

Original Research Article

TEMPORAL VARIATIONS IN DENSITY AND DISTRIBUTION OF NEUTROPHILS WITH ASSOCIATED MORPHOLOGICAL CHANGES IN UTERUS AT ALL STAGES OF ESTRUS CYCLE IN MICE

ABSTRACT

Uterus is an important reproductive organ that undergoes proliferative, degenerative, repair and regenerative changes in its histological tunics during different stages of estrous cycle. These changes are temporal and are tightly regulated by the ovarian hormones. Derangements in remodeling is the reason for pathological conditions such as endometritis, endometriosis etc., The present study was designed to report in detail the temporo-spatial events that occur in uterus with special reference to the difference in % relative endometrial surface area, neutrophil density and their distribution during estrus cycle in mice. Our studies revealed that histomorphological variations were more pronounced in endometrium than other tunics. Histomorphometric studies found that the % relative endometrial surface area was maximum during late proestrus and early estrus. It reduced to its minimum levels in the middle of metestrus and was immediately restored within 6hrs. Histological and immunohistochemical studies confirmed that neutrophils were present during all stages of the estrus cycle. Their influx and density was maximum at early metestrus and was minimum at late metestrus. Their density were moderate and static thereafter until early proestrus. These findings suggested that the rate of infiltration of neutrophils in to uterus is a controlled and stage specific process. Their complex role of inflammation, phagocytosis and endometrial repair in remodeling the uterus may be dependent on the uterine microenvironment at a given time point.

Key words: Uterus, estrus cycle, mice model, endometrium, myometrium, surface area, neutrophils.

INTRODUCTION

The laboratory mouse is a common animal and its uterus is widely used as a model to study mechanisms of steroid hormone signaling, reproductive toxicology, endometriosis, uterine cancer and implantation Yip *et al.*[1]. Estrus cycle is controlled by ovarian steroid hormones and oscillations in their circulating levels delineate four main stages namely: proestrus, estrus, metestrus, and diestrus Wood *et al.*[2].The endometrium of uterus undergoes regular cyclical changes during the estrus cycle especially in the absence of pregnancy and these include proliferation, secretion Yip *et al.*[1] and remodeling without shedding (menses) in mice Cousins *et al.*[3]

Endometrial remodeling during estrous cycle is with an increase in expression of inflammatory mediators and leukocyte infiltration in to uterus. Critchely *et al.*[4]. During these phases the endometrium is prone for microbial infection and the infiltration of immune cells helps to reduce the microbial load and overcome pathogen challenges Quayle [5].

Among the leukocytes, neutrophils, form a significant but varying population of immune cells in the uterus of many species Hunt [6]. Neutrophils perform numerous functions but not limited to innate immunity by phagocytosis and NETosis Mayadas *et al.*[7], angiogenesis Heryanto *et al.*[8], tissue remodeling Alhussein and Dang [9], pain & estrus cycle regulation via opiod peptides

Kobayashi [10] and Sasaki *et al.*[11], spermatozoa clearance Taylor *et al.*[12] and gestation Zhao *et al.*[13]etc.,

Neutrophils are short lived cells Alhussein and Dang.[9]. There seems to be mechanisms that tightly regulate their infiltration, localization and density within the uterus in normal physiological conditions. Absence of neutrophils/neutropenia impedes endometrial repair in mice Kaitu'u-Lino *et al.*[14]and also results in fetal abortions as they are critical for establishing utero-placental circulation Zhao *et al.*[13]. Overinfiltration and activation cause enhancement in the pathogenesis of endometriosis Wang *et al.*[15], cancer and auto immune diseases Mayadas *et al.*[7]. Therefore, for a better understanding of their regulation, role within the uterus, it is necessary to know their quantity, localization and distribution, during different stages of estrus cycle in physiological conditions.

This study therefore aims to detect, quantify and to record the distributional changes in uterine neutrophils and to correlate it with the micromorphological changes within the uterine environment in all stages of the estrus cycle in BALB/c mice.

