



Histoenzymic Localization of Phosphatases, Oxidoreductases and Nonspecific Esterase in the Uterus of Canines Collected After Ovariohysterectomy

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Abstract

The present investigation was aimed at elucidating the localization patterns of the phosphatases, oxidoreductases, and non-specific esterase in the uterus of canines collected after ovariohysterectomy and correlating these enzymes with the stage of reproduction and disease conditions. Cryostat sections of 6-7 μ thickness at -20°C were obtained on glass slides with cryostat microtome and incubated with substrates of different enzymes. A strongly positive reaction of phosphatases and oxidoreductases was seen in the epithelium lining the endometrium and endometrial glands, and blood vessels in the prepubertal, cyclical, and gravid uterus. There was spatial localization of enzymes some of the enzymes (AKPase) were localized in the basal region of epithelium and glands which might be indicative of cellular transport towards the basal compartment while most of the enzymes were localized in the apical region of the cells or in dispersed in the cytoplasm of the cells. The reaction of these enzymes in the diseased uterus was strong to intense in papillary endometrial hyperplasia, cystic papillary endometrial hyperplasia, and cystic endometrial hyperplasia and that could be due to degenerative changes occurring due to infections. It was concluded that the reactivity was higher in the epithelium, glands of pubertal and pregnant animals as compared to the prepubertal group, while the reactivity of most of the enzymes was stronger in diseased animals.

Keywords: Canines; Esterase; Histoenzymic; Oxidoreductases; Phosphatases; Uterus

Abbreviations

AKPase: Alkaline Phosphatase; G-6-Pase: Glucose-6-Phosphatase; G-6-PD: Glucose-6-Phosphate Dehydrogenase; LDH: Lactic Dehydrogenase; SDH: Succinate Dehydrogenase; NADHd: Nicotinamide Adenine Dinucleotide Diaphorase; NADPHd: Nicotinamide Adenine Dinucleotide Phosphate Diaphorase; NS: Nonspecific Esterase

Introduction

The dog is the most important companion animal because of its docility, reliability, and enjoyable disposition. Thus, the dog is a very important patient at the veterinary clinic. In recent years, the involvement of veterinarians is escalating in the field of canine reproductive assessment and care. Successful reproduction in canines requires a healthy genital tract. The canine patients are

brought to the veterinary hospital for ovariohysterectomy either for neutering or for treatment of disease. Neutering is usually advised in canines after one year of age but is sometimes chosen by owners earlier too. A disorder called cystic endometrial hyperplasia - pyometra complex (CEH-P) is the most severe and most often diagnosed clinical condition of the uterus in canines.

The uterus is a muscular tube, which is one of the most important organs in the body in terms of reproduction. This provides a suitable atmosphere for the implantation of the fertilized ovum and successive development of the placenta. This is how the developing fetus is nurtured throughout pregnancy. At the time of birth, intense contractions of the muscular layer of the uterus expel the fetus through the lower part of the uterus, the uterine cervix, into the birth canal. The uterine environment is modified because of its tissues comprising luminal and glandular epithelia, stroma, and immune cells and the influence of ovarian steroids which determine the availability.

Enzymes are essential for most of the processes of cell metabolism. Phosphatases are the crucial enzymes in liberating and reprocessing the phosphate molecules that are essential for biological processes like cell differentiation and proliferation, gene expression, and several metabolic processes. The role of phosphatases is implicated in the process of implantation in rodents [1]. Roy, *et al.* have reported the presence of phosphatase enzymes in the uterine secretions of buffalo [2].

So, keeping in view the paucity of literature available, this study is proposed to elucidate the histoenzymic localization of phosphatases, oxidoreductases, and nonspecific esterase in the uterus of canines collected after ovariohysterectomy.

Materials and Methods

Materials

The uteri from female canines (n = 54) were presented to the teaching Veterinary and Clinical Complex, GADVASU, Ludhiana for neutering and disease conditions where ovariohysterectomy performed. The permission for the use of study materials was received from the institutional animal ethics committee (IAEC), Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana (Registration no. 497/GO/Re/SL/02/CPCSEA) before the study and the protocol number was (GADVASU/2021/IAEC/59/01).

Cryo-sectioning

Fresh unfixed tissues were placed in a tissue freezing medium (Leica) and frozen in liquid nitrogen. Cryostat sections of 6-7 μ

thickness at -20°C were obtained on glass slides with cryostat microtome as described earlier [3].

