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Research Article

DYNAMICS OF INTERMEDIATE FILAMENT PROTEIN EXPRESSION IN THE BUFFALO UTERUS DURING THE FOLLICULAR AND LUTEAL PHASE OF THE ESTROUS CYCLE

Sagarika Dehury[1 *], Varinder Uppal[2], Devendra Pathak[2], Anuradha Gupta[2]

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ABSTRACT: Laminin and vimentin are two intermediate filaments that play a pivotal role in uterine cellular proliferation, differentiation, maintaining cellular integrity or stability, receptivity, and the implantation process. In the buffalo uterine tissue, the immunoreactivity in terms of optical density (OD) value for both the intermediate filaments was assessed during the follicular and luteal phases of the estrous cycle. During the luteal phase, the endometrial immune expression for both the intermediate filaments in term of OD value was significantly higher ($p<0.05$) than the follicular phase, suggesting that they actively participate in implantation and uterine receptivity. In contrast, the myometrium's immunoexpression did not differ significantly between the two phases. Immunoelectron microscopy was also used to locate the vimentin antibody. Despite the lack of vimentin immunoexpression in glandular epithelial cells under light microscopy, vimentin-positive immune gold particles were seen ultrastructurally on the microfilaments of these cells.

Keywords: Laminin, Vimentin, Optical density, Immunoexpression, Immunoelectron microscopy, Ultrastructure.

INTRODUCTION

In ruminants, despite the synepitheliochorial nature of the placenta, effective implantation requires extracellular matrix remodelling, as observed in rodents and humans [1]. Mesenchyme and epithelium interact to generate many organs whose development is controlled by steroidal sex hormones [2]. For implantation to occur, the receptive endometrium must undergo endometrial extracellular matrix (ECM) remodelling [3]. ECM remodelling plays a fundamental role in regulating cell function, differentiation, migration, and proliferation, along with specific impacts on trophoblast invasion, placentation, cell death, and the development of an appropriate and functional implantation chamber surrounding the embryo [4].

The transition of mesenchymal to epithelial (MET) occurs when cells change from migrating mesenchymal to polarized epithelial stages [5]. Multiple studies have demonstrated that the migration and differentiation of epithelial cells are also influenced by the extracellular matrix, particularly the basal lamina [2]. In every instance, laminin was found in the endometrial gland and vascular epithelial basement membranes of the uterus [6]. It is a significant adhesive glycoprotein essential for attaching epithelial cells to the basement membrane [7]. Basement membrane laminins (LNs) have been demonstrated to influence cellular phenotypes and differentiation throughout organogenesis in vivo and in vitro [8]. Laminins are a strong promoter of growth, morphogenesis, and differentiation. They also impact on cellular differentiation adhesion, migration, phenotypic maintenance, and cell survival [8, 9, 10]. In human laminin deficiency, impaired decidualization [11].

Another intermediate filament protein, vimentin, a 57 kDa protein, is found in the cytoplasm of mesenchymal cells and serves structural stability [12]. Vimentin is well known for playing a crucial part in maintaining intracellular structure. It has been acknowledged for its mechanical function as a stress absorber and in cell plasticity. The assembly and disassembly of vimentin

[1]*Department of Veterinary Anatomy and Histology, College of Veterinary Science and Animal Husbandry, Odisha University of Agriculture and Technology, Bhubaneswar, Odisha, 751003, India.*
[2]*Department of Veterinary Anatomy, College of Veterinary Science, Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana, Punjab - 141004, India.*
** Corresponding author. e-mail: sagarikad@ouat.ac.in*

is a crucial structural requirement for carrying out cellular processes like cell migration and cell division [13]. In human endometrial glands' proliferation and/or differentiation during decidualization was influenced by vimentin [14]. Vimentin influenced the implantation process [15], uterine receptivity, and cellular integrity or stability [16]. The present study examined the dynamic role of laminin and vimentin intermediate filament protein expression during the follicular and luteal stages of the estrous cycle to evaluate their role in endometrial receptivity in the buffalo uterus.

MATERIALS AND METHODS

Sample collection

Samples were collected from uterine horn and body region from twenty-four (n=24) adult cyclic healthy Murrah buffalo, aged 2-6 years, twelve (n=12) during the follicular phase and twelve (n=12) during the luteal phase of estrous cycle from the GADVASU Ludhiana slaughterhouse and post-mortem hall. No institutional ethical committee permission is required if the sampling was done from a slaughterhouse or during post-mortem examination. Only those animals who came for treatment to the college veterinary clinical complex and succumbed due to accidental injury were considered for sampling during post-mortem examination. By looking at the corpus luteum or matured follicle on the surface of the ovary, it is possible to determine the luteal and follicular phases [17,18]. Following collection, the tissue samples were preserved in 10% neutral buffered formalin for the immunohistochemistry investigation. For the immuno-electron microscopy (IEM) study, fresh buffalo endometrial tissue samples of 1mm ×1mm thickness were fixed with 4% Paraformaldehyde + 0.1% glutaraldehyde for 2-4 h at 4°C.