MATERIALS AND METHODS

Mice selection and estrus synchronization

BALB/c mice for this study were kindly provided by the institutional laboratory animal unit, Konkuk university, South Korea. All the procedures for conducting this study were in strict accordance to the Animal Care and Use Committee, Seoul, South Korea. Adult cycling, healthy postpubertal, virgin BALB/c mice that weighed 25 ± 3.2 gms were selected for the present study. About 190 mice were caged in 19

cages @10 mice/cage. These mice were fed ad libitum and housed as per the recommendations. All the mice in 18 cages received PMSG injections as per Wei *et al.*[16] for synchronized estrus. These animals were checked as below and were sacrificed for their uterus. One set of mice was utilized for performing bilateral ovariectomy (data not shown).

Estrus Detection

Two fold detection for estrous was performed. Firstly, by Visual method as per Champlin *et al.*[17]; Byers *et al.*[18] and Secondly, by Vaginal cytology method as per Felicio *et al.*[19].

Histology & Histometry

Mice that were synchronized for estrus and those that were detected positive were alone utilized for the present study whereas the rest were salvaged back to laboratory animal unit. The uterus from these positive animal pool were collected carefully at 0 hrs and every 6 hrs interval from 6 number of mice thereafter. Uterine tube was cut at the middle of its length and sagittal tissue pieces from it were washed with saline and fixed in 10% neutral buffered formalin. The tissues were then processed for paraffin embedding and paraffin sections. Sections of 4 -5 micrometer thickness were cut using a microtome and were utilized for standard hematoxylin and eosin staining technique Bancroft and Stevens [20]. Micro morphological and Quantitative histological observations on these sections were performed using astereo microscope aided with AxioVision 4.6.3.0 software.

Data were presented as mean \pm standard deviation (SD) or as indicated in figure legends. Significance was determined with 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 $p \leq 0.001$.

Immunohistochemistry

Fresh uterine tissues were collected in duplicate for immunohistochemical analysis. These tissues were embedded in OCT compound and frozen in the freezing chamber. Frozen sections of 6 micrometer thickness were cut using a cryostat. Sections of fresh frozen uteri were fixed in 96% ethanol and immunostained with neutrophil specific mouse monoclonal anti Ly6G primary antibody which was detected by Texas red conjugated rat anti mouse secondary antibody.

RESULTS AND DISCUSSION

The uterine wall of mice had inner endometrium, middle myometrium and an outer perimetrium (Fig 1a). The surface epithelium was formed of simple columnar epithelial cells. These cells turned into high columnar during estrus with numerous mucous secretory granules in their apical regions as reported by Kaeoket *et al.*[21] in sows. They became shorter at diestrus. Sub epithelial capillary plexus were seen immediately below the basement membrane. No neutrophils were found within the surface epithelium during the entire cycle in mice. Whereas, Kaeoket *et al.*[21] reported that neutrophils transmigrate and occur occasionally among the epithelial cells in sows. In contrary to humans, the lamina epithelialis was intact at all stages of the estrus cycle and neither desquamated cells nor neutrophils were found inside the lumen of the uterus at any stage of the estrus cycle in mice (Fig 1a).

The Lamina propria was made of connective tissue, glands and stromal cells. It possessed capillary plexus underneath the surface epithelium. These subepithelial plexus were found well developed and prominent during estrus but regressed at diestrus stage. Infiltration of neutrophils from these plexus was found higher at late estrus and early metestrus. The infiltrated neutrophils localized closer to the basal lamina of the surface epithelium and didn't migrate into the lumen (Fig 2a). Salinas-Munoz *et al.*[22] in their study reported that, E2 prevented this transepithelial migration of neutrophils into the lumen to favour survival of sperms for fertilization. The surface area of endometrium was higher during proestrus. New blood vessels started emerging from the existing subepithelial capillary plexus and made them prominent and extensive during estrus. The density of neutrophils increased within the uterus as they migrated from these plexus. The angiogenesis and neovascular sprouting in the existing capillary plexus could have been influenced by the expression of VEGF by the migrating neutrophils as reported by Mueller *et al.*[23]. Further, these neutrophils formed the first line of innate immunity and are the most prominent line of cellular defense against invading microorganisms in uterus Alhussein and Dang[9]

In late estrus and early metestrus, the density of neutrophils peaked in uterus and were found maximum. The neutrophils that migrated later may of proinflammatory subsets that induced a physiological inflammation within the uterus. These proinflammatory neutrophils generated intercellular gaps during transmigration for the passage of serum proteins (such as cytokines, antibodies, and complement) and generated edema fluid Mayadas *et al.*[7].