Localization of enzyme

The cryosections were incubated with different substrates for the demonstration of phosphatases, oxidoreductases, and esterases. The phosphatases included alkaline phosphatase (AKPase) and glucose-6-phosphatase (G-6-Pase) whose activity was tested as suggested by Barka and Anderson [4]. The oxidoreductases included succinic dehydrogenase (SDH), lactate dehydrogenase (LDH), glucose-6-phosphate dehydrogenase (G-6-PD), and reduced nicotinamide adenine dinucleotide phosphate diaphorase (NADPH-diaphorase), and reduced nicotinamide adenine dinucleotide diaphorase (NADH-diaphorase) whose activity was tested using the protocol described [5]. The positive and negative controls were used wherever possible.

Evaluation of staining intensity

Staining intensity was evaluated blindfolded by two of the co-authors, an anatomist (corresponding author) and a pathologist (Dr. Kuldip Gupta). Based on the observations, staining intensity was classified as no staining (0), weak staining (+), moderate (++), strong (+++) and intense staining (++++).

Results and Discussion

Phosphatases

The relative intensity of localization of phosphatases has been recorded in table 1.

Alkaline phosphatase

A strongly positive reaction of AKPase was seen in the epithelium lining the endometrium and endometrial glands of prepubertal animals (Figure 1A-B). The localization of enzyme was in the basal region of epithelium and glands which might be indicative of cellular transport. Alkaline phosphatase is a lysosomal enzyme that catalyzes various reactions in the body and is involved in the active transport of protein and DNA turnover in the nucleus [6]. A weak to the moderate reaction was observed in the stroma (Figure 1C) and muscle layers (Figure 14D) in prepubertal animals. During the pubertal life, a moderate positive reaction was observed in the epithelium of the endometrium and a strong reaction in the glands (Figure 1E). Like the prepubertal group, the reaction was localized in the basal compartment of the glands. A moderate to weak positive reaction was seen in the connective tissue. The variable reaction was seen in the blood vessels. In the cystic pyometra uteri, the cyst wall and papillary projections also showed a strong positive

reaction. The reaction was also strong in larger muscular vessels. AKPase is known to play a role in the metabolism of phosphate esters [7]. The activity of alkaline phosphatase in the uterus of Marwari goats has been investigated [8]. On the second day of ovulation, alkaline phosphatase activity was lowest in all tissues, then increased sharply from the third day and peaked in uterine tissues on the ninth day. A mild alkaline phosphatase response was detected at the distal edge of the surface epithelium of a cow's uterus [9]. Alkaline phosphatase activity was shown to be inversely related to the uterine glands. Similar observations were recorded regarding AKPase localization in the gravid uterus of goats [10]. It has been proposed that phosphatases in buffalo uterine tissue might be involved in anaerobic glycolysis since alkaline phosphatases are known to promote glucose transfer for endometrial carbohydrate metabolism [11].

| Group | Layer | Sub-compartment | AKPase | G-6-Pase |
|-------------|-------------|----------------------|--------|----------|
| Prepubertal | Endometrium | Lamina epithelialis | +++ | ++ |
| | | Stroma | ++ | ++ |
| | | Glands | ++++ | ++++ |
| | Myometrium | Inner circular | ++ | ++ |
| | | Outer longitudinal | + | + |
| | | Stratum vasculare | +++ | +++ |
| Perimetrium | | 0/+ | 0/+ | |
| Pubertal | Endometrium | Lamina epithelialis | +++ | +++ |
| | | Stroma | ++ | ++ |
| | | Glands | ++++ | ++++ |
| | Myometrium | Inner circular | ++ | ++ |
| | | Outer longitudinal | ++ | ++ |
| Perimetrium | | | | |
| Pregnant | Endometrium | Lamina epithelialis | ++++ | ++++ |
| | | Stroma | ++ | ++ |
| | | Glands | ++++ | ++++ |
| | Myometrium | Inner circular | + | + |
| | | Outer longitudinal | + | + |
| Perimetrium | | +++ | +++ | |
| Diseased | Endometrium | Cyst wall | +++ | +++ |
| | | Papillary projection | ++++ | ++++ |
| | | Stroma | +++ | +++ |
| | Myometrium | Inner circular | + | + |
| | | Outer longitudinal | ++ | ++ |
| Perimetrium | | + | + | |

Table 1: Histoenzymic localization of phosphatases in the canine uterus.

