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Research Article

Histoenzymic Studies on Localization of Diaphorases and Esterases in Cyclic Corpus Luteum of Indian Buffalo

Kritima Kapoor*, Opinder Singh and Devender Pathak

Department of Veterinary Anatomy, College of Veterinary Sciences, Ludhiana, GADVASU Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana, India

***Corresponding Author:** Kritima Kapoor, Department of Veterinary Anatomy, College of Veterinary Sciences, Ludhiana, GADVASU Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana, India.

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Abstract

The current study was conducted on corpus luteum (CL) from healthy buffalo ovaries (n = 40) collected from local slaughterhouses. The CL was cleaned, observed grossly, and categorized into early luteal (stage I, 1 to 5 days), mid-luteal (stage II, 6 to 11 days), late luteal (stage III, 12 to 16 days), and regressing phase (stage IV, 17 to 20 days) based on their gross morphology, having ten ovaries in each group. The cryosections of CL obtained were incubated with substrates to study the distribution pattern of NADPH-diaphorase, NADH-diaphorase, and Non-specific esterase (NSE). The NADH-diaphorase activity was moderate within the developing luteal cells and weak in connective tissue in corpus haemorrhagicum, with strong to intense reaction in the luteal cells of mid-luteal phase CL and negligible activity in luteal cells of regressing phase. However, luteal cells of mid-luteal phase CL exhibited mixed activity for NADPH-d. In the corpus albicans phase, the NADPH-d activity was almost nil. NSE activity was moderate in the developing luteal cells in the corpus haemorrhagicum phase, strong in the luteal cells in mid-luteal phase, and moderate to weak activity for NSE within the cytoplasm of the regressing luteal cells. Therefore, it can be concluded that increased NADPH during the mid-luteal phase might be correlated to the increased secretory activity of cells as the enzyme is responsible for the conversion of cholesterol to progesterone and fatty acid synthesis. Histoenzymic localization of diaphorases and esterases in cyclic CL of buffalo, therefore, indicated their subsequent role in steroidogenesis.

Keywords: Buffalo; Corpus Luteum; Diaphorases; Esterases; Histoenzyme; Reproduction

Introduction

The corpus luteum is a temporary endocrine gland and is one of the body's most vascular tissues, with its endothelial cells representing over 50% of the total cells. The different phases of the CL lifecycle are accompanied by dynamic changes in vasculature, luteal cell populations, and cell-cell interactions. During its development, the CL undergoes a period of tremendously fast growth that involves hypertrophy, proliferation, and differentiation of the steroidogenic cells, as well as extensive angiogenesis [1]. The primary function of the corpus luteum is the production of progesterone. Progesterone production reaches a maximal plateau phase in the mature CL. The steroidogenic cells, i.e., small and large luteal cells, are associated with the production of steroid hormones, i.e., progesterone. For any steroid-producing cell, including luteal cells, the initial step for the production of progesterone is to obtain the precursor, i.e., cholesterol [2]. The majority of mammals have in their luteal tissue a number of non-steroidogenic cells, such as endothelial cells, fibroblasts, and the cells of the immune system, which secret a variety of regulator factors of ovarian function, such as prostaglandins, growth factors, or lipoxygenase products. As the immune cells like macrophages and T lymphocytes become active, they begin to synthesize the cytokines TNFα type [3], 1β interleukin, and γ interferon, factors that have mainly positive effects on the endocrine function of the ovary which led to ovarian follicles and

corpus luteum regression [4]. During the beginning of degeneration, luteal cells are separated by collagen fibers and exhibit regressive changes such as lysis, vacuolization, and shrinkage of the cytoplasm, as well as lysis and rhexis of their nuclei. Inactive corpora lutea are not deficient in stores of cholesterol but may be low in the enzyme system necessary for converting this probable precursor in progesterone synthesis [5]. Several enzymes like dehydrogenases are involved in the cellular conversion of cholesterol into progesterone. Dehydrogenases of glycolytic and tricarboxylic acid (TCA, i.e., Krebs cycle) pathways play an essential role in providing the energy needed for various metabolic activities of somatic and germ cells [6]. The localization of diaphorases and esterases has not been studied and correlated with respect to its function in buffalo CL.

