area in control group was 214.3 µm (43.3%) and 215.2 (44.2%) µm in 13th and 14th week respectively. Whereas, in OVX mice it measured 151.3 µm (54.4%) and 145.2 µm (54.7%) after 1st and 2nd week of surgery (Table 2 & 3). This myometrial reduction accounted 29.4% and 33.5% for thickness. In contrary, its % relative surface area was 11% and 10.5% higher than the control groups. These findings therefore indicate that myometrium undergoes involutory/atrophic changes in OVX mice at a lower degree than its endometrium (Chart 2).

Estrogen has proliferative influence on myometrial smooth muscle and its deficiency inhibited growth and proliferation of endometrial and myometrial cells in OVX mice (Utami *et al*, 2017) [24]. This agreed with our present findings However, in order to compare the relative involutory/atropic changes between endometrium and myometrium in OVX mice, we determined the Myometrial: Endometrial ratio in these groups. The values were higher in OVX mice than control groups indicating that the myometrial involutory changes is comparatively lower than the endometrial part in these ovariectomised mice (Chart 3). The outer perimetrium was static without any change in both groups.

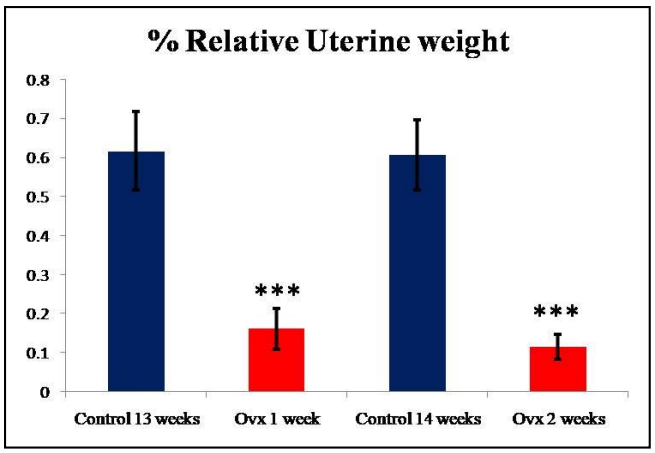


Chart 1: Chart showing reduction in the % relative uterine weight in OVX mice post Ovariectomy

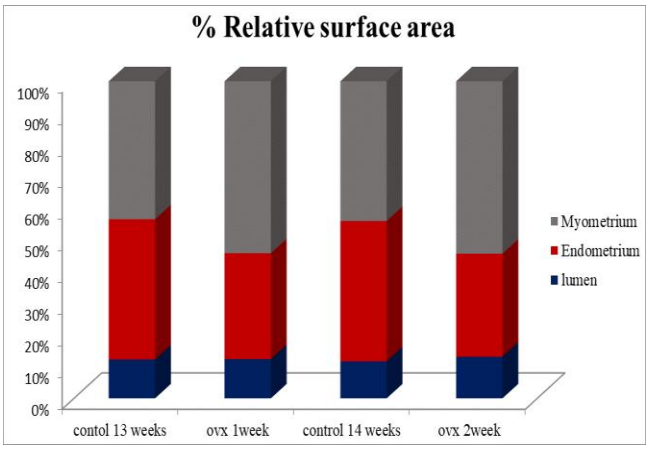


Chart 2: Chart showing differences in the distribution of % relative surface area of uterine layers in controls and OVX mice.