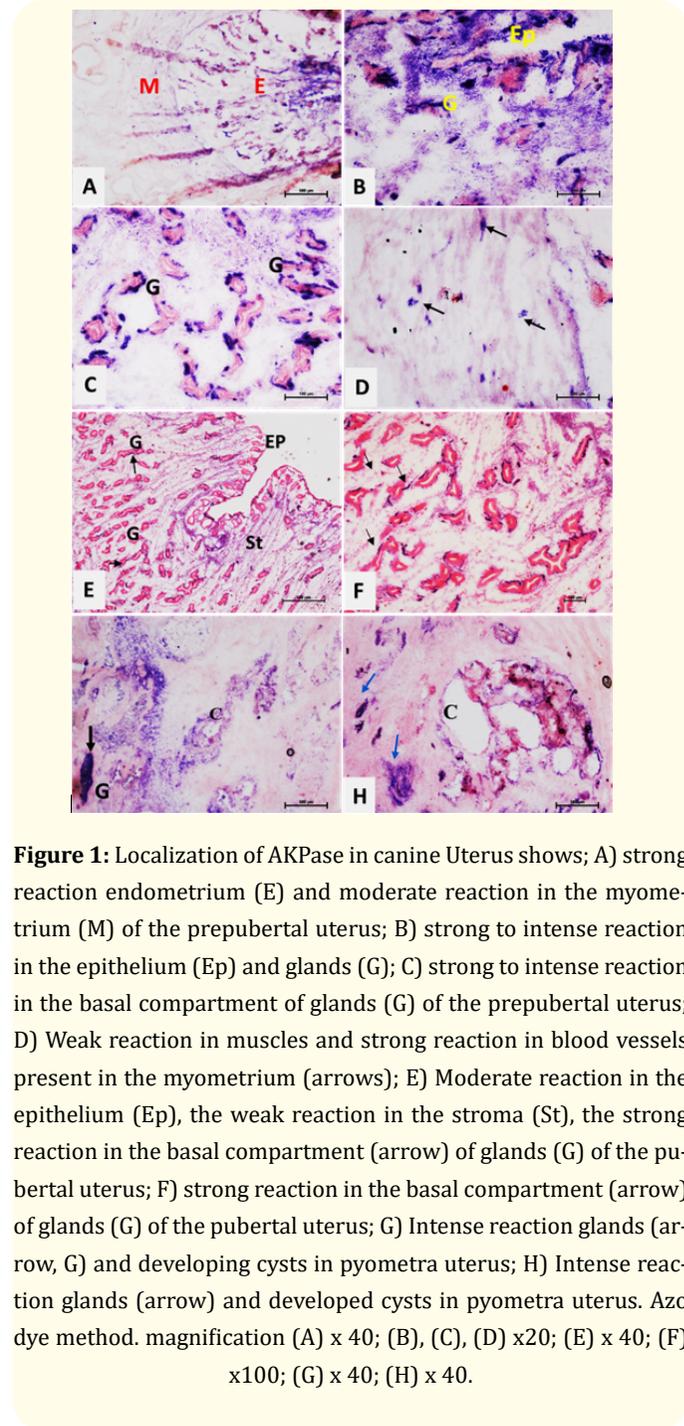


Figure 1: Localization of AKPase in canine Uterus shows; A) strong reaction endometrium (E) and moderate reaction in the myometrium (M) of the prepubertal uterus; B) strong to intense reaction in the epithelium (Ep) and glands (G); C) strong to intense reaction in the basal compartment of glands (G) of the prepubertal uterus; D) Weak reaction in muscles and strong reaction in blood vessels present in the myometrium (arrows); E) Moderate reaction in the epithelium (Ep), the weak reaction in the stroma (St), the strong reaction in the basal compartment (arrow) of glands (G) of the pubertal uterus; F) strong reaction in the basal compartment (arrow) of glands (G) of the pubertal uterus; G) Intense reaction glands (arrow, G) and developing cysts in pyometra uterus; H) Intense reaction glands (arrow) and developed cysts in pyometra uterus. Azo dye method. magnification (A) x 40; (B), (C), (D) x20; (E) x 40; (F) x100; (G) x 40; (H) x 40.

Glucose-6- phosphatase (G-6-Pase)

The activity of glucose-6-phosphate dehydrogenase was found to be moderate to strong in the luminal epithelium, the glandular epithelium (Figure 2A-B), and moderate in the circular and longitudinal layers of the myometrium, and weak reaction in perimetrium

