



RESEARCH ARTICLE

# Endometrial Nerve Fibers in Cycling Rhesus Monkeys: Immunohistochemical Study with Frozen Sections

Tatsuo Tomita <sup>1</sup>, Kunie Mah <sup>2</sup>

<sup>1</sup> Department of Integrative Biosciences, Oregon Health and Science University, Portland, OR

<sup>2</sup> Oregon National Primate Research Center, Beaverton, OR



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## ABSTRACT

The presence of nerve fibers in the eutopic primate and human endometrium is unsettled. Fine nerve fibers are present mostly at the endometrial-myometrial interphase, but nerve fibers have not been detected in the functionalis in the normally cycling primate and human endometrium. There are no reports on nerve fibers in the cycling primate and human endometrium. Fine nerve fibers in the functionalis have been reported from women with endometriosis but not women without endometriosis. Several authors claim that immunohistochemical findings of nerve fibers in the functionalis is diagnostic for endometriosis without mentioning menstrual cycle from the women studied for nerve fibers. The current study aimed to detect nerve fibers in the artificially cycling rhesus with uterine frozen sections using neurofilament, CD56 and PGP9.5 as markers for nerve fibers. We detected clumps of fine nonmyelinated nerve fibers together with a few myelinated nerves consistently at the basalis-myometrium interphase and in the basalis with partially spreading into the deep functionalis while there were no nerve fibers in functionalis from all menstrual phases. There were diffusely and thinly distributed fine nerve fibers in the myometrium from all cycling phases. In the rhesus endometrium, there were no nerve fibers in the upper and middle zone of cycling functionalis in both proliferative and secretory phases. The nerve fibers in the basalis grow into to the deep zone functionalis in the eutopic monkey endometrium, thus the normally present nerve fibers in the deep zone functionalis are not specific for endometriosis.

**Keywords:** endometrium, CD56, immunohistochemistry, neurofilament, nerve fiber, rhesus monkey

## Introduction

The presence of nerve fibers in the mammalian endometrium is still unsettled<sup>1</sup>. In rabbits, rats, mongoose and pig, nonmyelinated nerve fibers were detected in the endometrial-myometrium interphase mixed with few myelinated nerve fibers<sup>2</sup>. There were a few literatures on monkey and baboon endometrium revealing most nerve fibers at the endometrial-myometrium interphase and thinly and diffusely in the myometrium<sup>2-5</sup>. Most immunohistochemical studies had been previously performed in the routinely formalin-fixed and paraffin-embedded human endometrium: innervation of the human endometrium is significantly less than other parts of the uterus such as myometrium, horn and cervix<sup>2</sup>. There are small nonmyelinated nerve fibers at the endometrial-myometrium interphase and diffusely distributed small nerve fibers in the entire myometrium<sup>6-11</sup>. Many nerve fibers are located adjacent to the blood vessels, mostly arteries but some are freely distributed in the endometrial stroma while fine nerve fibers are located continuously from the basalis to the lower zone functionalis<sup>3</sup>. In women with endometriosis, there were significantly increased nerve fibers in the endometrial functionalis, which has claimed and used as a diagnostic marker for endometriosis<sup>7-16</sup>. Tokushige et al extensively studied immunohistochemical staining for nerve fibers in women with endometriosis using PGP9.5, NF and other markers for nerve fibers and claimed that this increased nerve fibers in the functionalis are diagnostic for endometriosis compared to the absent nerve fibers in women without endometriosis<sup>7,9-16</sup>. The others reported no correlation of the presence of functionalis nerve fibers in women with endometriosis<sup>17</sup>. In the eutopic women, the highest nerve density was reported in the myometrium with less density in the basalis and few in the functionalis<sup>7-12</sup>. There have been no reports on nerve fibers in the eutopic cycling women and rhesus monkeys and this study aimed to reveal nerve fibers in the artificially cycling rhesus monkeys. We used mostly neurofilament (NF) and CD56 as markers for nerve fibers. NF has been widely used as a highly specific marker for nonmyelinated A $\alpha$ , A $\beta$ , A $\delta$  and B fibers and myelinated adrenergic fibers<sup>18,19</sup>. CD56 is a marker for fine nerves, fine varicose and sensory nerve endings, cell membranes of ganglion cells and fetal striated muscle<sup>20</sup>. All previously reports on the human endometrium had been performed with routinely formalin-fixed and paraffin-embedded tissues<sup>8-17</sup>. We had previously demonstrated that immunostaining with frozen sections was superior to the formalin-fixed and paraffin-embedded tissues for immunostaining lymphatic vessels using LYVE-1 and blood vessels using von Willebrand factor<sup>21</sup> and extended immunostaining nerve fibers using frozen tissues from cycling and spayed rhesus monkeys.

