Indian Journal of Veterinary Anatomy 36(2): 90-94, December 2024

Immunoexpression of Estrogen receptor α (ERα) in buffalo cervix contributes to histochemical and histoenzymic changes during the estrous cycle.

Sagarika Dehury[1*], Varinder Uppal[2], Devendra Pathak[3] and Anuradha Gupta[4]
Department of Veterinary Anatomy, Guru Angad Dev Veterinary and Animal Science University, Ludhiana, Punjab 141004

Received: 21 September 2024; Accepted: 15 December 2024

ABSTRACT

The cervix is the part of the uterus that not only acts as a passage for sperm towards the site of fertilization but also, the functionality of the cervix contributes to sperm motility, viability, and transportation inside the birth canal. Twelve (12) buffalo cervix samples were collected, of which, six (6) were from the follicular phase and six (6) from the luteal phase of the estrous cycle. Analysis of estrogen hormone activity was done by immunohistochemical study for estrogen receptor α(ERα), histochemical study for acid mucopolysaccharide was done by Alcian blue staining at pH 1.0 and 2.5, histoenzymic activity of NADH-d and NADPH-d were studied in sections of buffalo cervix in both follicular and luteal phase of the estrous cycle. Estrogen hormones greatly influence the function of the cervix during estrous, which significantly influences the acid mucopolysaccharide content and NADH-d and NADPH-d activity of the cervix. These factors were more active during the follicular phase, improving the cervix's functional state. This state is essential for sperm survival and plays a significant role in buffalo reproduction.

Key words: Immunohistochemical study, ERα, Acid mucopolysaccharide, NADH-d, NADPH-d

INTRODUCTION

The caudal most portion of the uterus was formed by the fibromuscular canal known as the cervix. It was communicated with the uterine cavity through the internal os (ostium uteri internum) and opened into the vagina by the external os (ostium uteri externum). As part of the reproductive organ, the cervix's function is regulated by steroid hormones. Estrogens cause the cervical mucus to be thin and transparent, whereas progesterone renders it thick, opaque, viscid, and impenetrable to spermatozoa (Abarbarel, 1946). So, the cervix not only acts as a passage for migration of sperm towards the site of fertilization, but also, the functionality of the cervix contributes toward sperm motility, viability, and transportation inside the birth canal. Estrogen has a stimulatory effect on glycosaminoglycan (GAG) synthesis.Alcian blue was used to demonstrate acid mucopolysaccharides. GAG-like sulfated acid mucopolysaccharides (heparin, chondroitin, and dermatan sulfates) were stained with Alcian blue at pH 1.0, and both sulfated and non-sulfated acid mucopolysaccharides (hyaluronic acid) were stained with Alcian blue at pH 2.5 (Ferringer & Ko, 2014). Acid mucopolysaccharide content in the cervical mucous contributes to sperm motility. NADH-diaphorase is an enzyme that transfers hydrogen from reduced nicotinamide adenine dinucleotide (NADH) to other molecules, such as dyes, by which the activity of NADH is localized. NADH is involved in glycolysis and energy production (Chaudhry & Varacallo, 2023). The angiogenesis in the cervix was determined by performing Nicotinamide adenine dinucleotide phosphate-diaphorase (NADPH d) localization (Dehury et al., 2024).In this study, estrogen receptor-α (ERα) immunoexpression along with histochemical analysis of acid mucopolysaccharide and history enzymic study of NADH and NADPH d was done in buffalo cervix during the follicular and luteal phase of estrous cycle to determine the functional status of the cervix, i.e., required to optimize sperm survivability, which is a significant contributing factor in buffalo reproduction.

MATERIALS AND METHODS

Sample collection

Twelve (12) uterine cervix samples of the adult Murrah buffalo were collected from the GADVASU, Ludhiana slaughterhouse and post-mortem hall. Six (06) follicular and six (06) luteal phase samples were chosen based on the presence of corpus luteum or matured follicle on the surface of the ovary.

1. Veterinary Anatomy and Histology, College of Veterinary Science and Animal Husbandry, Odisha University of Agriculture and Technology, Bhubaneswar, 751003, 2&3. Professor, 4.Prof. & Head

*Corresponding Author: sagarikad@ouat.ac.in

Following collection, the tissue samples were preserved in 10% neutral buffered formalin for the histochemical and immunohistochemistry investigation. Fresh samples were collected and kept at -20˚C in a deep freezer for histoenzymic study.

