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Temporal Variations in Density and Distribution of Neutrophils with Associated Morphological Changes in Uterus at All Stages of Estrus Cycle in Mice

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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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 objective of the present study is to report on the % relative endometrial surface area, neutrophil density and their distribution in uterus at all stages of estrus cycle in normal mice to help understand the pathologies in unterine remodeling by abnormal neutrophil recruitment. All these temporo-spatial events are detected using Histomorphological, micro-morphometrical and immunohistochemical techniques. 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.

Keywords: Mice model; endometrium; myometrium; surface area; remodeling; neutrophils.

1. 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 [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[2]. The endometrium of uterus undergoes regular cyclical changes during the estrus cycle especially in the absence of pregnancy and these include proliferation, secretion [1] and remodeling without shedding (menses) in mice [3].

Endometrial remodeling during estrous cycle is with an increase in expression of inflammatory mediators and leukocyte infiltration in to uterus [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 [5].

Among the leukocytes, neutrophils, form a significant but varying population of immune cells in the uterus of many species [6]. Neutrophils perform numerous functions but not limited to innate immunity by phagocytosis and NETosis [7], angiogenisis [8], tissue remodeling [9], pain & estrus cycle regulation via opiod peptides [10] and [11], spermatozoa clearance [12] and gestation [13] etc.

Neutrophils are short lived cells [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 [14]and also results in fetal abortions as they are critical for establishing utero-placental circulation [13]. Overinfiltration andactivation cause enhancement in the pathogenesis of endometriosis [15], cancer and auto immune diseases [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[16].

This study therefore aims to investigate the density of neutophils, to record their distribution within uterus and to correlate it with the micromorphological and histometric changes to understand their recruitment and regulation for uterine remodeling in BALB/c mice.

2. MATERIALS AND METHODS

2.1 Mice Selection and Estrus Synchronization

BALB/c mice for this study were kindly provided by the institutional laboratory animal unit, Konkuk university, South Korea. Adult cycling, healthy postpupertal, virgin BALB/c mice that weighed

25± 3.2gms were selected for the present study. About 190 mice were caged in 19 cages @10 mice/cage. These mice were fed adlibitum and housed as per the recommendations. All the mice in 18 cages received PMSG injections as per [17] 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).

2.2 Estrus Detection

Two fold detection for estrous was performed. Firstly, by Visual method as per [18]; [19] and Secondly, by Vaginal cytology method as per [20].

2.3 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. 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 [21]. Micro morphological and Quantitative histological observations on these sections were performed using astereo microscope aided with AxioVision 4.6.3.0 software.

2.4 Statistical Analysis

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 $p \leq 0.001$.

2.5 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.

3. 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 granuels in their apical regions as reported by [22] 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, [22] reported that neutrophils transmigrate and occurr occasionallyamong 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). E2 prevented this transepithelial migration of neutrophils into the lumen to favour survival of sperms for fertilization [23]. 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 [24]. Further, theseneutrophils formed the first line of innate immunity and are the most prominent line of cellular defense against invading microorganisms in uterus [9].