## Materials and Methods

Animal care for the rhesus monkeys (*Macaca mulatta*) was provided by the veterinary staff of the Division of Animal Resources of the Oregon Primate Research Center, Beaverton, OR in accordance with NIH policy for the care and use of laboratory animals. Female rhesus monkeys were oophorectomized and treated sequentially with estradiol (E2) and progesterone (P) to induce menstrual cycles described before<sup>22-26</sup>. To induce cycles, the oophorectomized animals were first treated with 3cm Silastic capsules (Dow Corning Corp, Midland, MI) packed

with crystalline E2 (Sigma) to stimulate development of an induced proliferative phase. After 14 days, a 6cm Silastic capsule packed with crystalline P (Sigma) was implanted sc, and both implants remained in place for 14 days to induce the secretory phase. To induce menstruation, the P implant was removed while the E2 implant was left in place. Menstrual bleeding normally occurs 2-4 days after the P implant was removed. The human endometrium according to the exact menstrual cycle is hard to study from the ethical points of view and this study was undertaken with the artificially cycling rhesus monkeys. The numbers of the animals studied were as follows: the induced menstrual phase (3 days after removing P capsule, E-3p): 5 animals, early proliferative phase (E-7p): 4 animals, late proliferative phase (E-7p): 3 animals, early secretory phase (7 days after placing P capsule, E+ 7P): 3 animals and late secretory phase (E+14P): 4 animals to total 19 animals. A few animals were spayed for 5 months to simulating the postmeno-pausal state in women. Uteri were procured by necropsy under the appropriate anesthesia at the Oregon National Primate Center. With each uterine histologic sections, the endometrial thickness including the functionalis, basal and total thickness was measured on the H and E-stained tissue sections. For frozen sections, small fresh uterine tissues including myometrium (1 x 1 x 0.5cm) were embedded in OCT matrix (Fisher Scientific, Pittsburgh, PA) and were frozen in liquid propane in the liquid nitrogen as described before<sup>22-25</sup> and were frozen sectioned at 7 to 10  $\mu$ m sections. Frozen sections were mounted on Super Plus slides (Fisher Scientific), microwave-irradiated on ice for 3 sec, fixed in 2% paraformaldehyde in phosphate buffer at pH 7.4 for 10 to 15 min at room temperature and immersed twice for 2 min in 85% ethanol (22-25). To inhibit endogenous peroxidase activity, sections were incubated with a solution containing glucose oxidase (1U/ml) and sodium azide (10mml/ml) in PBS for 45 min at 25°C<sup>22-25</sup>. Then, sections were incubated with each diluted primary monoclonal NF and monoclonal CD56 and rabbit PGP9.5 antibodies, respectively at 4°C overnight as reported before<sup>22-25</sup>. After rinsing and immersion in blocking serum again, sections were incubated with second antibody (1 : 200 dilution) for 30 minutes at room temperature. Final visualization was achieved with the ABC kit (Vector Laboratory, Burlingame, CA) and 0.025 diaminobenzidine tetrahydrochloride in Tris-buffer pH7.6, 0.03 H<sub>2</sub>O<sub>2</sub> to induce brown color. The other sets of frozen sections were processed in the same way in the peroxidase method, then the final visualization was achieved by the alkaline phosphatase Vector Blue kit (SK 5300, Vector Laboratories, Burlingame, CA) for blue coloring. The source of the primary antibodies and each dilution of the antibody were listed as follows: Monoclonal human NF (clone 2F11), Dako System, Carpinteria, CA 1 :100 dilutions Monoclonal CD56, (clone 1E8C9), Dako System, Carpinteria, CA 1:100 Rabbit PGP9.5 (TGX 17039), Gene Tex, CA 1: 100

The Day 28 specimen (E + 14p) revealed the longest functionalis and total endometrial length (Table 1), and this specimen was studied with 30 $\mu$ m thick sections for peroxidase method and 10 $\mu$ m sections for alkaline phosphatase method, respectively for nerve fibers since this specimen had the most reliable possibility to show nerve fibers in case that nerve fibers were present in the functionalis.

Zonation of rhesus endometrium has been divided as: zone I-luminal epithelium, zone II-upper straight epithelium, zone III-middle gland segment and zone IV-bottom of the bland, basalis (3). In this study, we divided the zonation into three zones: zone I-upper functionalis, zone II-middle straight functionalis and zone 3-basalis as used in the zonation of the human endometrium (2).