Sample processing

The samples underwent a standard acetone-benzene schedule processing for paraffin sectioning (Luna, 1968). The paraffin sections were at a thickness of 4-5 µm. Following dewaxing and rehydrating, concentrated (10X) EZ-Antigen Retrieval buffer (Manufacturer: Biogenics) was diluted ten times to facilitate heat-induced antigen retrieval. The endogenous peroxidase activity was inhibited by submerging the sections in 3% (v/v) H2O2 and washing them in 0.1M phosphate-buffered saline (pH 7.4). Sections were blocked with 2.5% normal horse serum to avoid nonspecific antibody binding. Then, the sections were incubated with ready-to-use primary antibodies ER α (Manufacturer-Biogenics) at 4˚C overnight in a humidified chamber. The sections were treated with a secondary antibody (ImmPRESSTM HRP Universal Antibody-Anti-Mouse IgG/Anti-Rabbit) following washing in 0.1M phosphate-buffered saline (at pH 7.4). The chromogen 3, 3'-diaminobenzidine tetrahydrochloride (DAB) (ImmPACT® et al. -HRP) was used. Counterstaining was done using Gill's III hematoxylin. After being rinsed under running tap water, the sections were dehydrated, cleared, and mounted with DPX. For immunohistochemical observations,the photomicrographs of stained sections were captured using a microscope, a connected camera, and photographic equipment (Eclipse 80i, Nikon, Japan). Alcian Blue at pH 1.0 and 2.5 staining of the paraffin sections was undertaken for histochemical study to localize the acid mucopolysaccharides qualitatively. Fresh, unfixed tissue with a thickness of 10 µm was cryosectioned at -20 ºC using a cryostat microtome for the histoenzymic investigation of NADH-d and NADPH-d activity. The cryosection slides were incubated in the medium (pH 7.0-7.1) containing NADH-d and NADPH-d as substrate at 37° C for 10-15 min. Then, the slides were fixed with 15% formol saline and mounted with glycerin jelly for observation.

RESULTS AND DISCUSSION

The immunoexpression of the estrogen receptor (ERα) was detected in the lining epithelium, lamina propria submucosa, tunica muscularis, and tunica serosa layers (Fig.1). In a positive reaction, the nucleus took the immune stain. The ERα immunostaining was more intense in the epithelium during the follicular phase (Fig.1a) than in the luteal phase (Fig.1b). Similarly, Rodríguez-Piñón *et al.* (2008) in sheep and Sagsoz et al. (2010) in bovine cervix reported the ERα immunostaining was more intense in the epithelium and tunica muscularis during the follicular phase,whereas intense staining was observedin the epithelium during the luteal phase. In the sub-epithelial connective tissue part, mostly immunopositive cells were found, with few negative cells during the follicular phase. The connective tissue component was negative for the immune staining in the luteal phase. The immunoexpression of ERα during the estrous cycle showed that the estrogen hormone activity was

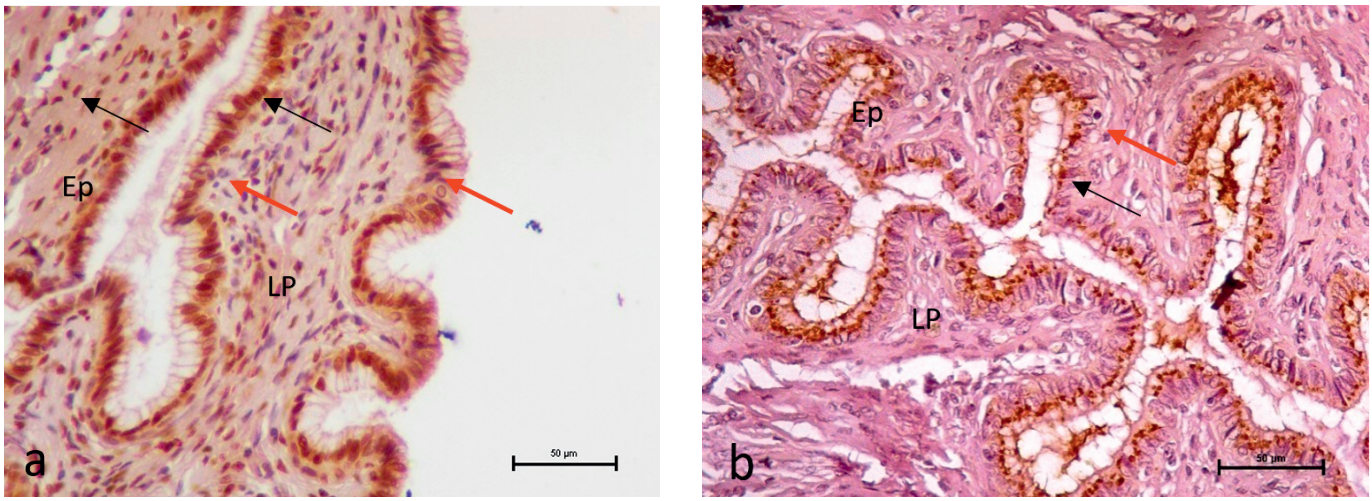

Fig 1. Localization of ERα receptor (positive cells with black arrow, negative cells with red arrow) in buffalo cervixepithelium (Ep) and lamina propria (Lp) during (a) follicular phaseX400, (b) luteal phase X400

