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PGP 9.5- Immunoreactivity in The Ovary at Different Stages of Oestrous Cycle in Rats

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ABSTRACT

The reproductive process in female mammals is characterized by cyclic alteration in the female tract. During the oestrous cycle, the primordial follicle will be developed into the Graafian follicle before the ovulation and it takes place under the influence of hormonal control. This work was carried out to study the general pattern of innervation in the ovary at different stages of the oestrous cycle. Twenty-four adult female Sprague Dawley rats were used to perform the goal of the study. These rats were sacrificed after the detection of their cycle stage by vaginal smears and the ovaries were fixed and section using a frozen cryostat. Detection of nerve fibers was done using the immunohistochemistry technique. The results from this study showed that there is innervation in the ovary throughout the oestrous cycle indicated by the presence of PGP 9.5-immunoreactive nerve fibres. The number of nerve fibres found during each stage of the oestrous cycle significantly varies; the nerve fibre count during the oestrus stage was significantly higher ($P < 0.05$) than the nerve fibre count at proestrus, metestrus, and diestrous stage. In conclusion, immunohistochemistry PGP 9.5 marker was a useful approach and indicator for nerve fibers distribution in organs which can be changed with different physiological conditions.

INTRODUCTION

The oestrous cycle of laboratory rodents has been most extensively studied (Goldman et al., 2007; Byers et al., 2012). Rats and mice are considered ideal examples of poly-estrous mammals. In the rats, the oestrous cycle lasts about 4 to 5 days. There are a lot of changes that occur in the ovary throughout an oestrous cycle. The ovulation will take place at the later stage of oestrus and it has been postulated as an inflammatory process.

Many recent immunohistochemical approaches were applied to investigate possible changes of immunoreactive nerve fibers such as Protein Gene Product (PGP) 9.5 in different tissues and organs of the body in both humans and large, small, or lab animals. The PGP 9.5 was considered excellent indicators for such innervations changes occurred due to normal or pathological events in different organs, i.e. intestine (Vento and Soinila, 1999), cervix (Tingaker et al., 2006), skin (Tokushige et al., 2006), tonsils (Yamaoka et al., 2007), synovial joints (Al-Saffar et al., 2011a; Al-Saffar et al., 2011b), uterus (Zagólski et al., 2016), etc. In fact, Protein Gene Product 9.5 is a general neuronal marker to mark nerve fibers. It is a neuron-specific protein, structurally and immunologically distinct from neuron-specific enolase. Standard immunohistochemical techniques have demonstrated the presence of PGP 9.5 in neuron and nerve fibers at all levels of the central and peripheral nervous system, in many neuroendocrine cells, in segments of renal tubules, in spermatogonia and leydig cells of the testis, ova and in some cells of both pregnant and non-pregnant corpus luteum (Wilson et al., 1988). However, very little or nothing is stated about innervations during oestrous cycle.

The objective of the current study is to identify by immunohistochemical approach the innervations in the ovarian parenchyma during different stages of the oestrus cycle by using PGP 9.5 marker as an indicator for ovarian immunoreactive nerve fibers in the rat's ovary.

MATERIALS AND METHODS

Animals:

Twenty-four adult female Sprague Dawley rats, regularly cycling were used in this study. They were kept in two polypropylene cages with wire mesh top designed to hold food and water bottle, sized 40 x 25 x 15cm, and fed *ad libitum* with commercial feed. Their tails were labelled with non-toxic permanent marker pens for identification. The procedure conducted for this experiment was approved by the Animal Care and Use Committee of Universiti Putra Malaysia (Ref. No. UPM/FPV/PS/3.2.1.551/AUP-R105).

Stages of Oestrous Cycle:

Vaginal smears were taken to identify the different stages of the oestrous cycle based on the type of cells present in the smears (Marieb, 1997). During oestrus stage, a very high number of large cornified non-nucleated epithelial cells were present. However, during the proestrus stage, there were more of nucleated epithelial cells but very few keratinized epithelial cells. Leucocytes were also present during this stage. During the metestrus stage, very few keratinized cells and leukocytes were present compared to the proestrus stage.