## Results

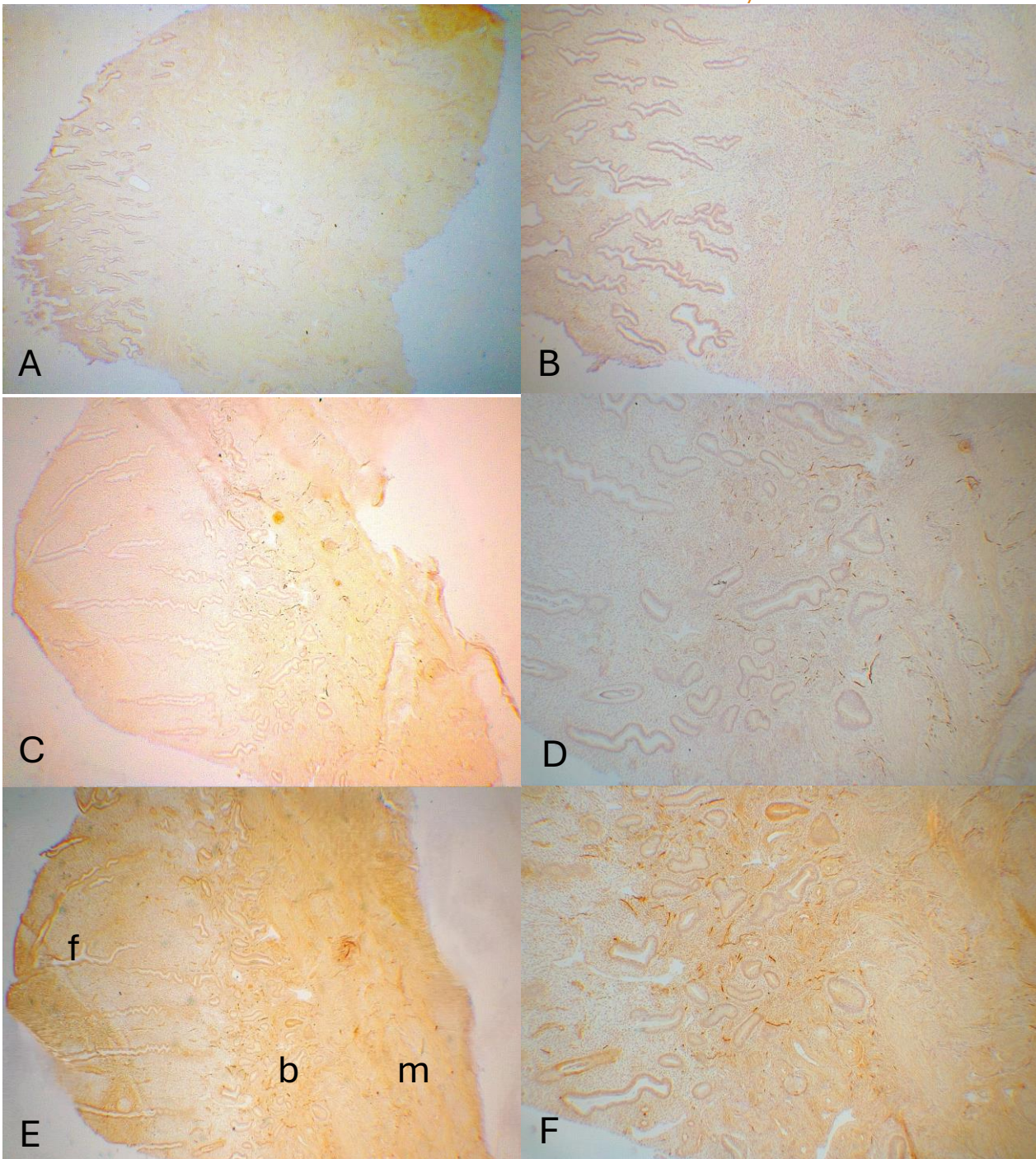
The E capsule has been placed during the entire cycling phases as listed as E. The three day after P capsule was removed stated as E – 3P while the 14<sup>th</sup> day with P capsule was placed after removing the previously placed p capsule as E + 14P. The day 3 (E – 3P, E continuously placed, and P removed for 3 days) endometrium consisted of thin sloughed off endometrium, corresponding to the remnant of basalis, which measured  $438 \pm 38 \mu\text{m}$  in thickness (Table 1). There were few or no NF immunostained small linear nonmyelinated vertical nerve fibers (20-40 $\mu\text{m}$  in length) by CD56 in the sloughed off residual basalis while there were consistently present thin, linear, nonmyelinated nerve fibers liner nerve fibers scattered in the entire myometrium with relatively more nerve fibers revealed by CD56 than by NF immunostaining (Fig. 1-A and -B). Day 7 (E – 7P) endometrium revealed elongating functionalis ( $1,575 \pm 75\mu\text{m}$ ) (Table 1) where there were a few linear nonmyelinated nerve fibers at the basalis-myometrium interphase and in basalis with scattered linear nonmyelinated nerve fibers in the myometrium using NF as a marker (Fig. 1-C and -D) and using PGP 9.5 as a marker (Fig. 1-E and -F). In the Day 14 (E – 14P) specimen, functionalis was more elongated ( $1,700 \pm 58\mu\text{m}$ ) and there were a few myelinated nerve fibers and several nonmyelinated nerve fibers at the basalis-myometrium interphase and nonmyelinated nerve fibers in the myometrium using both NF (Fig. 2-A and -B) and CD56 (Fig. 2-C and -D). The Day 21 (E + 7P) specimen showed more elongated functionalis ( $1,800 \pm 47\mu\text{m}$ ) (Table 1) with a few nonmyelinated nerve fibers and more

linear nonmyelinated nerve fibers in the basalis-myometrium interphase with a few nonmyelinated nerve fibers spreading into the superficial myometrium using NF (Fig. 2-E and -F) and DC56 (Fig. 2-G and -H). In the Day 28 ( $E \pm 14P$ ) specimen, there were most elongated functionalis ( $2,225 \pm 131\mu\text{m}$ ) (Table 1) with numerous thick nerve fibers in the 20  $\mu\text{m}$ -thick section immunostained with CD56 (Fig. 3-A and -B) and saw-tooth appearance at the upper luminal functionalis in the 10 $\mu\text{m}$  thick sections with a few myelinated nerve fibers and more nonmyelinated nerve fibers at the basalis-myometrium interphase with NF immunostaining using alkaline phosphatase (fig.3-C and -D) and diffusely, thinly scattered small linear nonmyelinated nerve fibers in the myometrium with both CD56 and NF immunostaining (Fig. 3-A to -D). Comparing immunostaining between peroxidase and alkaline phosphatase methods, alkaline phosphatase method detected fine non-myelinated nerve fibers (\*), which peroxidase method did not detect although the both methods detected thick non-myelinated and myelinated nerve fibers as well (Fig. 3-E and -F). In the above four cycling stages, there were no myelinated and nonmyelinated nerve fibers in the upper and middle zone functionalis using both NF and CD56 with peroxidase and alkaline phosphatase methods (Fig. 3-A to -D). In the specimens from rhesus spayed for 5 months, the endometrium was simulated for the postmenopausal endometrium where there were extremely thickened basalis (500-750 $\mu\text{m}$ ) with no attached functionalis, and there were thin, diffusely increased fine linear vertical nonmyelinated nerve fibers in the thickened basalis (Fig. 4- B to -D) by NF and CD56 immunostaining but there were no immunostained nerve fibers in the basalis using PGP9.5 staining (Fig. 4-E and -F). In the myometrium, there were markedly and diffusely increased fine linear nerve fibers with large nerve bundles, myelinated and numerous nonmyelinated nerve fibers around the cross sections of arteries with NF and CD56 (Fig. 4-A to -D) but partially immunostained by PGP9.5 around the arteries (Fig. 4-E and -F).