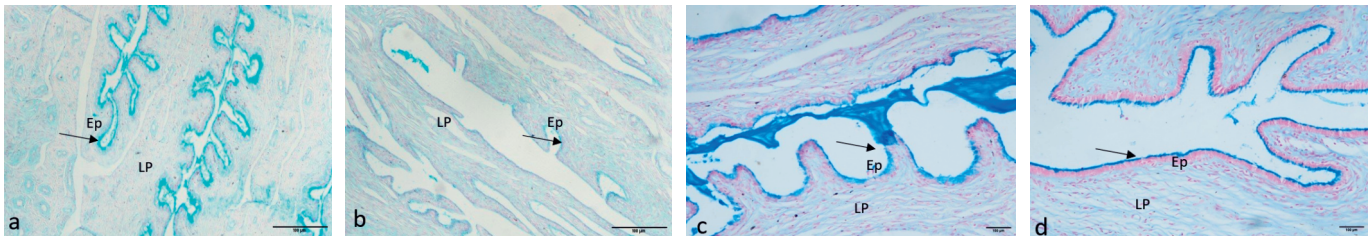

Fig 2. Localization of acid mucopolysaccharides (arrow mark) in the luminal epithelium (Ep) of buffalo cervix with Alcian blue stain (pH 2.5) X 100 during (a) follicular phase, (b) luteal phase; buffalo cervix with Alcian blue stain (pH 1.0) X 200(c) follicular phase, (d) luteal phase.

higher in the follicular phase than in the luteal phase of the estrous cycle.

Alcian Blue at pH 1.0 and 2.5 staining for acid mucopolysaccharide displayed a strong reaction in the lining epithelium of the cervix during the follicular phase (Fig.2a & Fig.2c), but the reaction was moderate during the luteal phase (Fig.2b & 2d) as reported by Pathak *et al.* (2012) in sheep. The granular form of alcinophilic material in the supranuclear zone of the cervical epithelium (Fig.2a-2d) was also observed. Fateh El-Bab and El-Naggar (1975) in buffalo also demonstrated acid mucopolysaccharides materials on the apical surface of the cervical epithelium, and the reactivity was higher during the proestrous and estrous as compared to the diestrous phase. Underlying subepithelial connective tissue showed weak reactivity in both estrous cycle phases (Fig.2a-2d). Wordinger *et al.* (1972) reported a predominance of sulfated acid mucous substances in non-ciliated cells of the bovine cervical epithelium. The lamina propria submucosa, tunica muscularis, and tunica serosa were weakly positive for the acid mucopolysaccharides throughout the estrous cycle. Greater reactivity during the follicular phase is due to the estrogenic stimulatory effect on GAG synthesis. The increased hyaluronic acid concentration in the follicular phase resulted in increased hydration, cervical mucous viscosity, and disorganization of collagen fibers in the extracellular matrix (Akgul *et al.,* 2012). GAGs are active in the process of the opening of the uterine cervix (Golichowski *et al.,* 1980; El Maradny *et al.,* 1997) by increasing water content and reorganization of collagen fibers in the cervix **(**Soh *et al.,* 2012**).** Lee & Ax (1984) in bovine reported increased chondroitin sulfates in the cervical mucus during estrous when compared to the cervical mucus during the di-estrous phase. Cubas et al. (2010) in rat cervix reported increased sulfated glycosaminoglycan (GAG) during estrous as the synthesis of GAG was stimulated by estrogen, not progesterone. From the immunohistochemical study, it was evident that higher estrogenic activity during the follicular phase directly influenced the acid mucopolysaccharide content in the cervix that would take part in sperm capacitation (Lee and Ax, 1984; Mahmoud and Parrish, 1996), sperm motility (Mahmoud and Parrish, 1996), sperm survivability as it is vital as a physiological barrier to microbial infection (Cubas et al.,2010).