(Figure 2C). An intense reaction was observed in the epithelium and glands of the pubertal canine uterus (Figure 2D). This enzyme is known to be involved in glucose metabolism. Higher activity of these phosphatases was implicated in higher secretory activity. The activity was more intense in the basal region of cells of glands (Figure 2E). A moderate to weak positive reaction was seen in the connective tissue. A weak to moderate reaction was observed in the myometrium (M) and perimetrium (P) of the pubertal uterus (Figure 2F). The variable reaction was seen in the blood vessels. There was an intense reaction in the lining epithelium (Ep) and cysts (C) cysts in the pyometra uterus (Figure 2G) and a strong reaction in cysts (C) and papilla (P) in the pyometra uterus (Figure 2H). Uniformly moderate reactions were observed in lamina epithelialis, endometrial glands, and myometrium of buffaloes [11]. The activity of glucose-6-phosphate dehydrogenase was found to be high in the luminal epithelium (4.7 ± 0.1), the glandular epithelium (4.1 ± 0.1), and caruncles (4.3 ± 0.1) layers of the myometrium, and moderate in the circular (3.0 ± 0.1) and longitudinal (2.6 ± 0.1) layers [12].

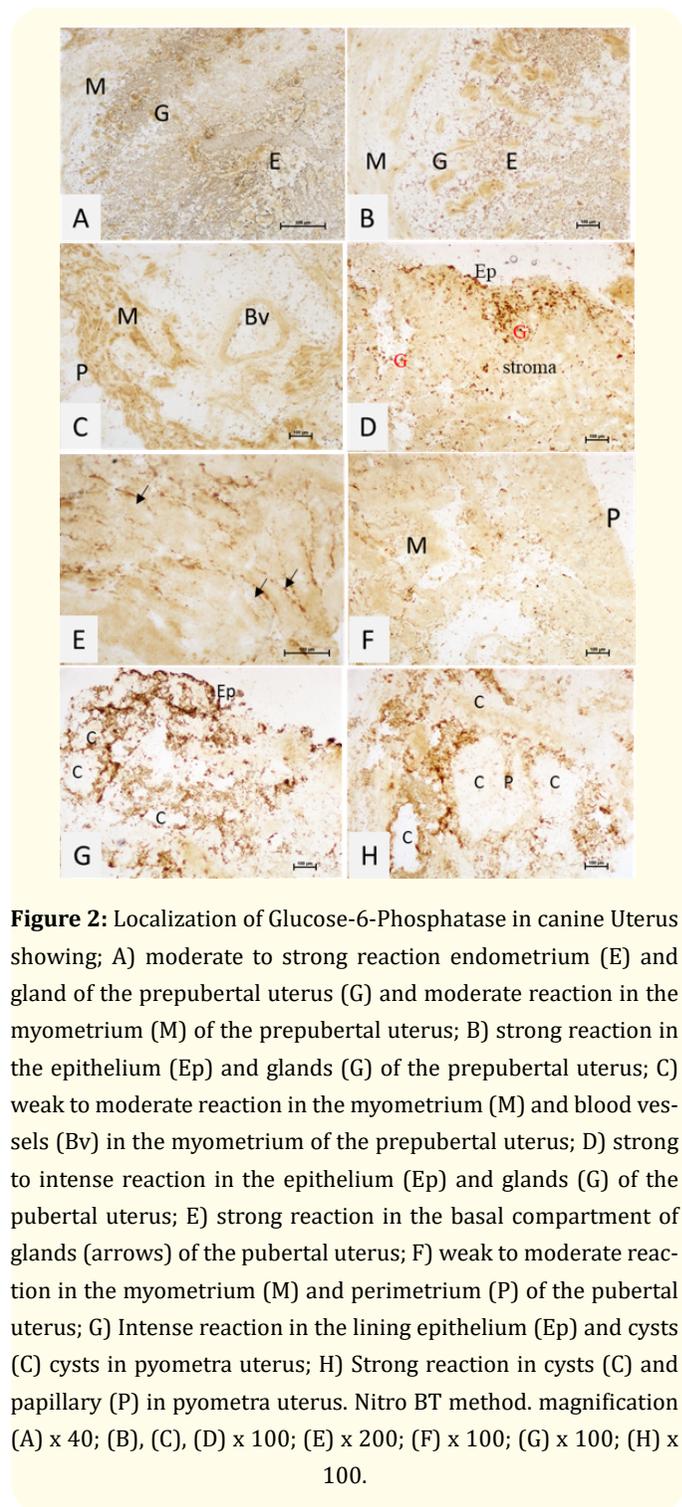
Oxidoreductases

Glucose-6-Phosphate dehydrogenase (G-6-PD)

The intense reaction was observed in the epithelium and glands of pubertal and pregnant animals (Figure 3A-B). The activity was more intense in the apical region as compared to the basal region of cells. A moderate to weak positive reaction was seen in the connective tissue and myometrium (3C). Strong to intense reactions were observed in cystic endometrial hyperplasia (Figure 3D-E) and papillary cystic hyperplasia (Figure 3F). Uniformly moderate reactions were observed in lamina epithelialis, endometrial glands, and myometrium of buffalo uterus [11].