Vaginal smears of 24 rats were evaluated at 0800 daily whereby the outer surface of the vagina was flushed with a few drops of normal saline. The normal saline was smeared on a clean glass slide and allowed to dry. The slides were then stained with Giemsa stain for 20 minutes before observing the cells under a light microscope at 40X magnification. The different phases of the oestrus cycle were determined by observing the proportion of cells present in the vaginal smear. Leucocytes were predominantly present during the diestrous phase (Fig. 1A), nucleated epithelial cells were predominant during proestrus (Fig. 1B), oestrus phase (Fig. 1C) had a majority of keratinized cells and metestrus phase (Fig. 1D) had an equal proportion of nucleated epithelial cells, keratinized cells, and leucocytes in the vaginal smear.

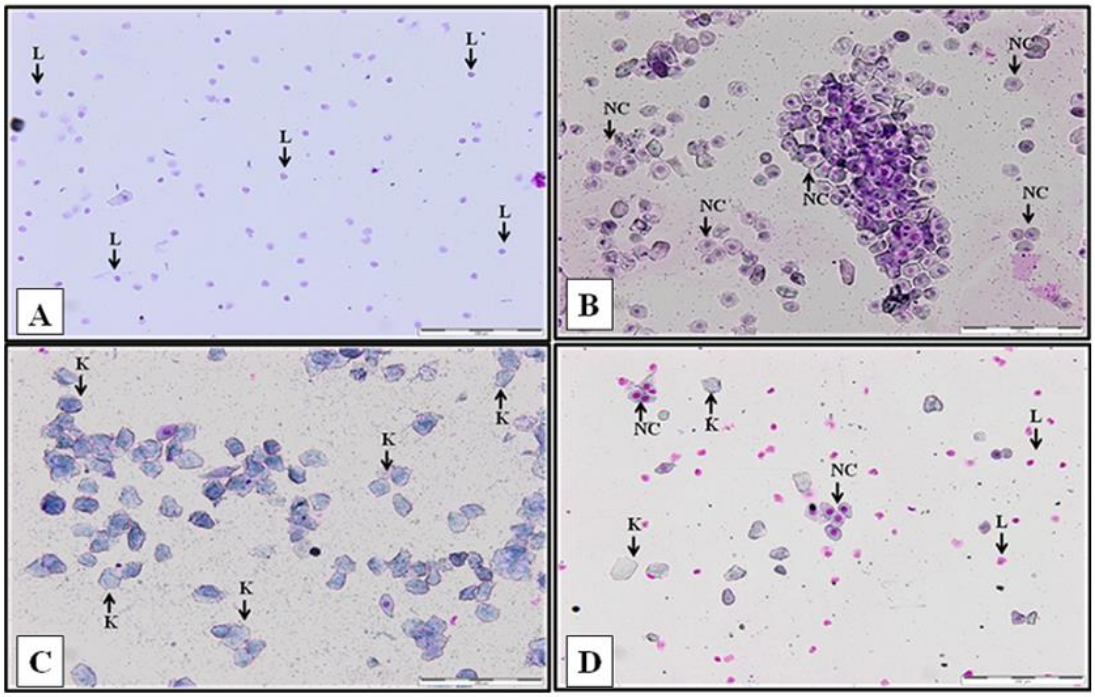

Fig. 1: Vaginal smears stained with Giemsa at different stages of oestrus cycle of rats. A: Diestrous stage showing predominance of leucocytes (L), 40X. B: proestrus rat showing predominance of nucleated epithelial cells (NC), 20X. C: Oestrus rat showing predominance of keratinised cells (K), 20X. D: metestrus rat showing an equal amount of leucocytes (L), keratinised cells (K), and nucleated epithelial cells (NC), 40X.

Ovaries Preparation:

Six rats from each stage of the estrous cycle were euthanized and their ovaries were collected and fixed in 4% paraformaldehyde for 24 hours. The ovaries were then transferred into 20% Sucrose for 2 to 3 hours and subsequently sectioned using a frozen cryostat**.** Each section (8µm thickness) was placed onto the chromium alum gelatin-coated glass slides.