**Table 1.** Endometrial Thickness of Cycling Rhesus Monkeys

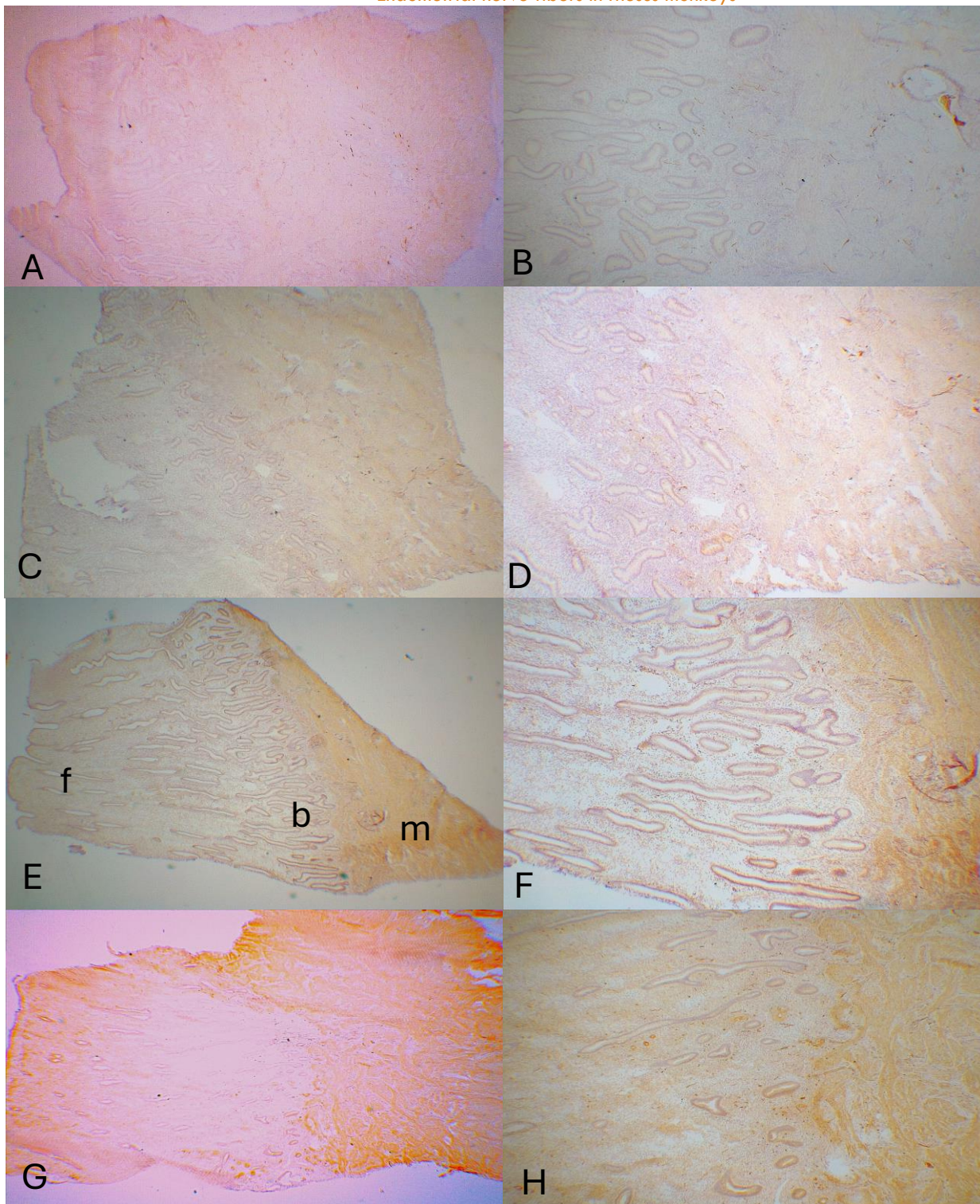
Menstrual phase (4) E-3p			
Animals	Basalis ( $\mu\text{m}$ )	Functionalis ( $\mu\text{m}$ )	Total length ( $\mu\text{m}$ )
1	400 $\mu\text{m}$	0 $\mu\text{m}$	400 $\mu\text{m}$
2	500	0	500
3	500	0	500
4	350	0	350
Mean $\pm$ SE	$438 \pm 38 \mu\text{m}$	$0 \pm 0$	$438 \pm 38 \mu\text{m}$
Early proliferative phase (4) E-7p			
1	400 $\mu\text{m}$	1,000 $\mu\text{m}$	1,400 $\mu\text{m}$
2	440	1,260	1,700
3	300	1,200	1,500
4	200	1,500	1,700
Mean $\pm$ SE	$335 \pm 47 \mu\text{m}$	$1,240 \pm 103 \mu\text{m}$	$1,575 \pm 75 \mu\text{m}$
Late proliferative phase (3) E-14p			
1	400 $\mu\text{m}$	1,000 $\mu\text{m}$	2,200 $\mu\text{m}$
2	400	1,600	2,000
3	300	1,700	2,000
Mean $\pm$ SE	$367 \pm 27 \mu\text{m}$	$1,700 \pm 58 \mu\text{m}$	$2,067 \pm 115 \mu\text{m}$
Early secretory phase (3) E +7p			
1	440 $\mu\text{m}$	1,700 $\mu\text{m}$	2,200 $\mu\text{m}$
2	400	1,800	2,200
3	200	1,900	2,100
Mean $\pm$ SE	$347 \pm 103 \mu\text{m}$	$1,800 \pm 47 \mu\text{m}$	$2,167 \pm 27 \mu\text{m}$
Late secretory phase (4) E+14p			
1	300 $\mu\text{m}$	2,000 $\mu\text{m}$	2,300 $\mu\text{m}$
2	300	2,100	2,500
3	400	2,200	2,600
4	400	2,600	3,000
Mean $\pm$ SE	$350 \pm 25 \mu\text{m}$	$2,225 \pm 131 \mu\text{m}$	$2,600 \pm 147 \mu\text{m}$