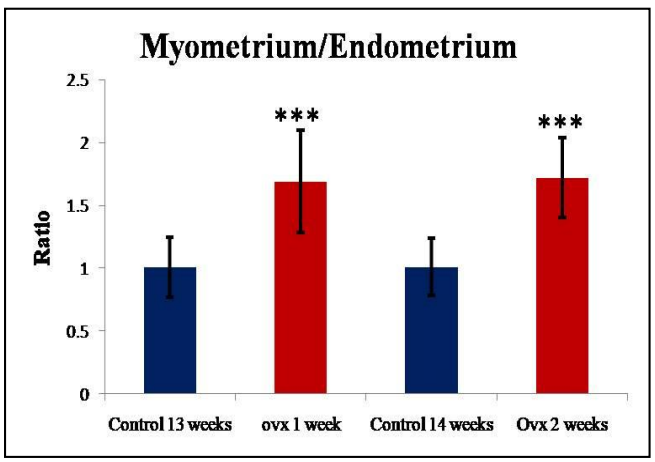
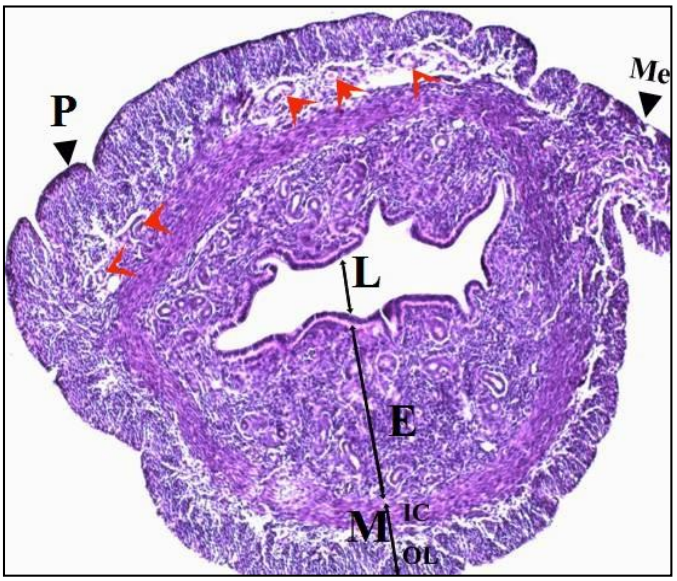
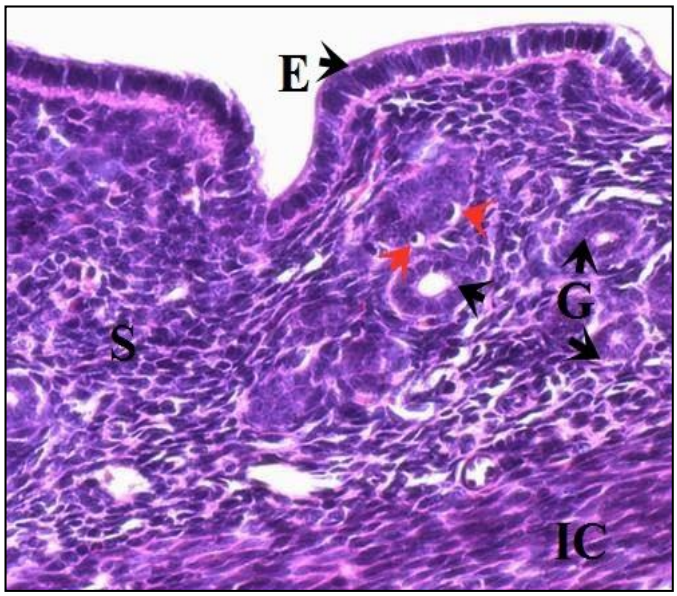


Chart 3: Chart showing increase in Myometrium/Endometrial ratio in OVX mice post Ovariectomy



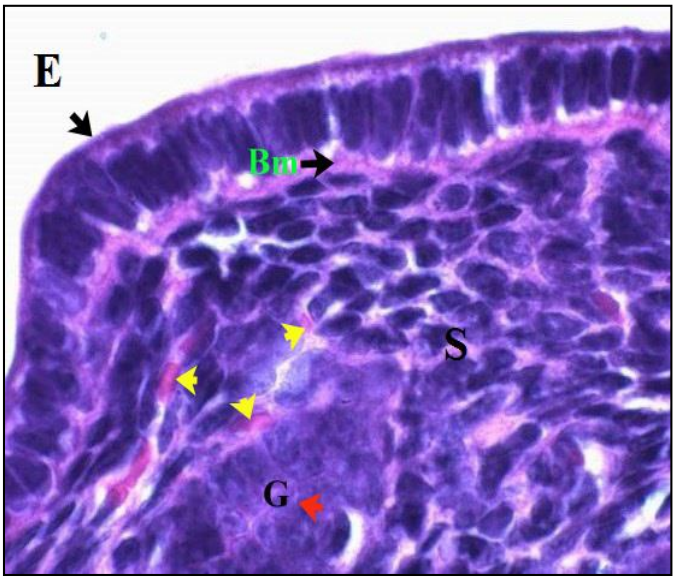
P- Perimetrium, L- Lumen, E – endometrium, M- Myometrium, IC- Inner circular layer, OL- Outer Longitudinal layer, Me- Mesometrial attachment, Blood vessels – red arrow heads

Fig 1: Cross section of the whole uterus of mouse 2 weeks after bilateral Ovariectomy (Hematoxylin and Eosin 100x).



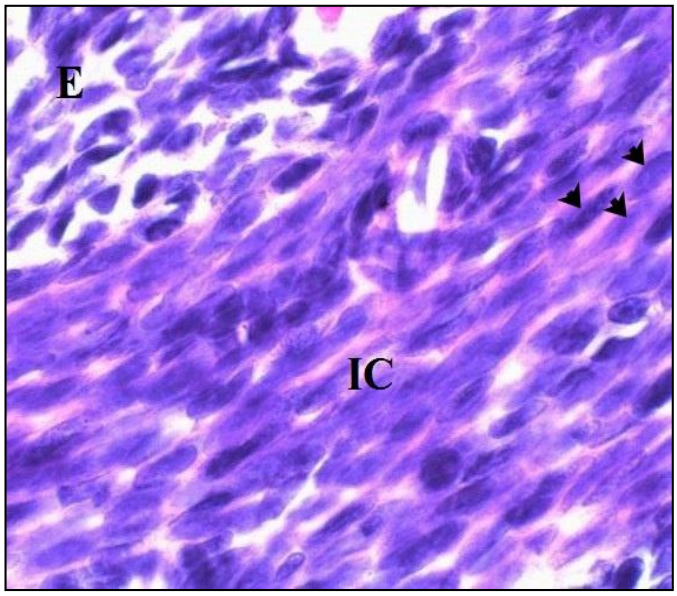
E- Endometrial surface epithelium, S – endometrial stroma, G – endometrial glands (black arrows), IC – Inner circular smooth muscle layer of myometrium, apoptotic cells –red arrow.