The NADH-d reactivity during the follicular phase in a blood vessel, glandular, and luminal epithelium of the cervix was very strong during the follicular phase(Fig.3a). However, a comparatively lesser reaction for NADH-d was observed during the luteal phase(Fig.3b). NADH staining in propria submucosa was weakly reactive during the follicular phase(Fig.3a), but a very weak reaction was observed during the luteal phase (Fig.3b). In the human cervix, the reactivity of NADH was higher in the epithelium and endocervical glands **(**Filipe and

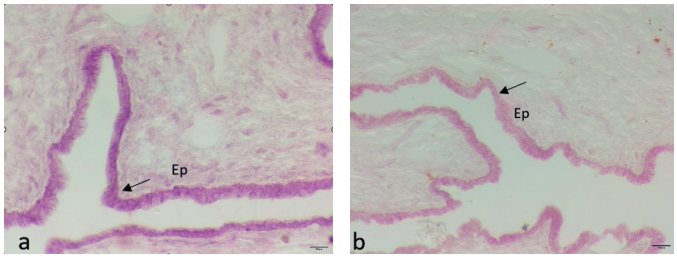

Fig 3. Localization of NADH-d activity (arrow mark) in the luminal epithelium (Ep) of buffalo cervix(a) follicular phase, (b) luteal phaseX 200

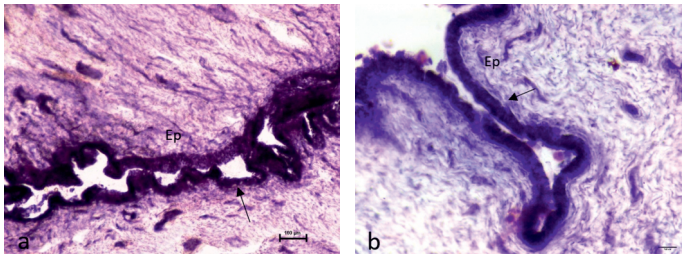

Fig 4. Localization of NADPH-d activity (arrow mark) in the luminal epithelium (Ep) of buffalo cervix(a) follicular phase, (b) luteal phaseX 200

Dawson, 1968). Uppal *et al.* (2008) observed that the NADH activity in the lamina epithelialis and muscularis had a strong activity. In contrast, lamina propria was moderately reactive, and weak activity was observed in the serosa in buffalo. The reactivity in the follicular phase was higher than in the luteal phase.Increased NADH-diaphorase reactivity indicated the cervix's increased functional status as well as sperm survival and movement inside the cervix as it requires glycolysis rather than respiration (Breckenridge and Pommerenke, 1958), and this enzyme is involved in glycolysis and energy production (Chaudhry and Varacallo, 2023).

In the follicular phase (Fig.4a), very strong NADPH-d reactivity in lamina epithelial and strong to moderate reactivity was observed in the propria submucosa part of the cervix. During the luteal phase (Fig.4b), the reactivity was strong to moderate in lamina epithelialis, and a weak reaction was observed in the propria submucosa part of the cervix. Localization of NADPH-d reaction was used to determine the angiogenic activity influenced by estrogen (Dehury *et al.,* 2024). Increased reactivity ofs NADPH-d and estrogen during the follicular phase by histoenzymic and immunohistochemical studies, respectively, indicated that a higher level of angiogenesis occurred during the follicular phase as compared to the luteal phase, that contributes to physiological edema during the time of estrous in animals.

The above study revealed that estrogen secretion during the follicular phase greatly influenced the acid mucopolysaccharide content and NADH-d and NADPH-d enzyme activity in the buffalo cervix. This improved the cervix's functional status and induced an optimum environment for sperm motility and survivability inside the female reproductive tract.

ACKNOWLEDGEMENTS

This study was part of the corresponding author's doctoral thesis, and the technical help rendered by the committee members has been duly acknowledged. The authors thankfully acknowledge the help rendered by the staff at the post-mortem hall, GADVASU Ludhiana, and slaughterhouse, Municipal Corporation Ludhiana.

REFERENCES

Abarbarel, R. 1946. Artificial reproduction of the cyclic changes in the cervical mucus of human castrates. *Endocrinology* 39 : 65-66.

Akgul, Y., Holt, R., Mummert, M., Word,A. andMahendroo, M. 2012. Dynamic changes incervical glycosaminoglycan composition during normal pregnancy and preterm birth. *Endocrinology* 153: 3493-503. https://doi: 10.1210/en.2011-1950

Breckenridge, M. A. B. and Pommerenke, W. J. 1951. Analysis of carbohydrates in Human cervical mucus. *Fertility & Sterility* 2: 29.