Lactic dehydrogenase (LDH)

Moderate LDH activity was seen in the epithelium, glands, and a weak reaction in the connective tissue layer of prepubertal animals (Figure 4A-B). A moderate to a strong positive reaction in the glandular epithelium of pubertal animals and a weak reaction in muscles were recorded. Strong reactions were observed in blood vessels present in the myometrium in the pubertal uterus (Figure 4C-D). Overall, increased activity of LDH was recorded in the diseased uterus in cysts and papillary projections. The enzyme is responsible for the conversion of glucose to acetyl coenzyme A, which is utilized for the active synthesis of fatty acids and steroids.



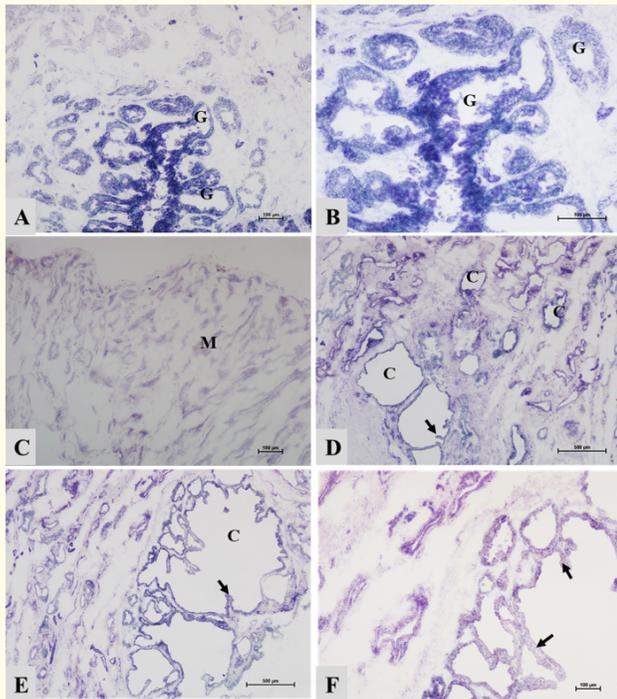


Figure 3: Localization of Glucose-6PD in canine uterus shows A) Intense reaction in the pregnant animals; B) intense reaction in the apical region of glandular epithelium C) A moderate to weak positive reaction in myometrium; D) Strong to intense reaction in cysts (C) in endometrial hyperplasia and papilla in cyst (arrow) E) Strong to intense reaction in cysts (C) in endometrial hyperplasia and papilla in cyst (arrow) F) Strong to intense reaction in papillary projections (arrows) in papillary cystic hyperplasia. Nitro BT method, Magnification; A x 100, B x 200, C x 100, D and E x 40, F x 100.

Succinate dehydrogenase (SDH)

Strong granular reactivity was recorded in the glandular epithelium in pubertal animals (Figure 4A), while a strong granular reactivity was also seen in glands and weak activity in muscles (M) in pregnant animals (Figure 4B). A weak reactivity in prepubertal and an overall strong reactivity were recorded in the diseased uterus. SDH is known to play important role in steroidogenesis as it is closely linked to the cytochrome system [13]. Succinate dehydrogenase activity was shown to be high in the luminal and glandular epithelia, and moderate in the caruncles and circular layer of the myometrium [12]. On day 0 and day + I, the activity of succinate dehydrogenase in the longitudinal layer of the myometrium was higher than at other phases of the cycle.

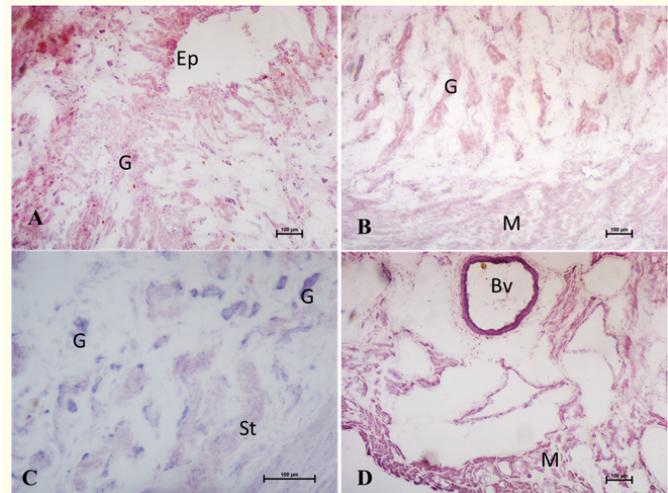


Figure 4: Localization of LDH in canine uterus showing A) moderate reaction in the epithelium (Ep) and glands (G) in prepubertal animals; B) moderate reaction in the glands (G) and weak reaction in the myometrium (M) in prepubertal animals C) A moderate to strong positive reaction in the glandular epithelium (G) of pubertal animals and weak reaction in muscles (M); D) Strong reaction blood vessels (Bv) present in the myometrium (M). Nitro BT method, Magnification; A x 100, B x 100, C x 200, D x 100.