The relative density and components of the stromal cells varied during different stages of the estrus cycle. In our study we found that during late estrus and

early metestrus, the endometrial stroma was influenced by an increase in infiltrated neutrophils that peaked during metestrus (Fig 2a, c). In contrary, Steffl [24] reported that maximum number of neutrophils migrated during estrus in sow. While, Lathbury and Salamonsen [25] reported that it is maximum during the immediate premenstrual and menstrual phases of the cycle in humans. These immigrated neutrophils accounted 6–15% of the total number of cells Evans and Salamonsen [26] and are responsible for the gross inflammation and edema in uterus Tibetts [27]. Other immune cells that were found with neutrophils in the endometrial stroma include eosinophils, lymphocytes, macrophages and mast cells as reported by Tassell *et al.*[28] in the endometrium of rats and McMaster *et al.*[29] in mouse. In our study we found that the population of neutrophils declined during late metestrus (Fig 3). This declination in the intra-uterine neutrophils was accompanied by a sharp reduction in the endometrial surface area. This could be due to the cessation of influx of the proinflammatory subsets and must be due enhanced removal of these subsets via apoptosis/NETosis as reported by Jorch and Kubes [30] and engulfment by macrophages as reported by Wang *et al.*[31] or They may have migrated reverse into the vascular vessels as found by Oliveira *et al.*[32]. These events favored resolution of inflammation during late metestrus

Fewer neutrophils were seen in the uterus post resolution i.e., in late metestrus and diestrus. (Fig 3). These neutrophils suggested that they were of anti-inflammatory/restoring phenotypes as their numbers were maintained until late proestrus. These anti-inflammatory subtypes may help clear cells and cellular debris as observed by Molina *et al.*[33] The endometrial surface area was found immediately restored during late metestrus within 6 hour interval and was maintained until early proestrus.

In myometrium blood vessels were seen interposed between inner circular and outer longitudinal smooth muscle layer and this resembled the stratum vasculare of other species (Fig 1b). These blood vessels were distinct and enlarged during late estrus and early metestrus and were found to convey a huge population of neutrophils in to the endometrium through myometrium. Neutrophils were seen migrating between smooth muscle cells towards the uterine lumen and their migration may help favor smooth muscle contraction as reported by Molina *et al.*[33] that the mediators from this neutrophils are responsible for labour and uterine contractility. The density and distribution of blood vessels in stratum vasculare were found less during late metestrus and thereafter. Neutrophils were not observed in the outermost perimetrium.

CONCLUSION:

Neutrophils were found in uterus during all stages of estrus cycle and therefore must be influenced by both the ovarian hormones estrogen and progesterone. The hormone and the uterine microenvironment at any particular stage therefore should favour neutrophils to perform their specific temporal functions at desired levels. Therefore, the molecular mechanisms of neutrophil recruitment and their initiation for any specific function in the uterus may be different. From our study we found that these variations in their density and possible functions is therefore controlled and timely regulated during different stages of the cycle. Neutrophils form Neutrophil Extracellular Trap (NET) in the endometrium especially during post-coitus, post partum and in endometritis. However, If neutrophils form NET in uterus during regular cycling is still to be studied. Our findings will help assess the status

of uterus and also to detect any uterine pathologies associated with neutrophil recruitment and homeostasis at their nascent stage.