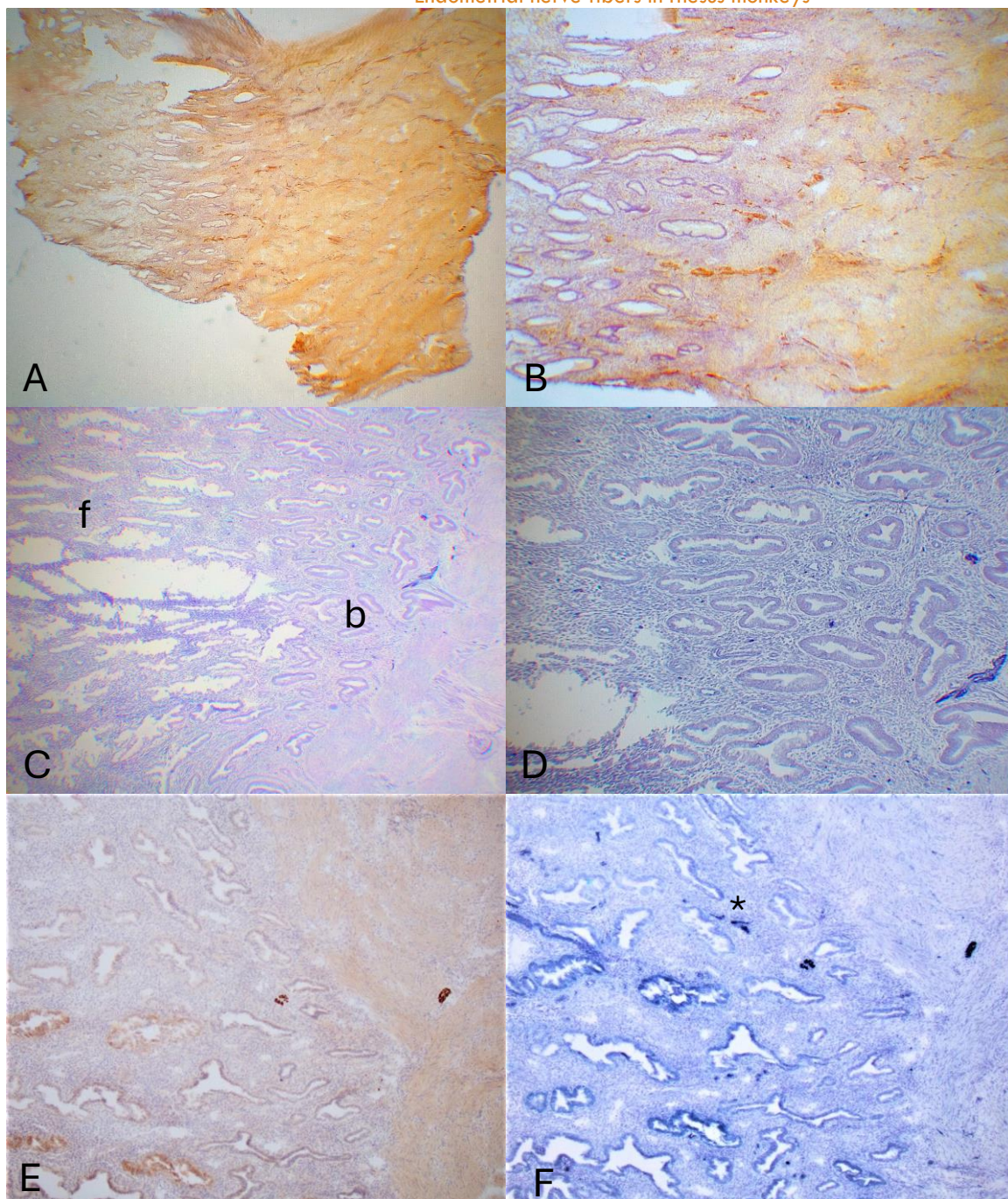
**Figure 1:** Day 3 (E - 3P) and Day 7 (E -7p) after removal of P In Day 3 endometrium, there were sloughed off thin remnants of basalis ( $438 \pm 38\mu\text{m}$ ) with few fine nerve fibers using CD56 while there were consistently distributed fine nonmyelinated nerve fibers in the myometrium (A-low power, B-high power). In Day 7, there were functionalis ( $1,240 \pm 103\mu\text{m}$ ) and several nonmyelinated nerve fibers at the basalis-myometrium interphase as well as scattered fine nerve fibers in the myometrium. (C-low power, D-high power) using NF. Similar nerve fibers were revealed at the basalis-myometrium interphase and myometrium using PGP9.5. (E-low power, F-high power) b:basalis, f:functionalis, m:myometrium A and B: CD56, C and D:NF, E and F: PGP9.5 immunostained