Materials and Methods

Tissue collection

The tissue samples of CL from healthy buffalo ovaries (n = 40) were collected from local slaughterhouses. The CL was extracted from ovaries and cleaned and observed grossly. The mature CL was either fully embedded in ovarian stroma or protruded on the surface with a well-marked neck separating the protruded crown from the ovary [7].

Based on their gross morphology, CL was categorized into early luteal (stage I, 1 to 5 days), mid-luteal (stage II, 6 to 11 days), late luteal (stage III, 12 to 16 days) and regressing phase (stage IV, 17 to 20 days) having ten ovaries in each group [2].

Cryopreservation and Cryosectioning

The tissue sample of fresh, unfixed CL collected from different stages of cyclic ovaries was immediately stored in liquid nitrogen or at -20°C. The tissue pieces of CL were placed on stubs to form blocks with cryogel and subjected to cryostat sectioning at -20°C [8]. Sections of 10-12 µm thickness were obtained on clean glass slides in double (Figure 1). The positive and negative controls were carried out wherever possible.

Enzyme incubation

The sections were incubated with different substrates to study the distribution pattern of Reduced nicotinamide adenine dinucleotide phosphate diaphorase (NADPH-diaphorase); Reduced nicotinamide adenine dinucleotide diaphorase (NADH-diaphorase) and Non-specific esterase (NSE) mentioned below in the following table 1.

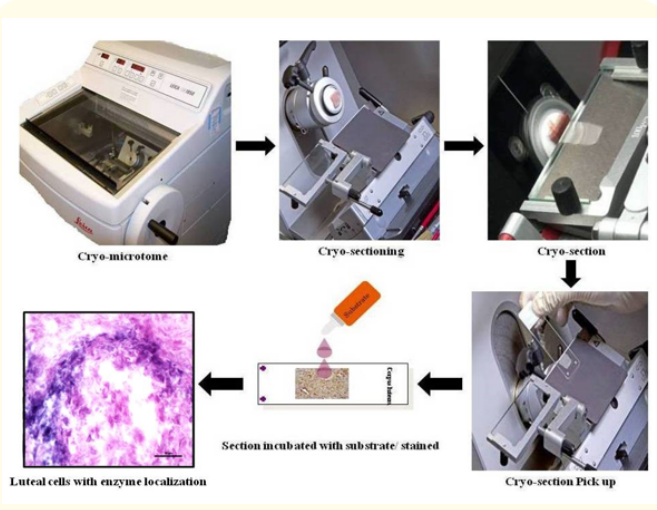

Figure 1: Illustration of cryosectioning protocol of CL tissue placed on stubs to form blocks and subjected to cryostat sectioning at -20°C and sections of 10-12 µm thickness are picked up on clean glass slides, incubated with suitable substrates for localization of enzymes.

Sr. No.	Enzyme	Substrate	Method	Reference	Incubation Time
Diaphorases					
(i)	Reduced nicotinamide adenine dinucleotide phosphate diaphorase (NADPH-diaphorase)	Co-enzyme (NADPH)	Standard method of bound enzyme by nitro BT method	[9]	35 min
(ii)	Reduced nicotinamide adenine dinucleotide diaphorase (NADH-diaphorase)	Co-enzyme (NADH)	Standard method of bound enzyme by nitro BT method	[9]	35 min
Esterases					
(i)	Non-specific esterase (NSE)	Alpha-naphthol acetate	Naphthol acetate method	[10]	10 min

Table 1: Histoenzymic techniques applied on cryosections of cyclic corpus luteum in buffalo.

Qualitative staining evaluation

Histoenzymic staining was evaluated qualitatively based on the observation of the intensity of enzyme localization. According to the scrutiny, the staining intensity was interpreted and graded as no staining (0), weak staining (+), moderate (++), and strong (+++) in different stages of CL.