REFERENCES

1. Yip KS, Suvorov A, Connerney J, Lodato NJ, Waxman DJ. Changes in mouse uterine transcriptome in estrous and proestrous. *Biol Reprod.* 2013;89(1):13, pp 1-12.
 2. Wood GA, Fata JE, Watson KLM, Khokha R. Circulating hormones and estrous stage predict cellular and stromal remodeling in murine uterus. *Reprod.* 2007;133:1035–1044
 3. Cousins FL, Murray A, Esnal A, Gibson DA, Critchley HOD, SaundersPTK. Evidence from a mouse model that epithelial cell migration and Mesenchymal-Epithelial transition contribute to rapid restoration of uterine tissue integrity during Menstruation. *PLoS ONE.* 2014; 9(1). e86378.
 4. CritchleyHO, KellyRW, Brenner RM, Baird DT. The endocrinology of menstruation—a role for the immune system. *Clin Endocrinol.* 2001; 55, 701–710.
 5. Quayle AJ. The innate and early immune response to pathogen challenge in the female genital tract and the pivotal role of epithelial cells. *JReprod Immunol.* 2002; 57:61-79.
 6. Hunt J. Immunologically relevant cells in the uterus. *Biol Reprod.* 1994; 50:461–466.
 7. Mayadas TN, Cullere X, LowellCA. The Multifaceted functions of Neutrophils. *Annu Rev Pathol: Mechanisms of Disease.* 2014;9:81-218.
 8. Heryanto B, Girling JE, Rogers PAW. Intravascular neutrophils partially mediate the endometrial endothelial cell proliferative response to estrogen in ovariectomised mice. *Reprod.* 2004;127: 613 – 620
 9. Alhussien MN, DangAK. Potential roles of neutrophils in maintaining the health and productivity of the dairy cows during various physiological and physiopathological conditions: a review. *ImmunolRes.* 2019;67: 21-38
 10. Kobayashi, Y. The novel roles of neutrophils via opioid peptides: regulation of the estrous cycle and pain. *Arch Immunol TherExp (Warsz).* 2013; 61(3):187-91
 11. Sasaki S, TamakiY, NagataK, KobayashiY. Regulation of the estrous cycle by Neutrophils via opioid peptides. *J Immunol.* 2011;187(2):774-80
 12. Taylor U, ZerbeH, SeyfertH, Rath D, Baulain U, Langner KFA, Schuberth H. Porcine spermatozoa inhibit post-breeding cytokine induction in uterine epithelial cells in vivo. *Anim Reprod Sci.* 2009;115, 1–4, 279-289
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13. Zhao H, Kalish F, Schulz S, Yang Y, Wong RJ, Steveson DK. Unique Roles of Infiltrating Myeloid Cells in the Murine Uterus during Early to Midpregnancy. *J Immunol.* 2015;194 (8): 3713–3722.
 14. Kaitu'u-Lino TJ, Morison NB, Salamonsen LA. "Neutrophil depletion retards endometrial repair in a mouse model," *Cell Tissue Res.* 2007;vol. 328, no. 1, pp. 197–206.
 15. Wang X, Jia Y, Li D, Guo X, Zhou Z, Qi M, Wang G, Wang F. The Abundance and Function of Neutrophils in the Endometriosis Systemic and Pelvic Microenvironment. *Mediators Inflamm.* 2023, <https://doi.org/10.1155/2023/1481489>
 16. Wei S, Gong Z, An L, Zhang T, Dai H, Chen S. Cloprostenol and pregnant mare serum gonadotropin promote estrous synchronization, uterine development, and follicle-stimulating hormone receptor expression in mice. *Genet Mol Res.* 2015;14 (2): pp 7184-7195
 17. Chaplin AK, Dorr DL, Gates AH. Determining the stage of the estrous cycle in the mouse by the appearance of the vagina. *Biol Reprod.* 1978;8: 491-494
 18. Byers SL, Wiles MV, Dunn SL, Taft RA. Mouse Estrous cycle identification Tool and Images. *PLoS ONE.* 2012;7(4):e35538. doi:10.1371/journal.pone.0035538
 19. Felicio LS, Nelson JF, Finch CE. Longitudinal studies of estrous cyclicity in aging C57BL/6J mice: II. Cessation of cyclicity and the duration of persistent vaginal cornification. *Biol Reprod.* 1984;31: 446-453
 20. Bancroft JD, Stevens A. Theory and practice of histological techniques. 1996; (4th edn), Churchill livingstone, New York.
 21. Kaeoket K, Persson E, Dalin AM. Corrigendum to "The sow endometrium at different stages of the oestrus cycle: studies on morphological changes and infiltration by cells of the immune system" *Anim Reprod Sci.* 2001; 65: 95–114.
 22. Salinas-Munoz L, Campos-Fernandez R, Mercader E. Estrogen receptor-alpha (ESR1) governs the lower female reproductive tract vulnerability to *Candida albicans*. *Front Immunol.* 2018;9:1033
 23. Mueller MD, Lebovic DI, Garrett E, Taylor RN. Neutrophils infiltrating the endometrium express vascular endothelial growth factor: potential role in endometrial angiogenesis. *Fertil Steril.* 2000;74 107–112.
 24. Steffl M, Telgen L, Schweiger M, Amselgruber WM. Estrous cycle-dependent activity of neutrophils in the porcine endometrium: possible involvement of heat shock protein 27 and lactoferrin. *Anim Reprod Sci.* 2010;121(1-2):159-66.
 25. Lathbury LJ, Salamonsen L. A. In-vitro studies of the potential role of neutrophils in the process of menstruation. *Mol Hum Reprod.* 2000;6 (10):899-906.
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26. Evans J, Salamonsen LA. "Inflammation, leukocytes and menstruation," Reviews in Endocrine & Metabolic Disorders.2012; vol. 13, no. 4, pp. 277–288.
27. Tibetts TA, ConneelyOM, O'Malley. Progesterone via Its Receptor Antagonizes the Pro-Inflammatory Activity of Estrogen in the Mouse Uterus. Biol Reprod.1999; 60, 1158–1165.
28. Tassell W, Slater M, Barden JA, Murphy CR. Endometrial cell death during early pregnancy in the rat. Histochem J. 2000; 32, 373–379
29. McMaster MT, Newton RC, Dey SK, Andrews GK. Activation and distribution of inflammatory cells in the mouse uterus during the preimplantation period. J Immunol.1992;148, 1699–1705.
30. Jorch SK, Kubes P. An emerging role for neutrophil extracellular traps in noninfectious disease. Nat Med. 2017; 23(3):279–287. doi:10.1038/nm.4294.
31. Wang J, Hossain M, Thanabalasuriar A, Gunzer M, Meininger C, Kubes P. Visualizing the function and fate of neutrophils in sterile injury and repair. Science.2017;358(6359):111–116.doi:10.1126/science.aam9690
32. de Oliveira S, Rosowski EE, Huttenlocher A. Neutrophil migration in infection and wound repair: going forward in reverse. NatRev Immunol.2016; 16(6):378–391. doi:10.1038/nri.2016.49.
33. Molina BG, Muller I, Kropf P, Sykes L. The Role of neutrophils in Pregnancy, Term and Preterm Labour. Life.2022;12, 1512. <https://doi.org/10.3390/>