**Figure 2:** Day 14 (E-14p) after removal of P and Day 21 (E+7p) 7 days placing P Day 14 functionalis was more elongated ( $1,700 \pm 58\mu\text{m}$ ) containing several nonmyelinated nerve fibers with a few myelinated nerve fibers at the basalis-myometrium interphase and diffusely distributed nonmyelinated nerve fibers and one myelinated nerve fiber in the myometrium using NF (A-low power, B-high power) and slightly more nerve fibers revealed using CD56 (C-low power, D-high power). Day 21 functionalis was slightly more elongated ( $1,800 \pm 147\mu\text{m}$ ) containing several nonmyelinated nerve fibers at the basalis-myometrium interphase and diffusely distributed fine nonmyelinated nerve fibers in the myometrium using NF (C-low power, D-high power) and slightly more nerve fibers using CD56 (E-low power, F-high power). b:basalis, f:functionalis, m:myometrium A and B:NF, C and D:CD56, E and F: NF, G and H:CD56 immunostained





**Figure 3:** Day 28 (E + 14P) 14 days placing P Day 28 specimen was cut at 25 $\mu$ m in thickness to reveal more nerve fibers using CD56. The functionalis was the longest ( $2,225 \pm 13\mu$ m) and there were thick, darkly stained nerve nonmyelinated nerve fibers at the basalis-myometrium interphase and deep basalis using CD56 (A-low power, B-high power). In the 10 $\mu$ m sections with alkaline phosphatase method, one nonmyelinated nerve fiber (\*) and one myelinated nerve fiber was at the basalis-myometrium interphase and another myelinated nerve fiber was in the myometrium using NF (C-low power, D-high power). To compare the immunostaining between peroxidase method and alkaline phosphatase method using NF, both methods revealed myelinated nerve fibers (E-peroxidase, F-alkaline phosphatase) and only alkaline phosphatase method revealed one nonmyelinated nerve fiber (\*) (F). b:basalis, f:functionalis A and B:CD56, C and D: NF, E: NF with peroxidase method, F: NF with alkaline phosphatase method.