Table 1. % 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*

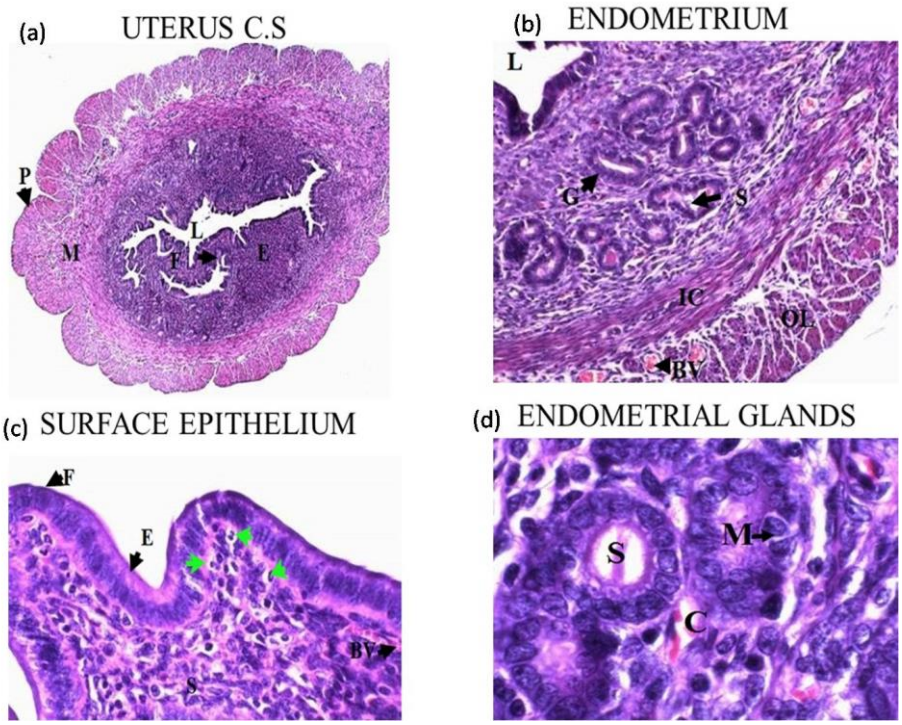

Fig. 1. Cross section of the uterus of mice showing its histomorphological features during estrus cycle. (Hematoxylin & Eosin)

(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- Supepithelial capillary plexus, apoptotic neutrophils – green arrows. (200X)
(d)S- Secretions inside the lumen of glands, M- Mitotic figure in glandular cells, C – Capillaries.(400X)

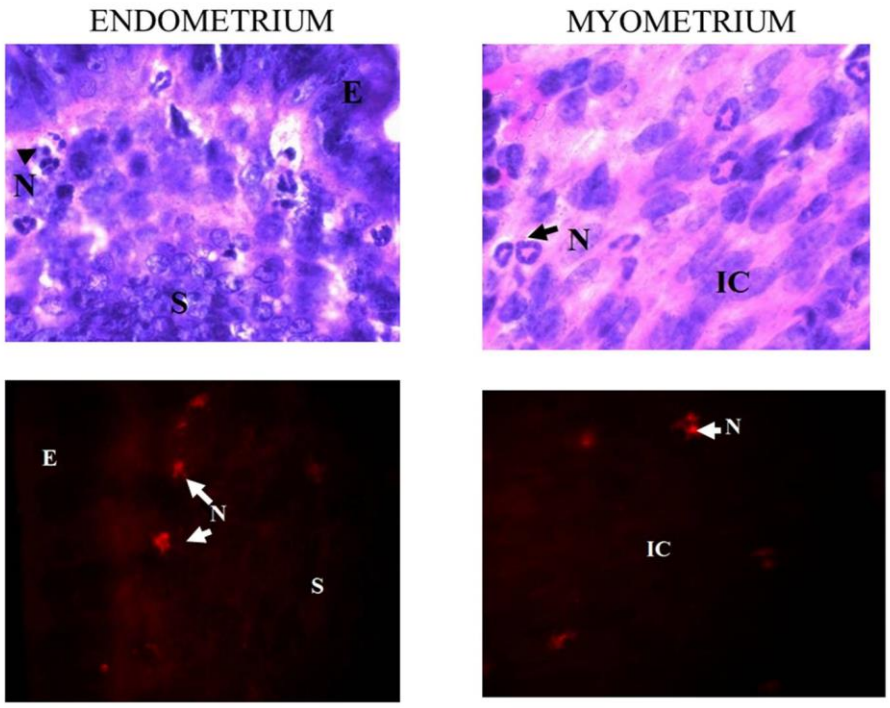

Fig. 2. Neutrophil infiltration in to the uterus during early metestral stage of estrus cycle

(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)