Tables1: % relative endometrial surface area and neutrophil density during different stages of estrus cycle.

Stage	nx6hrs	%Endometrial surface area	Neutrophil density (no's)
Proestrous	1	43.7±3.33 ^{c-r}	7.7±2.7 ^{d-r}
Estrous	2	43.83±2.48 ^{c-r}	6±1.4 ^{d-f,h-l}
	3	36.33±3.08 ^{abgh}	7.7±1.4 ^{d-r}
Early Metestrous	4	33.5±2.35 ^{abgh}	10.2±1.2 ^{a-c, f-r}
	5	34±2.28 ^{abgh}	12.3±1.2 ^{a-c,f-r}
	6	32.5±2.51 ^{abgh}	16.3±1.6 ^{a-e,g-r}
	7	31.8±2.4 ^{abgh}	4.8±1.2 ^{a,c-f, i-l}
Late Metestrous	8	25.8±2.14 ^{a-g,j-r}	3.5±0.8 ^{a-f,j}
	9	23.8±2.48 ^{a-g,j-r}	1.16±0.75 ^{a-g, m-r}
	10	33±2.1 ^{abhi}	0.67±0.52 ^{a-h, m-r}
	11	33.17±1.72 ^{abhi}	2.3±0.82 ^{a-g,m,o}
	12	34.17±1.94 ^{abhi}	2.3±0.52 ^{a-g, m,o}
Diestrous	13	33.17±1.83 ^{abhi}	5±0.89 ^{a,c-f,i-l}

	14	34±2.28 ^{abhi}	3.7±0.52 ^{a,c-f, ij}
	15	34.16±2.31 ^{abhi}	5.2±0.98 ^{a,c-f, i-l}
	16	33.83±1.94 ^{abhi}	4.5±0.55 ^{a,c-f, ij}
	17	33.5±2.07 ^{abhi}	4.2±0.75 ^{a,c-f, ij}
Proestrous	18	36.1±2.14 ^{abhi}	4.3±0.52 ^{a,c-f, ij}