## Discussion

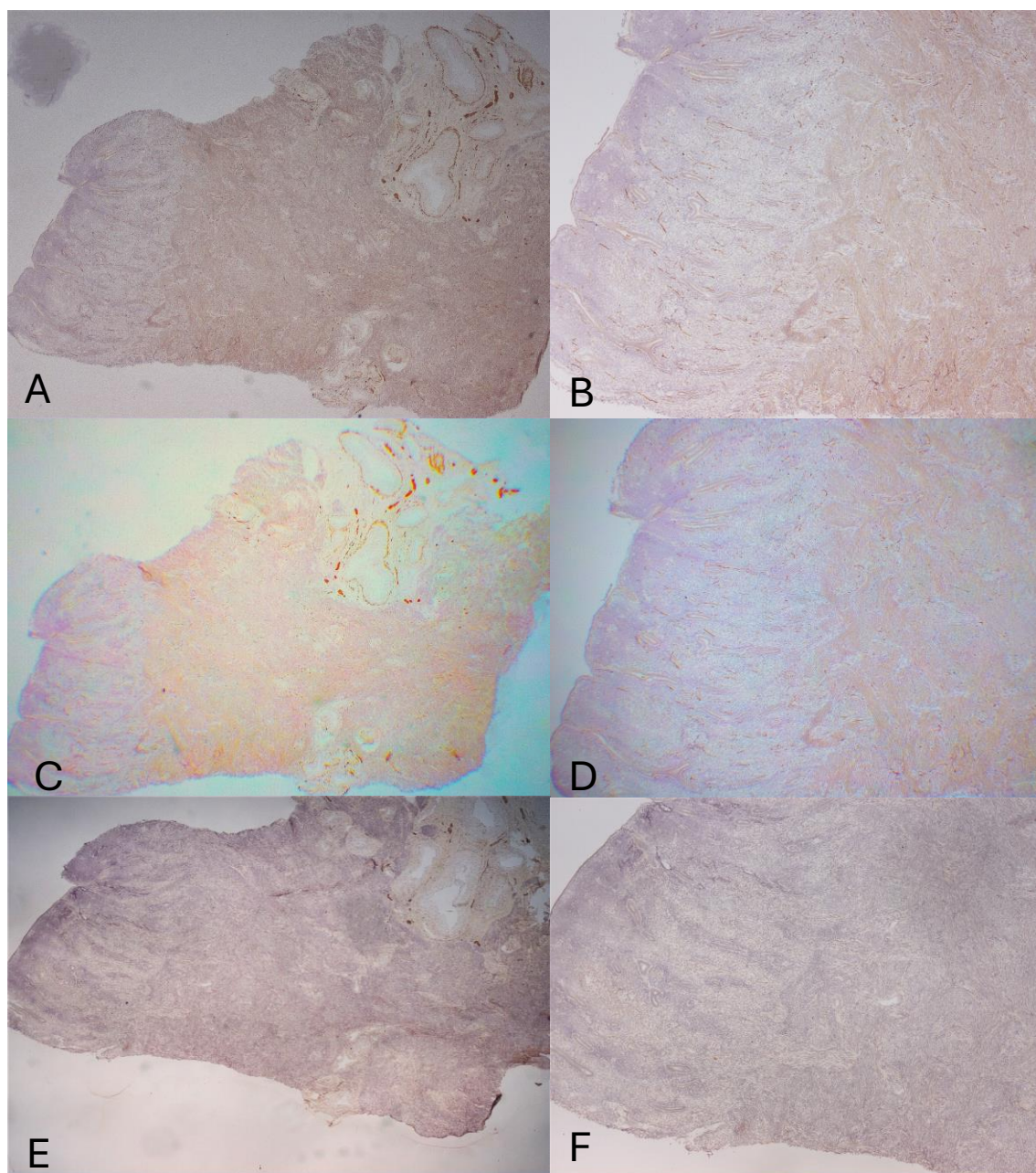
The presence of nerve fibers in the primate and human endometrium has not been settled. Nerve fibers in the endometrium are less than those in cervix and vagina<sup>30</sup>. In the eutopic rhesus monkey and baboons, nerve fiber density was the highest in the myometrium and there was no reported study of nerve density between basalis and myometrium<sup>4,5</sup>. In this study, rhesus monkeys were induced with menstrual cycle placing E capsule throughout the cycle, producing the proliferative phase, and placing P capsule to induce the secretory phase, then removing P

capsule to induce menstruation<sup>21-26</sup>. We studied the entire 28-day cycle studying nerve fibers in the endometrium and myometrium. In the cycling rhesus endometrium, there was consistent presence of fine nonmyelinated and occasional myelinated nerve fibers at the basalis-myometrial interphase, and myometrium was diffusely innervated with fine nonmyelinated fine nerve fibers. We did not detect nerve fibers in the upper and middle functional zones in the entire menstrual cycle in the rhesus endometrium. In the human uterus, there was consistent presence of nonmyelinated fine nerve fibers at the basalis-myometrial interphase and diffusely present fine nerve fibers in the



myometrium<sup>7-15</sup>. Many authors reported that nerve fibers were detected in the functionalis using NF and PGP9.5 in the endometrium from women with endometriosis but not in women without endometriosis<sup>7-15</sup>. A few authors reported no difference of nerve fibers in the functionalis between women with endometriosis and women without endometriosis<sup>8,10-16</sup>. Those who reported the presence of nerve fibers in the functionalis used it as a diagnostic tool for diagnosing endometriosis<sup>8,10-16</sup>. By laparoscopic biopsy, Boker et al and other further claimed that the presence of nerve fibers in the functionalis from women with mild and moderate endometriosis was at 95% sensitivity and 100% specificity<sup>13</sup>, to which we are skeptical since it is practically impossible to histopathologically define zonation of functionalis on the fragmented laparoscopy specimens. This claim must be verified by more study in a detailed study of nerve fibers regarding zones of functionalis and basalis since their biopsy-obtained specimens were not reliable for precise zonation of functionalis for

nerve fibers in the endometrium. The previously reported studies all used routinely formalin-fixed and paraffin-embedded tissues, which have proven to be not the best way to detect nerve fibers for lymphatic and blood vessels<sup>7-16</sup>. We used frozen section to detect nerve fibers in this study since immunohistochemical staining for nerve fibers has shown much more prevailing using frozen section compared to using formalin-fixed and paraffin-embedded tissues<sup>27</sup>. We used NF as a specific marker for nerve fibers, which immunohistochemically stains nonmyelinated A, B fibers and small fibers and we added CD56, which immunohistochemically stains thin sensory nerve fibers, fine varicose and sensory nerve endings and cell membrane of ganglion cells with better immunostaining for small nonmyelinated nerve fibers than NF in our experience<sup>27</sup>. PGP9.5 is a pan neural marker originally isolated from whole human brain<sup>28</sup> as is found neurons, peripheral nerves and neuroendocrine cells but is not specific for neural and neural sheath tumors<sup>29</sup>.