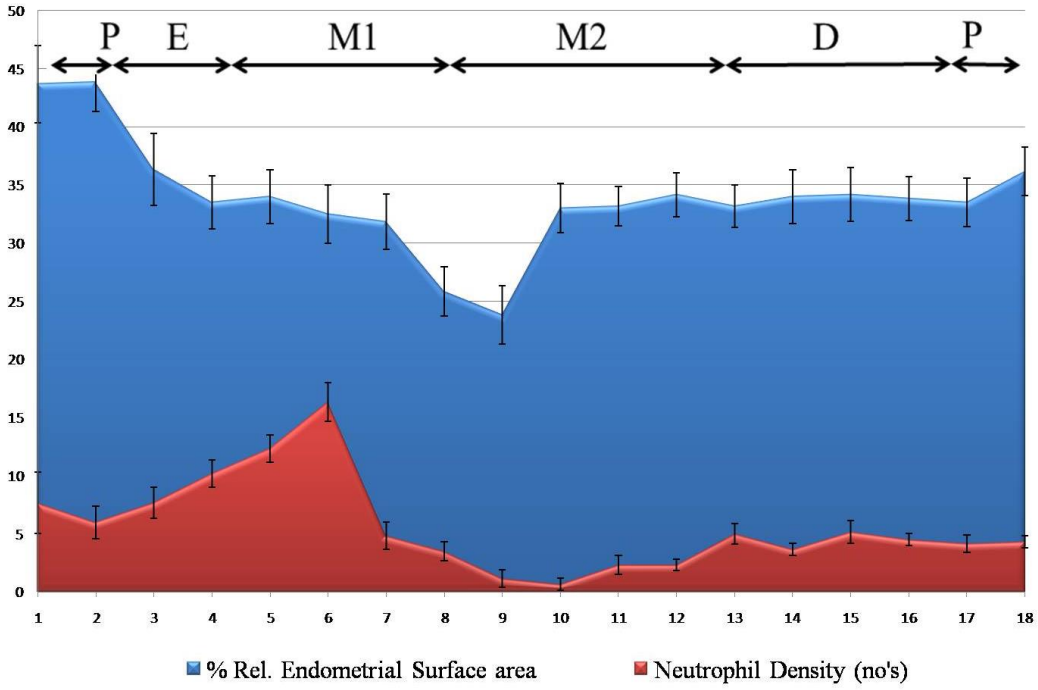

Fig. 3. Temporal changes in the Endometrial surface area and Neutrophil density during different stages of the estrus cycle

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

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 [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, maximum number of neutrophils migrated during estrus in sow [25]. While, it is maximum during the immediate premenstrual and menstrual phases of the cycle in humans [26]. These immigrated neutrophils accounted 6–15% of the total number of cells [27] and are responsible for the gross inflammation and edema in uterus [28].

Other immune cells that were found with neutrophils in the endometrial stroma include eosinophils, lymphocytes, macrophages and mast cells as in the endometrium of rats [29] and in mouse [30]. In our study we found that the population of neutrophils declined during late metestus (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 [31] and engulfment by macrophages [32] or They may have migrated reverse into the vascular vessels [33]. These events favored resolution of inflammation during late metestrus.

Fewer neutrophils were seen in the uterus post resolution ie., in late metestus 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 [34] 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 statum 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 [34] 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.

4. CONCLUSION

In our study, neutrophils were found present at all stages of estrus cycle in the uterus of mice. Their influx and distribution within the uterus was dynamic with changes in endometrial surface area. From our study we found that the variations in neutrophil density is tightly controlled and is timely regulated during different stages of the cycle. These findings will help detect any uterine pathologies associated with neutrophil recruitment and homeostasis at their nascent stage in other experimental research. However, neutrophil recruitment and their activation to perform specific function such as angiogenic, proinflammatory, anti inflammatory and immune function etc., should be dependent on the microenvironment in uterus as influenced by the ovarian hormones. Molecular mechanisms that control these numerous functions of neutrophils were to be unveiled to better understand their derangements in pathological conditions.

DISCLAIMER (ARTIFICIAL INTELLIGENCE)

Author(s) hereby declare that NO generative AI technologies such as Large Language Models (ChatGPT, COPILOT, etc) and text-to-image generators have been used during writing or editing of manuscripts.

ETHICAL APPROVAL

All the procedures for conducting this study were in strict accordance to the Animal Care and Use Committee, Seoul, South Korea.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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