Results and Discussion

Diaphorases

Reduced nicotinamide adenine dinucleotide diaphorase (NADH- diaphorase)

NADH-diaphorase activity was observed to be moderate within the developing luteal cells and weak in the connective tissue in corpus haemorrhagicum. However, the enzyme activity was high in luteal cells at the periphery of developing luteal parenchyma (Figure 2A). The blood vessels surrounded by the developing luteal cells and within connective tissue septa depicted moderate enzyme activity (Figure 2B-C). The enzyme localization is strong specifically at the margin of small and large luteal cells which is correlated to initiation of active steroidogenesis (Figure 2C). The luteal cells exhibited strong to intense uniform granular reaction of NADH-diaphorase in corpus luteum during its mid-luteal phase (Figure 2D). It is documented that hamster CL contained diaphorase activity on all days of the cycle, which was maximum on day 2, decreased progressively and essentially the same on all days of the process in the interstitium [11]. Similarly, intense NADH-d activity was reported in goat CL during late pregnancy [12]. The strong NADH-d activity was observed within the perinuclear area of the luteal cells (Figure 2E). However, the blood vessels depicted moderate to low enzyme activity. A weak to moderate activity was also observed in connective tissue components in this phase (Figure 2F). However, in late luteal phase, the regressed luteal cells had negligible activity of NADH-d, whereas few intact luteal cells and blood vessels in parenchyma as well as septa had moderate NADH-d activity (Figure 3A). In the corpus albicans phase, the NADH-d activity was observed as moderate to strong activity only in occasional luteal cells and the thick blood vessels that were prominent at this stage (Figure 3B; Table 2). Similar observations were recorded in Indian buffaloes [13].

Reduced nicotinamide adenine dinucleotide phosphate diaphorase (NADPH- diaphorase)

Moderate NADPH-diaphorase activity was observed in the cytoplasm of the luteal cells located at periphery in the developing stage of corpus luteum (Figure 4A). However, the luteal cells at the centre strongly reacted to NADPH-d activity during this phase.

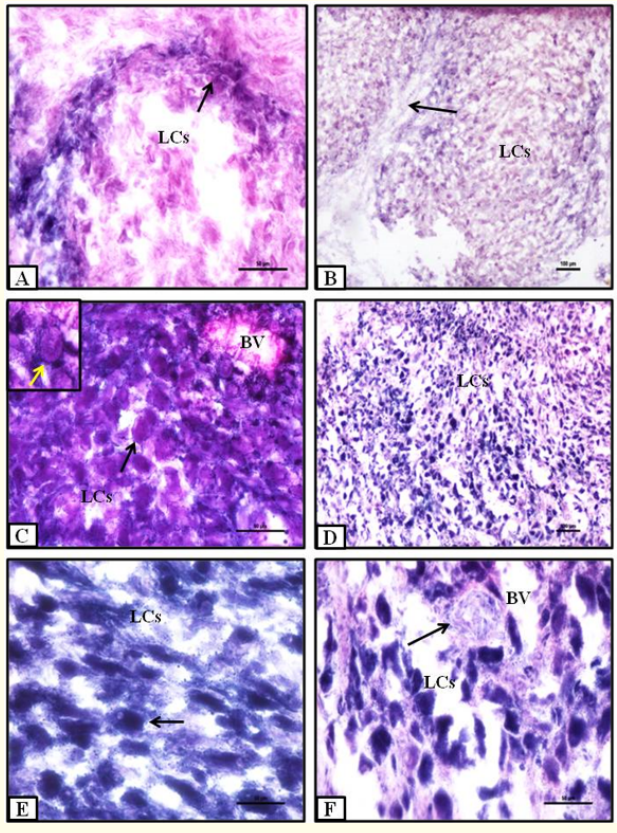

Figure 2: Photomicrograph of (A) Corpus haemorrhagicum (CL-H) showing moderate NADH-d activity (arrow) in the developing luteal cells (LCs) at periphery and weak in the connective tissue. X400. (B) CL-H with moderate NADH-d activity in blood vessels (arrow) around luteal cells (LCs) and within connective tissue septa. X100. (C) CL-H with strong NADH-d activity at margin (arrow) of small and large luteal cells (LCs; Inset) and moderate in blood vessels (BV). X400. (D) mid-luteal CL with intense uniform granular NADH-d reaction in luteal cells (LCs). X100. (E) strong NADH-d activity within the perinuclear area (arrow) of luteal cells (LCs) in mid-luteal CL (F) blood vessels (BVs; arrow) around luteal cells (LCs) showing moderate to low NADH-d activity. Nitro BT method X400.