**Figure 4:** Uterus Spayed for 5 Months In the spayed uterus, the basalis was thickened (500 to 700 $\mu$ m) and there were diffusely distributed fine vertical nonmyelinated nerve fibers immunostained using both NF (A-low power, B-high power) and CD56 (C-low power, D-high power) but not immunostained using PGP9.5 (E-low power, F-high power). And B:NF, C and D: CD56, E and F: PGP9.5 immunostained

Tokushige et al extensively studied the presence of nerve fibers in the endometrium from women with endometriosis compared to women without endometriosis, mainly using NF and PGP9.5<sup>7-11</sup>. Tokushige et al further used alkaline phosphatase immunostaining method for nerve fibers since nerve fibers are very difficult immunohistochemically stain using peroxidase method, and alkaline phosphatase is more sensitive than peroxidase albeit the more nonspecific staining than peroxidase staining<sup>30</sup>. They reported as follows: compared to the normal peritoneum, there were increased nerve fibers in the peritoneal endometriotic lesions, which are dislocated basalis with no attached functionalis<sup>30</sup>. These nerve fibers were unmyelinated sensory C, a mixture of sensory A $\delta$  and myelinated adrenergic fibers<sup>8,30</sup>. However, there were much more nonmyelinated fine nerve fibers in the basalis compared to occasional presence of myelinated nerve in the rhesus monkeys in our study. They used CGRP (calcitonin gene-related peptide), neuropeptide Y (NPY) and substance P (SP) as markers for sensory C and sensory A $\delta$ <sup>8,30</sup>. They further used Vacht (vesicular acetylcholine transporter) for cholinergic, VIP (vasoactive polypeptide) for parasympathetic nerve and tyrosine hydroxylase for myelinated adrenergic nerve marker<sup>8,30</sup>. Tokushige et al further published nerve density of nerve fibers in the women untreated for endometriosis as follows: functionalis;  $11 \pm 5 \text{ mm}^2$ , basalis;  $18 \pm 8 \text{ mm}^2$  and myometrium;  $3 \pm 1 \text{ mm}^2$ <sup>9</sup>. Endometriosis is a dislocation of basalis, which is normally innervated with fine nerve fibers<sup>31</sup>, thus endometriotic lesions represented by basalis are also innervated with fine nerve fibers and there are no functionalis elements in endometriotic lesions. The presence of nerve fibers in the endometriotic lesions supports endometrial/progenitor cell theory, that resides in the basalis bases and endometrial progenitor cells give rise to endometrial, endothelial, nerve and stromal cells<sup>32,33</sup>. The five-month spayed animals simulated the postmenopausal women. The endometrium revealed thickened basalis with no functionalis, showing extensively increased nonmyelinated fine nerve fibers in the basalis by NE and CD56 immunostaining while there was no immunostained fine nerves using PGP9.5 immunostaining (Fig. 4-A, -C and -E). There were numerous nerve fibers detected around arteries in the myometrium, among which immunostaining using NF and CD56 stained circular nerve fibers around the arteries but PGP9.5 immunostained nerve fibers partially, not completely surrounding the arteries (Fig. 4-F). There were diffusely increased nonmyelinated fine nerve fibers in the entire myometrium with myelinated thick nerves adjacent to arteries by all NF, CD56 and PGP9.5 immunostaining at relatively less immunostained by PGP9.5 compared to NF and CD56 immunostaining (Fig. 4-A, -C and -E). As shown in the spayed rhesus, PGP9.5 is a pan neural marker and is not the best marker for nerve fibers compared to NF and CD56 since small nerve fibers in the endometrial

basalis may not be adequately immunohistochemically stained as shown in the spayed rhesus (Fig. 4-F). Compared to the normal peritoneum, Tokushige et al claimed increased nerve fiber in the peritoneal endometriotic lesions, which is a dislocated basalis, and the normal basalis is also innervated with fine nerve fibers same as the dislocated endometrium in the peritoneal endometriotic lesions<sup>31</sup>. Tokushige et al's original reports were the presence of nerve fibers in the functionalis from women with endometriosis, and absent nerve fibers in the eutopic women without endometriosis, the latter finding was the same for the cycling rhesus monkeys in our study<sup>7-11</sup>. They published nerve fibers without the zonation of the functionalis and their high-power microphotographs did not provide the precise zone location of nerve fibers in the endometrium<sup>7-11</sup>. There has not been reports regarding endometrial nerve fibers from normally cycling women and rhesus monkeys. In the myometrium, there were diffusely distributed sensory C and sensory A $\delta$  and a few myelinated adrenergic nerve fibers, which transmit menstrual and chronic pelvic pain in tandem with uterine contraction. Comparing nerve fibers between endometrium and myometrium, myometrium is consistently distributed with fine nerve fibers throughout the menstrual cycle, while basalis also is distributed with fine nerve fibers continuous from the endometrial-basalis interphase. But there are no nerve fibers in the upper and middle zone functionalis in both proliferative and secretory phases. In the secretory phase, a few fine nerve fibers infiltrate to the lower zone functionalis adjacent to the basalis. Thus, this normal secretory functionalis is distributed with a few fine nerve fibers and the presence of nerve fibers in this lower zone functionalis is not diagnostic for endometriosis from the current study. Reduced total nerve density was reported during estrus and nerve density recovered during menstruation in the rat<sup>34,35</sup>. Since no nerve fibers were detected in the upper and middle zone functionalis, menstrual and chronic pelvic pain would be less likely transmitted by endometrial nerve but more likely transmitted through the diffusely distributed, sensory non-myelinated nerve fibers in the myometrium. Uterine innervation in the endometrium may play significant roles in various conditions such as menstrual pain, chronic pelvic pain with or without endometriosis<sup>36,37</sup>, infertility, spontaneous abortion and others<sup>38-40</sup>.

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