Immunohistochemistry:

The samples were fixed in 4% paraformaldehyde and then processed for immunohistochemistry. Briefly, the samples were frozen in isopentane, cooled in liquid nitrogen, and sectioned at 8µm in a cryostat. The sections were dehydrated in alcohol, rinsed in 0.1M phosphate-buffered saline, then incubated in the primary antisera: anti-protein gene product 9.5 [PGP 9.5] for 24 hours at 4ºC. Sections were incubated in secondary antiserum (for 1 hour), goat anti-rabbit IgG, followed by avidin- biotinylated horseradish peroxidase complex for another hour. Finally, sections were immersed in glucose diaminobenzidine nickel substrate, washed in distilled water, stained with eosin, and then mounted with DPX. The immunoreactive nerve fibers were identified using a light microscope under 10 x magnifications and the number of the nerve fibers present for each cycle stage was counted and recorded.

Statistical Analysis:

One-way ANOVA was used to analyse the number of immunoreactive nerve fibres that were present.

RESULTS AND DISCUSSION

Light microscopic examination of the ovarian sections that were processed by immunohistochemical technique and stained by PGP 9.5 marker revealed the presence of PGP 9.5-immunoreactive nerve fibres throughout the ovary at all stages of

the oestrus (Fig. 2). The nerve fibers were well distributed in the medullary and cortical parenchyma. They were obviously recognized adjacent to the medium (secondary) and large (tertiary) ovarian follicles. They were invested well in vascular supplements that were blood capillaries and sinusoids. The presence of immunoreactive nerve fibres was mainly distributed around the follicles, so it suggests that the innervations focus more on the maturation of ova and subsequent ovulation. The statistical analysis (Table 1) showed significant differences ($P<0.05$) in the number of nerve fibers during the fourth different stages of the oestrus cycle of the studied female Sprague Dawley rats. The number of PGP 9.5 immunoreactive nerve fibres were significantly higher in ovaries taken at oestrus stage (293.17 ± 1.56), followed by proestrus (273.00 ± 1.37), diestrous (230.54 ± 1.23) and metestrus (191.67 ± 1.05).

The previous postulation was considered ovulation as a similar process to that of the inflammation such as abscess formation. This thought was based on the fact that during abscess formation, the inflammatory process will be at its peak when the abscess is fully formed. This phenomenon is very similar to what usually found during follicular maturation, where the inflammatory surge is at its peak just before ova are released (Westwood, 2008). These records and considerations were further supported by the findings of the current study where during oestrus stage, a very high number of large cornified non-nucleated epithelial cells were present. However, during proestrus stage, there were more nucleated epithelial cells but very few keratinized epithelial cells. Leucocytes were also present during this stage. During the metestrus stage, there were very few keratinized cells and leukocytes were present compared to proestrus stage.

The existence of large number of immunoreactive nerve fibres around the immature and mature follicles (secondary and tertiary) appeared related well to the end process of the oestrous cycle i.e. the ovulation. These signs may be due to hormonal changes (FSH and LH) of the pituitary glands and their effects on follicular development and maturation (Al-Saffar and Almayahi, 2018). The reference recorded a high degree of vascularization around these above follicles in normal mature and immature does. The vascular system provided a good communication between follicles and the pituitary axis throughout the reproductive cycle.

Within the current decade, Al-Saffar *et al.* (2011a, b) recorded a significantly lowest number of PGP 9.5 immunoreactive nerve fibres in the synovial membrane of rat's knee joints suffered osteoarthritis. The researchers found a high number of PGP 9.5 immunoreactive nerve fibres in the joints of the normal control rats. Accordingly, a concept can be made that the PGP 9.5 marker plays an important role to explore density as well as changes in the number of immunoreactive nerve fibres in any organ or tissue. These markers are significant immunohistochemical indicators for innervations in both normal and pathological conditions.

The significant distribution of PGP 9.5-immunoreactive nerve fibers in the ovary of the rats was in good agreement with the records of Kimaro and Madekurozwa (2006) in their application of this indicator on the ovary of a sexually immature ostrich. They observed nerve bundles coursed through the ovarian stalk and extended into both medulla and cortex and specifically the PGP 9.5 immunoreactive nerve fibers were present in the thecal layer of the follicular wall.