Within each luteal cell, there was strong uniform peri-nuclear localization of the enzyme. The surrounding connective tissue elements however depicted moderate enzyme activity (Figure 4B). It was found that NADPH production increased for nucleic acid synthesis due to an increase in Glucose-6-phosphate dehydrogenase (G-6-PDH) enzyme activity during the proliferation of luteal cells in guinea pig CL [14]. In the mid-luteal phase of the corpus luteum,

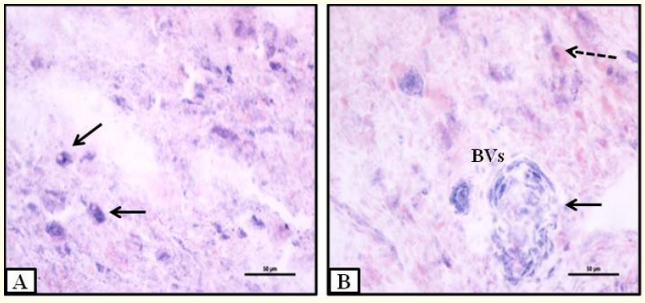

Figure 3: Photomicrograph of (A) late luteal phase CL showing moderate NADH-d activity in few intact luteal cells. X400 (B) corpus albicans phase showing moderate NADH-d activity in occasional luteal cells (dotted arrow) and strong in thicker blood vessels (BVs; arrow). Nitro BT method X400.

the luteal cells exhibited mixed activity for NADPH-d (Figure 4C). However, the peripheral cells mainly had strong NADPH-d activity (Figure 4D), whereas the central cells showed moderate reaction to the NADPH-d activity (Figure 4E; Table 2). The increased NADPH during the mid-luteal phase might be correlated to the increased secretory activity of cells as the enzyme is responsible for the conversion of cholesterol to progesterone and fatty acid synthesis [15,16]. The cytoplasm of most of the large luteal cells had strong NADPH-d activity, whereas small luteal cells depicted moderate activity for NADPH-d (Figure 4F). However, a strongly positive NADPH tetrazolium reductase reaction was observed in bovine large luteal cells during all stages of development and higher activity in small luteal cells than in large ones, whereas stromal cells show weak enzyme activity [17]. A weak to moderate activity was observed within the cytoplasm of a few intact luteal cells remaining in the late luteal phase. Although in our study, the enzyme reaction in most of the parenchyma at this stage was weak, it was still observed to be moderate to strong in the blood vessels (Figure 5A-B). On the contrary, similar enzyme activity of NADPH-d as of NADH-d was observed in all the phases of cyclic hamster CL [11]. In the corpus albicans phase, the activity was almost nil for NADPH-d within the shrunken parenchyma and weak to moderate activity within the remnant thicker blood vessels at the periphery and in connective tissue septae (Figure 5C-D). Similar observations were reported in buffaloes [13] and in sheep [18].