Sample processing for immunohistochemistry (IHC)

The samples were processed for paraffin sectioning by routine acetone benzene schedule [19]. The paraffin sections of 4-5 µm thickness were cut. After dewaxing and rehydration, the heat-induced antigen retrieval was done by 10 times diluting concentrated (10X) EZ-Antigen Retrieval buffer (Manufacturer- Biogenics). The endogenous peroxidase activity was blocked by immersing the sections in 3% (v/v) H2O2 and washing in 0.1M phosphate-buffered saline (at pH 7.4). To prevent nonspecific binding of antibodies, sections were blocked with 2.5% normal horse serum. The sections were incubated overnight with ready-to-use primary antibodies laminin and vimentin (Manufacturer-Biogenics) at 4°C in a humid chamber. After washing in 0.1M phosphate-buffered saline (at pH 7.4), the sections were treated with a secondary antibody (ImmPRESS® HRP Universal Antibody, Horse Anti-Mouse/Rabbit IgG, Polymer Detection Kit, Peroxidase (MP-7500), Vector Laboratories, USA). Again the washing is done with 0.1M phosphate-buffered saline (at pH 7.4) then the chromogen 3, 3'-diaminobenzidine tetrahydrochloride (DAB) (ImmPACT® DAB Substrate Kit, Peroxidase (HRP) (SK-4105), Vector Laboratories USA) and Gill's III hematoxylin was used for counterstaining. The sections were washed in running tap water, dehydrated, cleared, and mounted with DPX. Immunohistochemical observations and photomicrographs of stained sections were taken with a microscope, an attached camera, and photographic equipment (Eclipse 80i, Nikon, Japan). Immunoreaction intensity was measured by obtaining the optical density (OD) value of IHC photographs using Fiji Image's J colour deconvolution plug-in 1.7 [18, 20]. The statistical analysis was carried out utilizing the SPSS-16 program. An independent sample t-test was used to determine the significant difference in the mean OD values between the follicular and luteal phase of estrous cycle.

Sample processing for immunoelectron microscopy (IEM)

For immunoelectron microscopy (IEM), dehydration and clearing were done with graded alcohol and acetone, respectively. Embedding was done with LR white resin. After ultrathin sectioning, 80-100 nm thick sections were mounted on nickel grids. For immunolabelling, the ready-to-use primary antibody vimentin (Manufacturer—Biogenics) and secondary antibody anti-mouse IgG (Sigma) tagged with a 10-15 nm gold particle were used. Ultrastructure photographs were taken using a transmission electron microscope (Tecnai G20 HR-TEM 200kV) for IEM analysis.

RESULTS AND DISCUSSION

In buffalo uterus, laminin immunoexpression occurred in the cytoplasm of both the endometrial gland and surface epithelium, extracellular matrix of stroma, vessel wall, and myometrium in both follicular and luteal phase (Fig.1), but perimetrium was negative for laminin immunostaining. The optical density value for laminin immunostaining intensity in the endometrium and myometrium of buffalo uterus was summarized in Table 1. In the luteal phase (Fig.1b), significantly ($p<.05$) higher laminin endometrial staining in terms of optical density (OD) was observed than in the

follicular phase (Fig.1a); however, when both phases were considered, there was no significant difference in the OD value of laminin immunostaining in the myometrium. Laminin is a principal constituent of the epithelium basement membrane and a stromal extra-cellular matrix of the endometrium [21].

Table 1. Comparison of immunostaining intensity (OD) for different antibodies in follicular and luteal phases of estrous cycle in buffalo uterus.

Region of uterus	Mean Optical density (OD) value of immunostaining intensity			
	Laminin		Vimentin	
	Follicular	Luteal	Follicular	Luteal
Endometrium	0.16^{a}±0.006	0.23^{b}±0.01	0.42^{a}±0.02	0.62^{b}±0.02
Myometrium	0.20±0.02	0.18±0.01	0.19±0.02	0.23±0.02

OD value: Min-0 to max 2.4; Means with different superscripts differ significantly ($p<0.05$) between two columns under same antibody group.

