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Research Article

Immunolocalization of Progesterone Receptor (PR) During Different Phases of Estrous Cycle in the Oviduct of Water Buffaloes

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Abstract

The steroid hormone, progesterone, is a key modulator of the normal reproductive functions of animals. The goal of this study was to see how progesterone receptors were distributed in distinct segments of the buffalo oviduct during the follicular and luteal phases of the estrous cycle. Oviducts from twelve buffaloes (six each during both the phases) were collected from the local abattoir. Blood samples were collected before the slaughter of the animal to estimate levels of estrogen and progesterone hormones. The cellular distribution of progesterone receptor (PR) was examined using the immunohistochemical technique. The PR was localized in cells of mucosal lining, connective tissue cells, oviductal glands, muscular layer, and serosal covering in different segments of the oviduct. In lamina epithelialis, both ciliated and secretory cell types were positive for PR. Infundibulum and ampulla had a higher number of PR positive cells which as compared to utero tubal junction was statistically significant ($P < 0.05$). The infundibulum, ampulla, and isthmus showed more population of PR-positive cells during the follicular phase of the estrous cycle when compared to the luteal phase ($P < 0.05$). The data on the percentage of PR-positive cells and hormone analysis showed that the PR expression was higher during the estradiol dominance.

Keywords: Buffalo; Estrous Cycle; Immunohistochemistry; Oviduct; Progesterone Receptor

Abbreviations

DAB: 3,3'-Diaminobenzidine; DPX: Dibutyl Phthalate Polystyrene Xylene; HRP: Horseradish Peroxidase; IHC: Immunohistochemistry; PBS: Phosphate Buffer Saline; PR: Progesterone receptor

Introduction

Water buffaloes are the principal source of milk in India as they contribute more than 50% of the total milk yield, and thus play a very important role in the country's rural economy. The fundamental determinant of production is reproductive competence. The oviduct is a tubular conduit where fertilization of male and female gametes occurs leading to the formation of zygotes. It's also important for gamete transfer and storing, growth, initial progress of embryo, and transporting a growing embryo [1]. The lumen of the oviduct provides a suitable environment for the growth and development of zygotes during their early phase of development

[2]. These critical processes require not only an anatomically patent oviduct but also a functional tubal epithelium and tubal fluid. The lining epithelium of the buffalo oviduct consists of ciliated and secretory cells [3]. The ciliated cells are responsible for the transport, while, the secretory cells contribute to secretions. The secretory cells provide secretions, while the ciliated cells are accountable for conveyance. These cells and their physiology including the volume and composition of fluids are being governed by estrogen and progesterone hormones. Progesterone hormone is involved in modulating the anatomy and physiology of the oviduct [4]. It has been established in cows that progesterone controls the activities of the oviduct immediately after ovulation [5].

Progesterone hormone binds to receptors in cells and converts them into a form that can produce steroid-responsive genes [6]. The oviduct has four parts namely the infundibulum, ampulla, isthmus, and utero-tubal junction [3] and each of these segments has specific functions. The ampulla is involved in the transport of the oocyte-cumulus complex and fertilization, whereas the isthmus is responsible for the storage of sperm and transport of the embryo [4]. Improvement of reproductive efficiency in female water buffaloes requires a better understanding of their reproductive physiology under steroid hormonal control, during the estrous cycle. PR immunolocalization in the oviduct has been studied in cows [5], Swamp buffalo [7], pigs [8,9], canines [10-13], and sheep [14,15] but meager information is available on PR localization in the oviduct of water buffaloes. This paper discusses about how progesterone receptors are found throughout the oviducts of water buffalo during different phases, and how they vary depending on different hormones.

Materials and Methods

Animals

Six buffalo oviducts were taken shortly after slaughter, each at the follicular and luteal phases of their estrous cycle. To estimate the levels of estrogen and progesterone hormones, blood samples were also taken from the same animals.

In this study, the animals were divided into two groups: those with follicular ovaries (n = 6) and those with luteal ovaries (n = 6). The tissues were immersed in 10% neutral buffered formalin for one day for fixation. Tissue samples were washed with flowing tap water, dehydrated with acetone-benzene, and embedded in paraffin wax. Sections of the tissues were prepared at 4-5 microns thickness for histology staining.