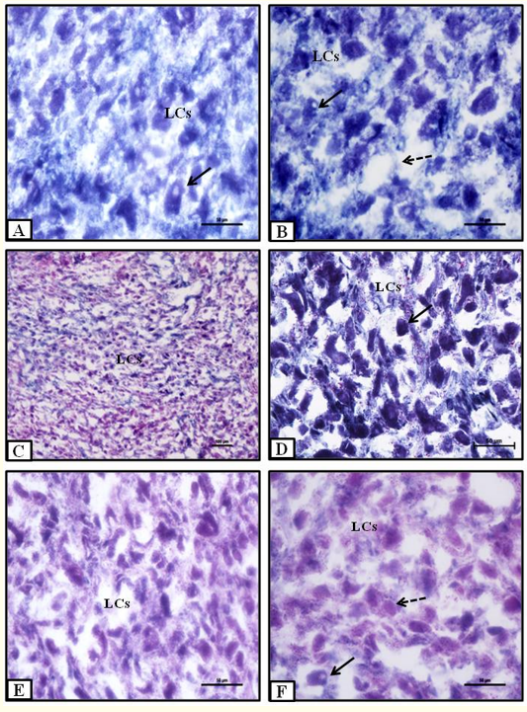

Figure 4: Photomicrograph of (A) CL-H showing moderate NADPH-d activity in cytoplasm (arrow) of luteal cells (LCs) located at periphery. X400 (B) strong uniform peri-nuclear NADPH-d activities in luteal cells (LCs) at centre during CL-H phase; moderate in surrounding connective tissue (dotted arrow). X400 (C) mid-luteal phase CL showing mixed activity for NADPH-d in luteal cells (LCs). X100 (D) mid-luteal CL showing strong NADPH-d activity in the peripheral luteal cells (LCs; arrow). X400 (E) moderate NADPH-d activity in central luteal cells. X400 (F) mid-luteal CL showing strong NADPH-d activity in large luteal cells (LCs; arrow) and moderate in small luteal cells (dotted arrow). Nitro BT method X400.

Non-specific esterases (NSE)

The intensity for NSE was moderate in developing luteal cells; observed within the parenchyma in corpus haemorrhagicum phase (Figure 6A). Mainly, the large luteal cells had moderate activity, whereas small luteal cells had weak activity for NSE in this phase (Figure 6B). However, the connective elements and septa had weak activity for NSE. In the mid-luteal phase, the activity for NSE was strong in the luteal cells within the luteal parenchyma (Figure 6C). Most of the luteal cells, i.e., both small and large had strong fine granular reactions within their cytoplasm in the perinuclear region

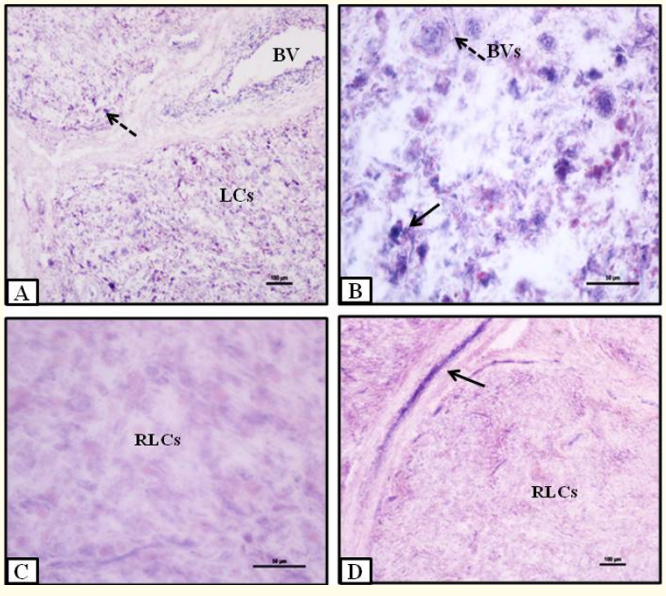

Figure 5: Photomicrograph of (A) late luteal CL showing weak to moderate NADPH-d activity in cytoplasm (dotted arrow) of few intact luteal cells (LCs) and in blood vessels (BVs). X100. (B) same phase CL showing moderate to strong in the blood vessels (BVs; dotted arrow) and few luteal cells (arrow). X400. (C) Corpus albicans showing no NADPH-d activity in regressing luteal cells (RLCs). X400. (D) same phase CL showing moderate NADPH-d activity in thicker blood vessels (arrow) at periphery around regressing luteal cells (RLCs) and in connective tissue septa. Nitro BT method X100.