The density of PGP9.5-immunoreactive nerve fibers which was found currently critical in the

normal physiological conditions of the rat's estrous cycle stages also recorded important in pathological conditions. It was recorded recently as a useful indicator in cases of peritoneal endometritis in women (Yao *et al.*, 2010). The reference recorded in endometriosis patients with pain higher density of PGP9.5-immunoreactive nerve fibers than those patients without pain.

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It can be concluded that the immunohistochemistry PGP 9.5 marker was a useful approach and indicator for nerve fibers distribution in ovarian tissue which can be changed with different physiological conditions and such characteristic features could be a useful technique for both physiological and pathological conditions

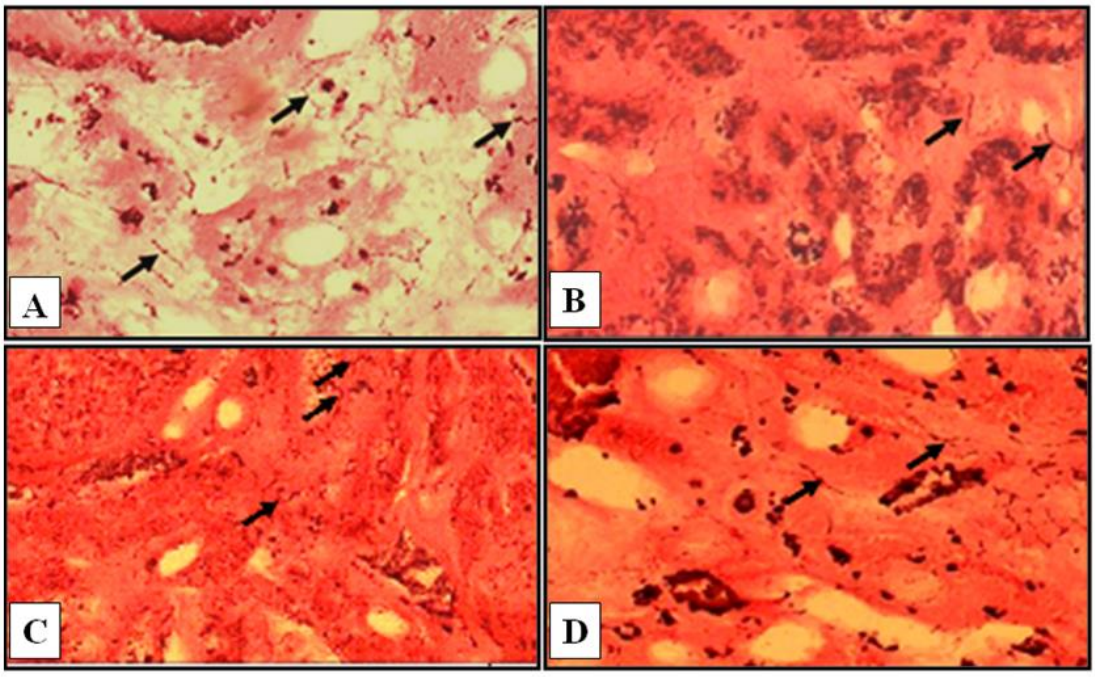

Fig. 2: Immunohistochemically stained ovarian sections at different stages of rat oestrus cycle showed immunoreactive nerve fibers for PGP 9.5 (arrow points) during proestrus (A), estrus (B), metestrus (C) and diestrous (D), X10.

Table 1. The mean (± SE) number of nerve fibres in the ovaries of a rat during different stages of oestrous cycle

Stages of oestrous cycle	No. of rats	Mean (±se) no. of nerve fibre
Proestrus	6	273.00 ± 1.37 [b]
Oestrus	6	293.17 ± 1.56 [a]
Metestrus	6	191.67 ± 1.05 [d]
Diestrous	6	230.54 ± 1.23 [c]

[a, b, c, d] Mean with different superscripts within the same column are significantly different ($P<0.05$)

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Conflict of Interest:

The authors declare no conflict of interest

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