NADH diaphorase

The intense reaction is seen in glands and blood vessels. Intense NADH reaction was seen in the *stratum vasculare* layer b/w two muscle layers. Intense NADH reaction is seen in the case of cyclical as compared to prepubertal. Cyst and papilla mixed up and shows intense NADH reaction. Intense reactions were seen in muscle layers and glands. It was observed that the reaction was more intense in case of disease conditions under the influence of progesterone which increases the activity of the enzyme. The activity of nicotinamide adenine dinucleotide-diaphorase has been correlated with mitochondrial activity within the cytoplasmic electron transport system based on the histoenzymic study on the uterus and early placentome of the sheep [14].

NADPH diaphorase

A strong to the intense reaction of NADPH diaphorase was recorded in epithelium and glands while a moderate reaction was noted in the myometrium of the pubertal canine uterus (Figs. 7 A-B). The reaction was granular (Figure 7B). There was a strong reaction in larger blood vessels (Bv) present in the myometrium

| Group | Layer | Sub-compartment | G-6-PD | LDH | SDH | NADHd | NADPHd | NSE |
|-------------|-------------|----------------------|--------|------|-----|-------|--------|------|
| Prepubertal | Endometrium | Lamina epithelialis | +++ | ++ | ++ | ++ | ++ | +++ |
| | | Stroma | ++ | ++ | ++ | ++ | ++ | ++ |
| | | Glands | ++++ | +++ | ++ | +++ | +++ | +++ |
| | Myometrium | Inner circular | ++ | ++ | + | ++ | ++ | ++ |
| | | Outer longitudinal | + | + | + | + | + | + |
| | | Stratum vasculare | +++ | +++ | ++ | +++ | +++ | +++ |
| Perimetrium | | 0/+ | 0/+ | 0/+ | 0/+ | 0/+ | 0/+ | |
| Pubertal | Endometrium | Lamina epithelialis | +++ | +++ | ++ | +++ | +++ | ++++ |
| | | Stroma | ++ | ++ | ++ | ++ | ++ | ++ |
| | | Glands | ++++ | +++ | +++ | ++++ | ++++ | ++++ |
| | Myometrium | Inner circular | ++ | ++ | + | ++ | ++ | ++ |
| | | Outer longitudinal | ++ | ++ | + | ++ | ++ | ++ |
| | Perimetrium | | + | + | + | + | + | + |
| Pregnant | Endometrium | Lamina epithelialis | ++++ | ++++ | ++ | ++++ | ++++ | ++++ |
| | | Stroma | ++ | ++ | ++ | ++ | ++ | ++ |
| | | Glands | ++++ | +++ | +++ | ++++ | ++++ | ++++ |
| | Myometrium | Inner circular | + | + | + | + | + | + |
| | | Outer longitudinal | + | + | + | + | + | + |
| | Perimetrium | | +++ | +++ | +++ | +++ | +++ | +++ |
| Diseased | Endometrium | Cyst wall | +++ | +++ | +++ | +++ | +++ | +++ |
| | | Papillary projection | +++ | +++ | ++ | ++++ | ++++ | ++++ |
| | | Stroma | +++ | +++ | ++ | +++ | +++ | +++ |
| | Myometrium | Inner circular | + | + | + | + | + | + |
| | | Outer longitudinal | ++ | ++ | ++ | ++ | ++ | ++ |
| | Perimetrium | | + | + | + | + | + | + |

Table 2: Histoenzymic localization of oxidoreductases in the canine uterus.

of pubertal animals (Figure 7C). An intense reaction of NADPH diaphorase was recorded in the glands, while a moderate reaction was recorded in muscles of the gravid uterus (Figure 7D). Similar observations were recorded in the pregnant uterus of sheep (15). In the case of a diseased uterus, a strong granular reaction was observed in the cysts and stroma of cystic hyperplasia (Figure 7E) and the papilla and cysts in cystic papillary hyperplasia (Figure 7F). The enzyme is known for steroidogenesis by converting cholesterol to progesterone and fatty acid synthesis. Isocitrate dehydrogenase (NADP+) activity in the glandular epithelium was confined primarily to superficial glands (12), with only minor activity identified in the epithelium of deep glands. The luminal epithelium had the most activity, followed by the superficial glandular epithelium and the caruncle.