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

Figures:

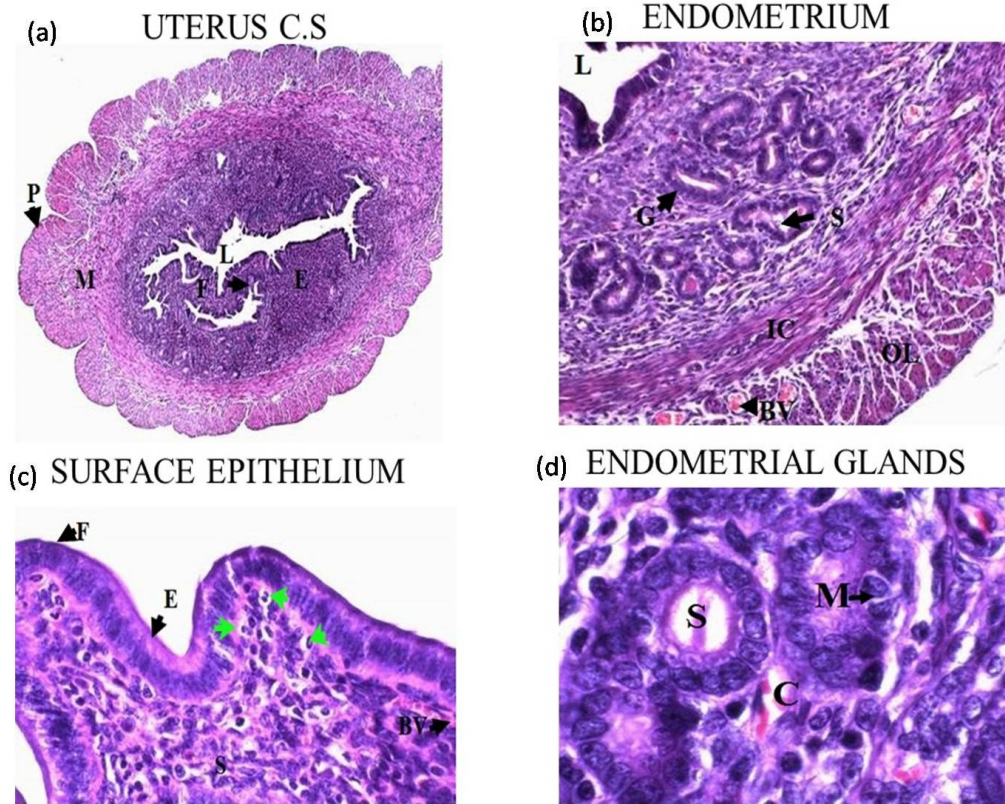


Fig 1. Cross section of the uterus of mice showing its histomorphological features during estrus cycle. (Hematoxylin & Eosin).

- (a) L- lumen, E- endometrium, F- fold, M- myometrium and P- Perimetrium (40X)
- (b) L- lumen, G- endometrial glands, S- secretions, IC- Inner circular smooth muscle layer, OI- Outer longitudinal smooth muscle layer, BV – Blood vessels. (100X)
- (c) F- Endometrial folds, E- Surface epithelium, S- Stroma, BV- Superepithelial capillary plexus, apoptotic neutrophils – green arrows. (200X)
- (d) S- Secretions inside the lumen of glands, M- Mitotic figure in glandular cells, C – Capillaries.(400X)