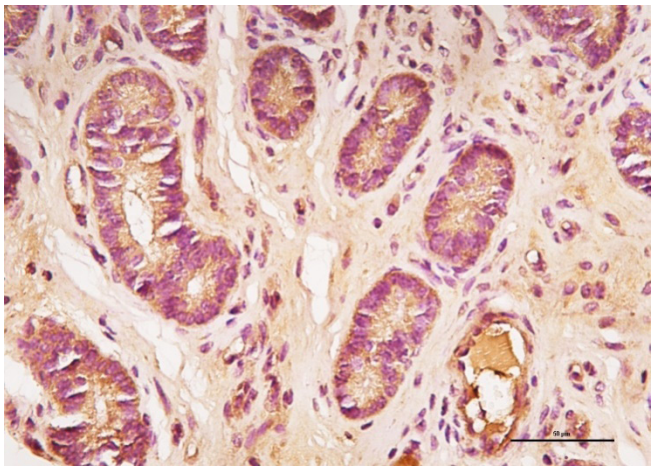

Fig. 1a.

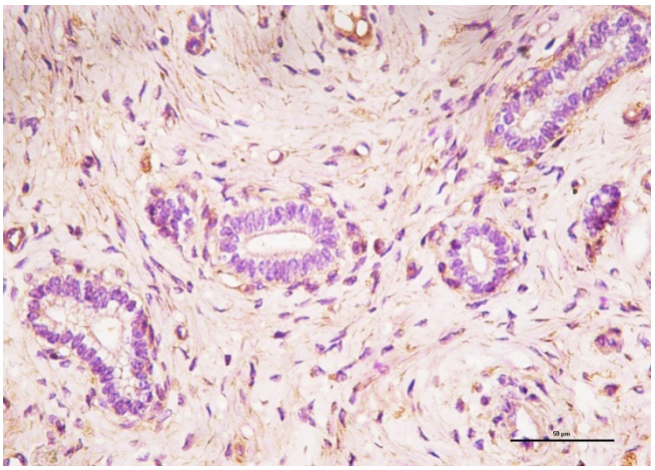

Fig. 1b.

Fig. 1. Photomicrographs of laminin expression in buffalo uterus. [1a. endometrium (Follicular Phase) × 400, 1b. endometrium (luteal phase) × 400.

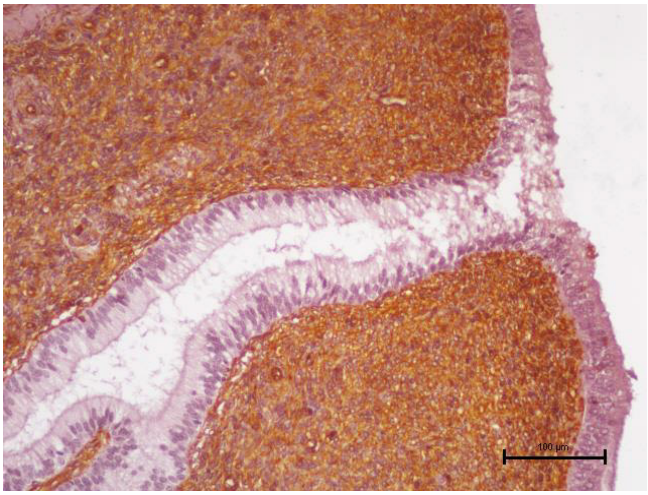

Fig. 2a.

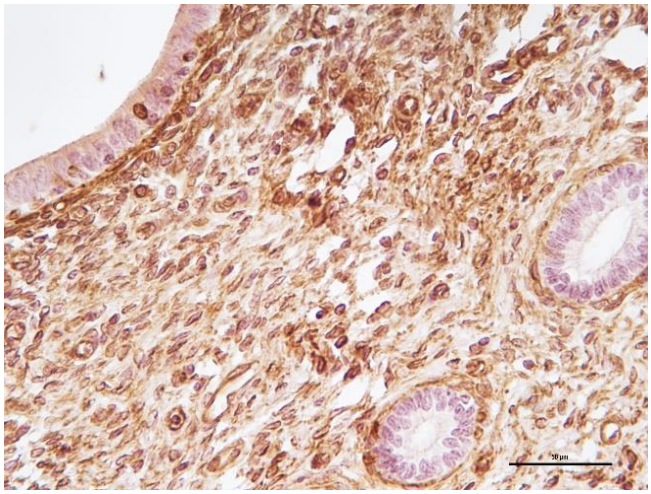

Fig. 2b.

Fig. 2. Photomicrographs of vimentin expression in buffalo uterus. [2a. endometrium (Follicular Phase) × 400, 2b. endometrium (luteal phase) × 400.