Nonspecific esterase (NSE)

A strong to the intense reaction of NSE was observed in the endometrial glands and a strong reaction in the myometrium (M) of the prepubertal canine uterus (Figure 8A). The activity was in the form of a granular reaction in glands (G) and a strong reaction in the myometrium of prepubertal animals (Figure 8B). The strong reaction was recorded in the smooth muscles in the myometrium (M) of prepubertal animals (Figure 8C). A strong reaction was also recorded in the epithelium and glands of pubertal animals (Figure 8D). The nature of the reaction was granular (Figure 8E). In the case of a diseased uterus, moderate reactions in the papilla and strong reactions in the cyst were observed in cystic papillary hyperplasia (Figure 8F) while a strong granular reaction was ob-

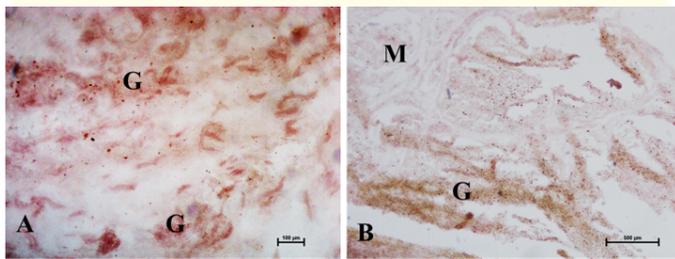


Figure 5: Localization of SDH in canine uterus showing A) strong granular reactivity in the glandular epithelium (G) in pubertal animals, B) strong granular reactivity in glands (G), and weak activity in muscles (M) in pregnant animals. Nitro BT method, Magnification; A x 100, B x 40.

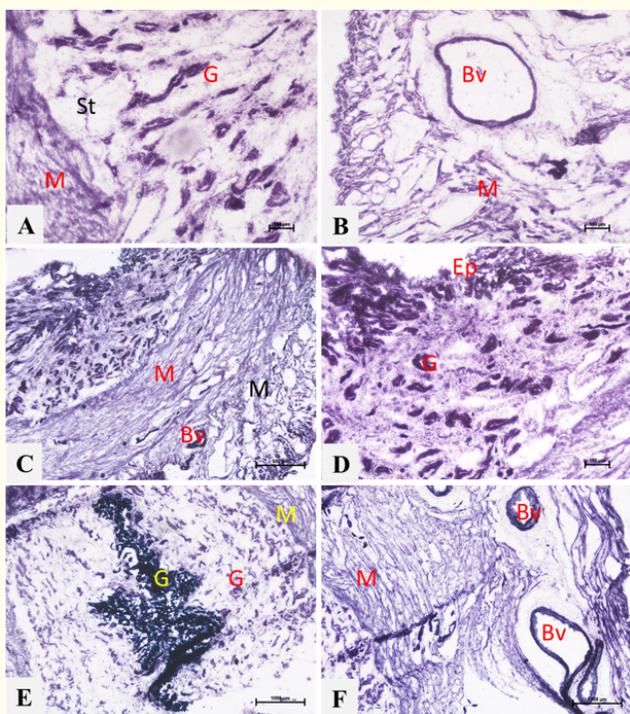


Figure 6: Localization of NADH diaphorase in canine uterus shows, A) strong to intense reaction in endometrial glands (G) and moderate reaction in the myometrium (M) of prepubertal animals; B) moderate to a strong reaction in the myometrium (M) and blood vessels (Bv) of prepubertal animals; C) strong reaction in the endometrium (E), blood vessels (Bv) present in the myometrium (M) of pubertal animals; D) Strong to intense reaction in the epithelium (Ep) and glands (G) of the gravid uterus; E) intense granular reaction in glands of the gravid uterus; F) moderate to a strong reaction in a blood vessel (Bv) and in muscle layer (M). Nitro BT method, Magnification A x 40, B x 200, C x 200, D x 40, E x 20, F x 200.