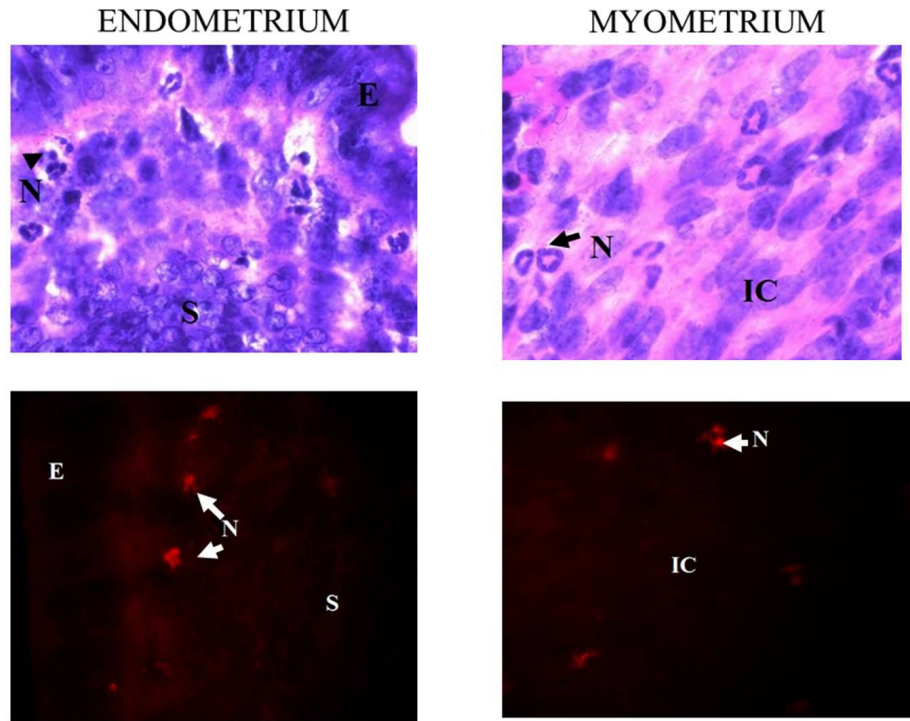


Fig2. Neutrophil infiltration in to the uterus during early metestral stage of estrus cycle

- (a) & (d) E- Surface epithelium, N- Neutrophils below the basal lamina, S – endometrial stroma (a- Hematoxylin & Eosin, b- Immunostaining – 1000X)
- (b) & (c) N- Neutrophils, IC – Smooth muscle cells of the Inner circular layer of smooth muscle.(b- Hematoxylin & Eosin, d- Immunostaining – 1000X)

Temporal changes in Endometrial Surface area and Neutrophil density in mice

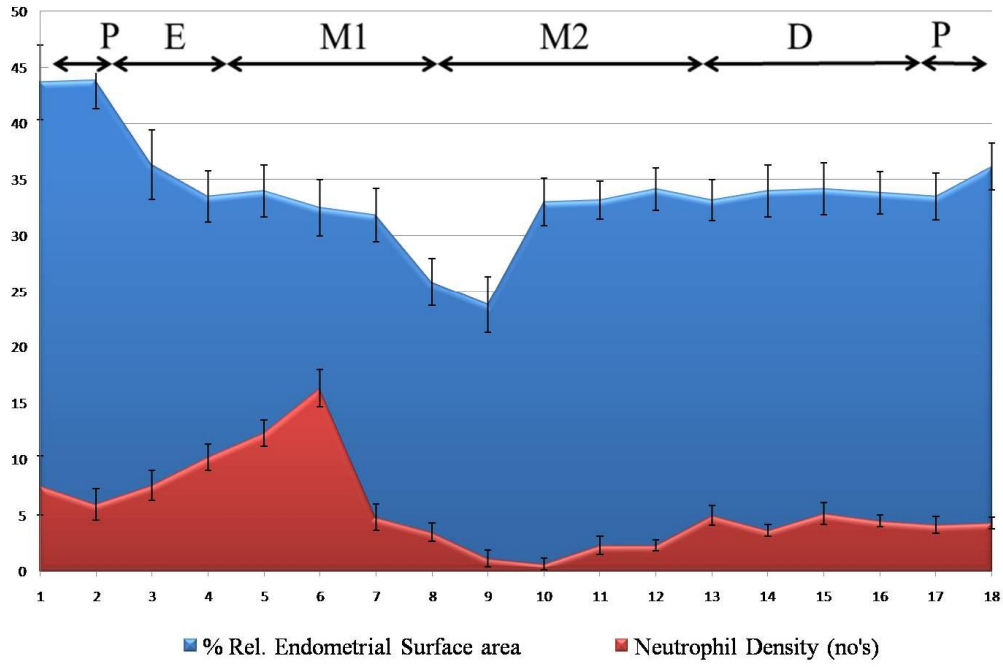


Fig 3. Temporal changes in the Endometrial surface area and Neutrophil density during different stages of the estrus cycle.

P- Proestrus, E- Estrus, M1 & M2 – Metestrus, D- Diestrus.

UNDER PEER REVIEW