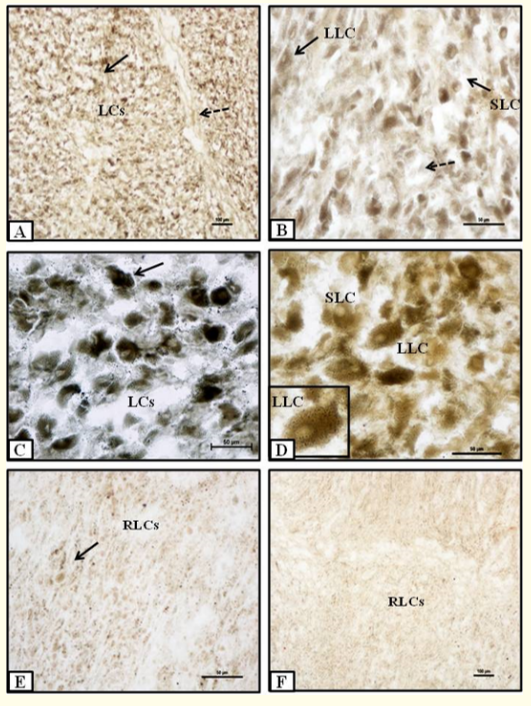

Figure 6: Photomicrograph of (A) corpus haemorrhagicum showing moderate NSE activity in developing luteal cells (arrow) and weak in septae (dotted arrow). X100 (B) same phase CL showing moderate NSE activity in large luteal cells (LLC) and weak in small luteal cells (SLC) and connective tissue elements (dotted arrow). X400. (C) mid luteal phase corpus luteum showing strong NSE activity in luteal cells (LCs; arrow). X400. (D) same phase CL showing fine granular NSE activity in both small (SLC) and large luteal cells (LLC; Inset). X400 (E) late luteal CL showing weak NSE activity in regressed luteal cells (RLCs; arrow). X100. (F) corpus albicans showing negligible NSE activity in regressed luteal cells (RLCs). X100. Naphthol acetate method.

(Figure 6D). The localization of nonspecific esterase was concentrated particularly in luteal cell cytoplasm in corpora lutea of all ages in rats [19]. Similar granular reaction of NSE was observed in myometrium glands in prepubertal dogs [20]. However, the late luteal phase had moderate to weak activity for NSE within the cytoplasm of the regressing luteal cells (Figure 6E). On the contrary, at this stage, the corpus albicans had weak to negligible activity for NSE in regressed luteal cells within the degenerating luteal parenchyma (Figure 6F; Table 2).

Conclusion

The histoenzymic localization of diaphorases and nonspecific esterases in different stages of cyclic CL thus depicted that it was strong and intense in early and mid-luteal phases while weak to negligible in late and the regressing phases. Therefore, it can be concluded that increased activity of these enzymes during the early and mid-luteal phase might be correlated to the increased secretory activity of cells as the enzyme is responsible for the conversion of cholesterol to progesterone, which is at peak during these phases of CL. Therefore, determining the role of these enzymes in different stages of cyclic CL can help in determining the level of progesterone synthesis during active phases of CL. This can thus, pave a basis for preventing production losses and reproductive failure in buffaloes.

S. No.	Group	CORPUS HAEMORRHAGICUM (Cl –H)			MID LUTEAL CORPUS LUTEUM (LCL)			LATE LUTEAL CORPUS LUTEUM			CORPUS ALBICANS (CL -AB)		
		LCs	Ct Septa	BVs	LCs	Ct Septa	BVs	LCs	Ct Septa	BVs	LCs	Ct Septa	BVs
1	NADH- d	++	+	+	+++	+/++	+/++	++	+	++	0/+	+	++/+++
2	NADPH-d	+++	+	+	++/+++	+/++	+/++	+/++	+	++	0/+	+	+/++
3	Non-Specific Esterase (NSE)	++	+	+	+++	+	+/++	+/++	+	0/+	0	0	0/+

Table 2: Histoenzymic distribution of Diaphorases and Non-Specific Esterases in cyclic corpus luteum of buffalo.

LCs: Luteal Cells; Ct: Connective Tissue; BVs: Blood Vessels

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