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 tunicsduring 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 areawas maximum during late proestrus and early estrus. It reduced toits minimum levels inthe 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 metestrusand was minimum at late metestrus. Their density were moderate and static thereafter until early proestrus. These findings suggestedthat the rate of infiltration of neutrophils in to uterus is a controlled and stage specific process. Theircomplex role of inflammation, phagocytosis and endometrial repair in remodeling the uterus may be dependent on the uterine microenvironment at a given time point.

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Comment [TECNO KH62]: Briefly describe methodology.

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

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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 miceCousins *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], angiogenisis 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 andactivation 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.

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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 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 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 ± 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 $p \leq 0.001$.

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collected from 6 mice

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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 cellsturnedinto high columnar during estrus with numerous mucous secretory granuels 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 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 werefound 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 infiltratedneutrophils localizedcloser 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 preventedthis transepithelial migration of neutrophils into the lumen to favour survival of sperms forfertilization.Thesurface 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, theseneutrophils formedthe first line of innate immunity and are the most prominent line of cellular defense against invading microorganisms in uterusAlhussein and Dang[9]

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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 generatededema 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 thatthe population of neutrophils declinedduring 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 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 inflammationduring 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/restoringphenotypes as their numbers were maintained until late proestrus. These anti inflammatory subtypes may help clear cells and cellular debrisas 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 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. Neutrophilswere 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 vesselsin 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 Extracellualr 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 uterusand also to detect any uterine pathologies associated with neutrophil recruitment and homeostasis at their nascent stage.

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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- 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)

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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)

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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.