Chaudhry, R. andVaracallo, M. 2024. *Biochemistry, Glycolysis.* StatPearls Publishing. https://www.ncbi.nlm.nih.gov/books/NBK482303/

Cubas, J. J. M., Simões, R. S., Oliveira-Filho, R. M., Simões, M. J., Baracat, E. C. and Soares-Jr, J. M. 2010. *Glycosaminoglycan Distribution in the Rat Uterine Cervix during the Estrous Cycle. Clinics* (Sao Paulo) 65(7): 703-708.

Dehury, S., Uppal, V., Pathak, D. andGupta, A. 2024.Exploring the Nexus of Steroidal Hormone Receptor, Uterine VEGF Expression and NADPH-d Interaction in Buffalo Uterus During Oestrous Cycle With Seasonal Variation. *Reproduction in domestic animal* 59 (8): https://doi.org/10.1111/rda.14710

El Maradny, E., Kanayama, N., Kobayashi, H., Hossain, B., Khatun and S., Liping, S. 1997. *The role of hyaluronic acid as a mediator and regulator of cervical ripening. Human reproduction* 12(5): 1080-1088.pp. 52-53, 333-335. Verlag Paul Parey, Berlin.

Fatehe- L-Bab, M. R. and El-Naggar, M. A. 1975. A histological and histochemical study of the cervix uteri of the buffalo cow during the estrus cycle. *Zentralblatt für Veterinärmedizin* 22: 164 -175.

Ferringer,T., and Ko**,** C. J. 2014**.** Chapter 1 - *The basics: Diagnostic terms, skin anatomy, and stains, Dermatopathology* (Second Edition). pp. 1-36. Elsevier Limited, Amsterdam, Netherlands.

Filipe, M. I. and Dawson, I. M. 1968. Qualitative and quantitative enzyme histochemistry of the human endometrium and cervix in normal and pathological conditions. *Journal of*

pathology and bacteriology 95 : 243-58. https://doi: 10.1002/path.1700950129.

Golichowski, A. M., King, S. R. andMascaro, K. 1980. Pregnancy-related changes in rat cervical glycosaminoglycans. *Biochemical Journal* 192(1): 1-8.

Lee, C. N. andAx, R. L. 1984. Concentrations of glycosaminoglycans in the female bovine reproductive tract. *Journal of Dairy Science* 67: 2006-2009.

Luna, L. G. 1968. *Manual of Histological Straining Methods of the Armed Forces Institute of Pathology*. 3rd ed. New York: McGraw-Hill.

Mahmoud, A. I. and Parrish, J. J. 1996. Oviduct fluid and heparin induce similar surface changes in bovine sperm during capacitation: a flow cytometric study using lectins. *Molecular Reproduction and Development* 43 : 554-60. https://doi: 10.1002/(SICI)1098-2795(199604)43:4<554:AID-MRD19>3.0.CO;2-Z.

Pathak, D., Nagpal, S. K. andSingh, G. 2012. Histochemistry of cervix of sheep in follicular and luteal phases of estrous cycle. *Indian veterinary Journal* 89 : 69 – 72.

Rodriguez-pinon, M., ztasende, C., Puime, P. andGaroalo, E. G. 2008. Estrogen and progesterone receptor binding capacity and estrogen receptor alpha expression (ERα mRNA) along the cervix of cycling ewe. *Reproduction fertility and development* 20 : 350-356. https://doi: 10.1071/rd07186.

Sagsoz, H., Akbalik, M. E., Saruhan, B. G. andKetani, M. A. 2010. Localization of estrogen receptor a and progesterone receptor B in bovine cervix and vagina during the follicular and luteal phases of the sexual cycle. *Biotechnic & Histochemistry* 86 : 262–271. https://doi: 10.3109/10520291003795861.

Soh, Y. M., Tiwari, A., Mahendroo, M., Conrad, K. P. and Parry, L.J. 2012. Relaxin regulates hyaluronan synthesis and aquaporins in the cervix of late pregnant mice. *Endocrinology* 153 : 6054-6064.

Uppal, V., Roy, K. S. andBansal, N. 2008. Histoenzymic localization of phosphatases, oxidoreductases and esterases in the cervix of buffalo during fetal life. *Indian Journal of Animal Sciences* 78 : 1090-1093.

Wordinger, R. J., Dickey, J. F. andHill, J. R. 1972. Influence of under nutrition on the histology and histochemistry of the bovine endometrium. *Journal of Animal Science* 34 : 453-459. https://doi.org/10.2527/jas1972.343453