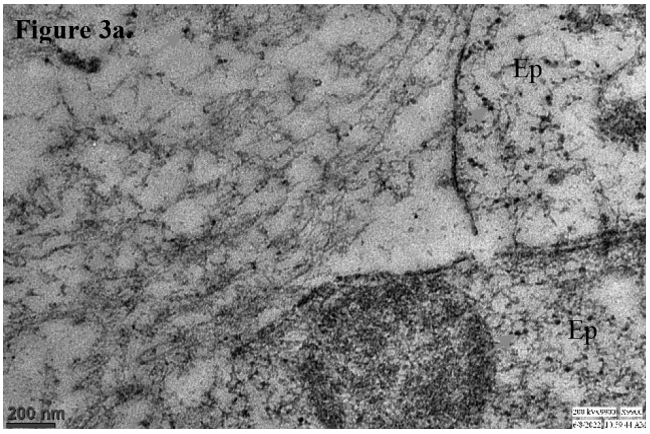

Fig. 3a.

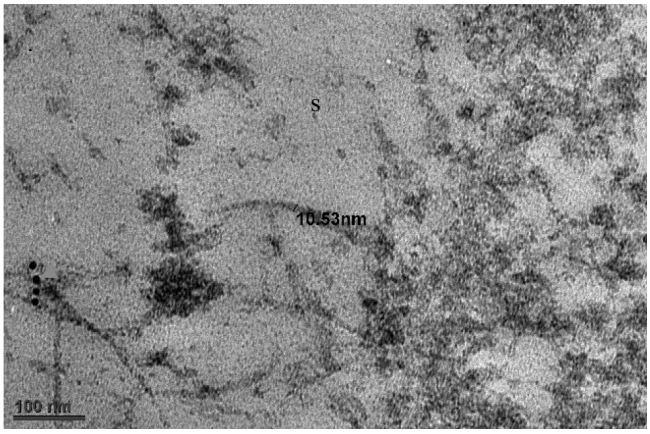

Fig. 3b.

Fig. 3. Photomicrographs of immunoelectron microscopic expression of vimentin in buffalo uterus. [3 a. Vimentin immunoreactivity (arrow) on microfilament of epithelial cell (Ep), 3b.Vimentin immunoreactivity (arrow) on microfilament of stroma (s).

Researchers found negative staining for the endometrial extracellular matrix during the proliferative stage [22] and the intensity of staining was gradually increased in the secretory phase, and maximum staining intensity was observed during the late secretory phase [23]. Progesterone [11] is likely an inducing factor for phase-wise laminin immunostaining variability of uterine endometrium, probably due to the progesterone- cAMP-PKA pathway [11].

The vimentin immunoreactivity was primarily restricted to the endometrial stroma, uterine blood vessel, myometrium, and perimetrium (Fig. 2). The endometrial gland and surface epithelium were negative for vimentin immunostaining. Similarly, in bovine [24] and in-vitro study [25], it also revealed negative expression for vimentin in the endometrial epithelium. On the contrary, in humans, the vimentin expression was observed in the surface and glandular epithelium, along with stromal and vascular components [12,14]. Vimentin antibody reaction triggers a robust response in the endometrial stroma. The reaction was more pronounced in the superficial stroma surrounding the glandular elements and beneath the luminal epithelium. The optical density value for vimentin immunostaining in the endometrium and myometrium of the buffalo uterus was summarized in Table 1. Significantly higher immunostaining intensity ($p \leq .05$) was observed in the endometrium during the luteal phase (Fig. 2b) compared to the follicular phase (Fig. 2a). In the myometrium, the immunostaining intensity for vimentin did not significantly differ between the follicular and luteal phases. During the luteal phase, the stromal reactivity (OD) for vimentin was higher as compared to the follicular stage, which indicated an increased vimentin reaction during the luteal phase, which might be helpful in the implantation process [15], as well as uterine receptivity and also cellular integrity or stability [16]. In humans, a stronger vimentin reactivity of stroma was observed during the secretory phase [12] but others observed no cyclical variation of vimentin expression in the endometrium [14].

Ultrastructurally, vimentin immunoreactivity could be seen on the microfilament of the stroma and also in glandular and surface epithelium (Fig. 3), in contrast to light microscopic observation where the vimentin immunoexpression was only found in the stroma but not in the glandular and surface epithelium. A cluster of 4-5 immunogold particles was detected on the microfilament (Fig. 3b). The average width of the microfilament was 10-11 nm.

CONCLUSION

During the luteal phase, the endometrial reactivity (OD) for laminin and vimentin intermediate filaments was significantly higher ($p \leq .05$) than in the follicular phase. Increased intermediate filament expression during the luteal phase indicated their role in implantation. Ultrastructurally, the vimentin immunoreaction was found on the microfilament of the stroma as well as in the endometrial surface and glandular epithelium. whereas, under light microscopy, no vimentin immunoexpression was observed in the endometrial epithelium.

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