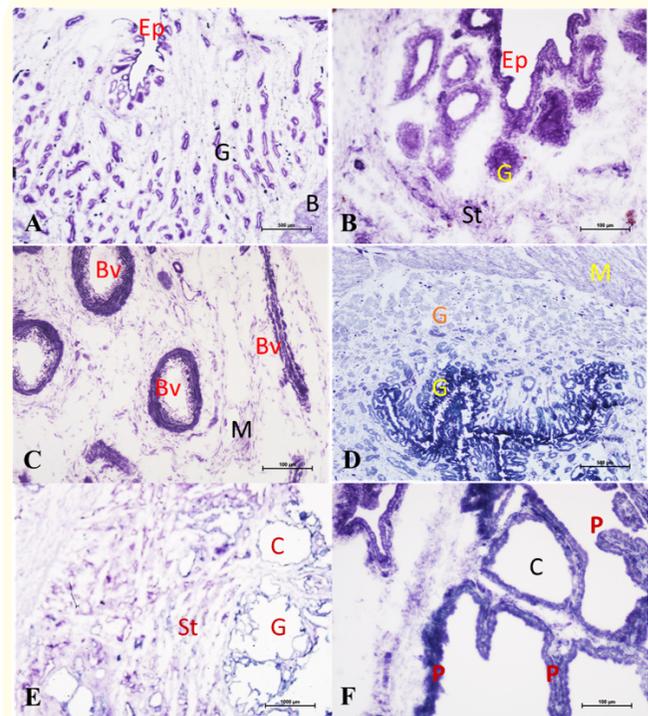


Figure 7: Localization of NADPH diaphorase in canine Uterus shows, A) strong to intense reaction in the epithelium (Ep) and glands (G) and moderate reaction in the myometrium (M) of pubertal animals; B) strong to intense granular reaction in epithelium and glands (G) and moderate in the stroma (St) of prepubertal animals; C) strong reaction in larger blood vessels (Bv) present in the myometrium (M) of pubertal animals; D) Intense reaction in glands (G) and moderate reaction in muscles (M) of the gravid uterus; E) strong granular reaction in cysts (C) and stroma of cystic hyperplasia; F) Strong reaction in papilla (P) and cyst (C) in cystic papillary hyperplasia. Nitro BT method, Magnification A x 40, B x 200, C x 200, D x 40, E x 20, F x 200.

served in cysts in cystic papillary hyperplasia (Figure 8G-H). The nonspecific esterase showed a consistent increase throughout the postpartum period in ewes [16]. Similar observations were also recorded in the buffalo uterus [11].

Conclusion

The present study concluded that the reactivity of enzymes was higher in the epithelium, and glands of pubertal and pregnant animals as compared to the prepubertal group, while the reactivity of most of the enzymes was stronger in diseased animals.

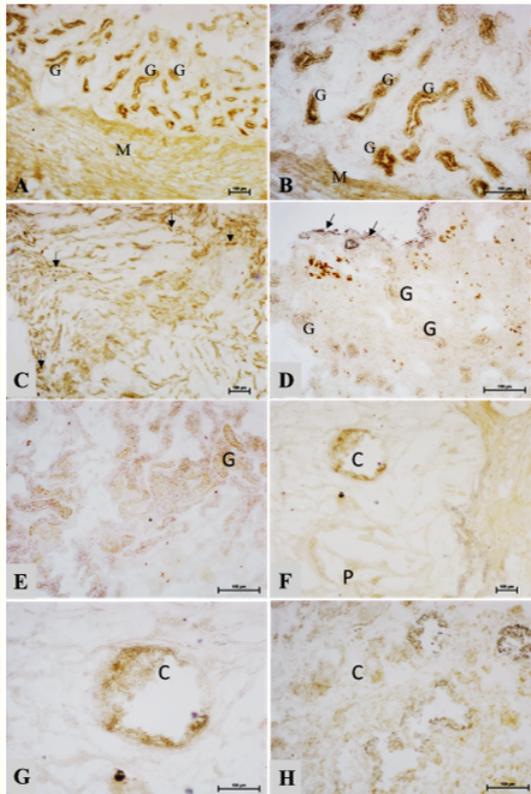


Figure 8: Localization of NSE in canine Uterus shows, A) strong to intense reaction in glands (G), the strong reaction in the myometrium (M) of prepubertal animals; B) A) strong to intense granular reaction in glands (G), the strong reaction in the myometrium (M) of prepubertal animals at higher magnification; C) strong reaction in the myometrium (M) of prepubertal animals; D) strong reaction in the epithelium (Arrow) and glands (G) of pubertal animals; E) strong granular reaction in glands (G) of pubertal animals; F) moderate reactions in papilla (P) and strong reaction in cyst (C) in cystic papillary hyperplasia G) strong granular reaction in cyst (C) in cystic papillary hyperplasia H) Multiple cysts with the strong granular reaction of NSE in cystic hyperplasia; α -Naphthyl acetyl method, Magnification A x 100, B x 200, C x 100, D and E x 200, F x 100, G and H x 200.

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