Fig 3: Endometrial morphology after 2 weeks in bilateral Ovariectomised mice (Hematoxylin and Eosin 200x)



E- Endometrial surface epithelium, S- endometrial stroma, G- Glands, Bm- Basement membrane, Sub-epithelial capillary plexus – Yellow arrow.

Fig 2: Endometrial surface epithelium in bilateral Ovariectomised mice after 1 week (Hematoxylin and Eosin 400x)



E- Endometrial stroma, IC – Inner circular smooth muscle layer of myometrium, Smooth muscle cells- black arrow.

Fig 4: Myometrial morphology in bilateral Ovariectomised uterus (Hematoxylin and Eosin 1000x)

Table 1: Changes in relative uterine weight after bilateral Ovariectomy in BALB/c mouse

Sl. no	Experimental group	Body weight (gms)	Uterine wet weight (mgs)	% relative weight
1	Control (13 weeks)	21.46±0.79	132.6±23.42	0.62±0.10
2	Ovx (1 week)	22.91±0.74	36.88±11.98	0.16±0.053***
3	Control (14 weeks)	21.90±0.82	133.5±22.01	0.61±0.09
4	Ovx (2 week)	24.31±0.68	28.1±8	0.115±0.032***

Note: Values are expressed mean ±standard deviation (n=8). Significance level is at ***p<0.001, **p<0.01 and *p<0.05, n=10

Table 2: Changes in histomorphometry of the uterus after bilateral Ovariectomy in BALB/c mouse

Sl.no	Parameter	Control (13 week)	Control (14 week)	Ovx (1week)	Ovx (2 week)
1	Height of the endometrial surface epithelium (µm)	31.2±3.7	32.3±3.9	14.3±2.3***	13.5±1.9***
2	Number of endometrial glands	45.8±5.2	50.1±5.1	35.7±6.5**	33.2±5.2***
3	Thickness of endometrium (dorsal & ventral) (µm)	259.8±33.6	271.7±35.7	95.1±7.4***	90.9±12.8***
	Thickness of endometrium (medial & mesometrial) (µm)	35.2±6.2	33.3±8.3	30.6±7.8	29±9
4	Thickness of the Tunica muscularis/ myometrium: ICM and OLM (µm)	104.2±14.1 110.1±12.2	106.5±16.7 108.7±15.1	72.2±16*** 79.1±12.5***	70.1±11.1*** 75.1±13.2***
5	Diameter of lumen(µm)	45.6±8.8	43.7±10	57±8.4*	59.5±9.4**
6	Total diameter (µm)	1071.3±72.9	1109.6±97.4	584.7±56.8***	567.3±51.8***

Note: Values are expressed mean ±standard deviation (n=8). Significance level is at ***p<0.001, **p<0.01 and *p<0.05, n=10

Table 3: Changes in the % relative area of the uterine layers after bilateral Ovariectomy in BALB/c mouse

Sl.no	Parameter	Control (13 week)	Control (14 week)	Ovx (1week)	Ovx (2 week)
1	% Endometrial surface area	44.0 ±7.4	44.5±5.7	33.5±7**	32.7±6**
2	% Myometrial surface area	43.3±8.1	44.2±8.7	54.4±7.4**	54.7±4.6*
3	% Luminal surface area	12.2±2.3	11.7±2.1	12.4±2.2	13.1±2.4
4	Myometrial/endometrial ratio	1.01±0.24	1.01±0.22	1.69±0.49***	1.72±0.32***

Note: Values are expressed mean ±standard deviation (n=8). Significance level is at ***p<0.001, **p<0.01 and *p<0.05, n=10

Conclusion

The wet uterine weight and its % relative weight declined sharply post ovariectomy. The rate of reduction was drastic in the first week group and the uterus appeared grossly thinner in these animals. Histological and histometric observations revealed reductional changes in both surface and glandular epithelium, subepithelial capillary plexus, inflammatory cells, number of endometrial glands, and endometrial thickness as a whole and supported these atropic changes. Decreased myometrial thickness and its vasculature were evident. Our results and findings may help other contemporary researchers to test and evaluate the uterotrophic effects if any of the numerous exogenous compounds effectively in these OVX models.

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