Immunohistochemistry

Polymer-based Horseradish peroxidase method was used for immunostaining as described earlier [16]. After the sections were deparaffinized and rehydrated, they were treated with heat-induced antigen retrieval. Sections were incubated with three percent H_2O_2 in methanol for 20 minutes to stop endogenic enzymes, and then non-specific protein hindering with Horse serum for 30 minutes. Tissue sections were treated in a diluted primary antibody (Santa Cruz Biotechnology, PR: SC-538 at 1:2000 dilutions) for one hour at laboratory conditions. After a PBS wash, slides were incubated in HRP-conjugated secondary antibodies for half an hour. Sections were washed with PBS and then treated with DAB for 1 minute before being brought back to the water. The sections were then stained with hematoxylin for 2 minutes, dehydrated, and mounted.

Image analysis and quantification of immunopositive cells

Pictures were captured of immunostained cells, with 10 images per slide per animal. 6-10 images were taken using a 40x objective lens per section. Pictures were processed and counted using a cell counter plugin of ImageJ (version 1.49d) [17]. The sections were assessed in detail to find the proportion of positively marked nuclei.

Estimation of estrogen and progesterone hormone in the blood samples

The estradiol levels of the blood samples were analyzed to estimate the estrogen hormone. The plasma progesterone was estimated by RIA [18].

Results and Discussion

To the best of our knowledge, the present study seems to be a pioneer in studying the PR localization in different segments of the uterine tube of water buffaloes. The purpose of the study was to determine if the progesterone receptor was present in different oviductal compartments at different points in the estrous cycle. The progesterone receptor was found to be in all four parts of the oviduct. In all the parts, PR was expressed in the tunica mucosa, tunica submucosa, tunica muscularis, and tunica serosa layers. PR was identified in both of the cell types that comprised the epithe-

lium in the oviduct. Similar observations have been recorded in the cow [19], heifers [20], sheep [21], and Thai Swamp buffalo [22].

In the infundibulum, during the follicular phase of the estrous cycle, expression of PR was recorded in the epithelial lining, connective tissue layer below the epithelium, muscular layer, and serosal layer (Figure 1A). At higher magnification immunoreactivity was distinctly nuclear. The nuclear localization was seen in both ciliated cells and secretory cells and cells in the connective tissue layer (Figures 1B, 1C and insets a and b). During the luteal phase also, the nuclear localization of PR was observed both in ciliated and secretory cells. The secretory blebs protruding out of the apical surface of the cells also showed a positive reaction. It indicated that the secretory blebs consisted of nuclear fragments.

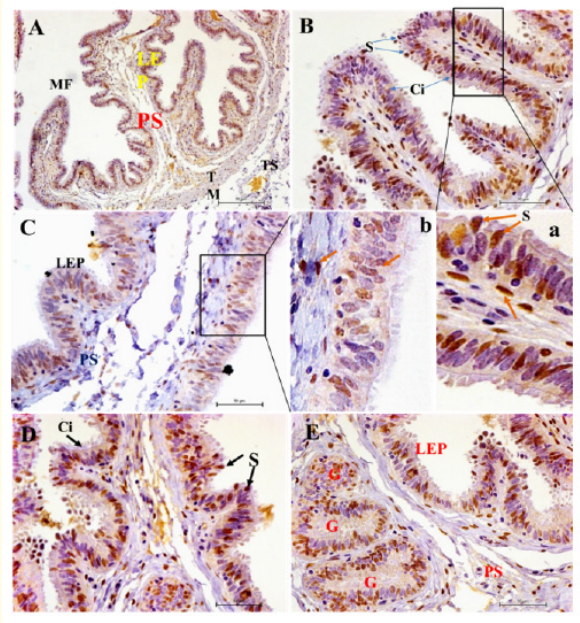

Figure 1: Immunostaining of infundibulum of buffalo with anti-PR antibody. A. During follicular phase, nuclear reaction in mucosaL fold (MF), lamina epithelialis (LEP). propria submucosa (PS), tunica muscularis (TM) and tunica serosa (TS). Polymer HRP method. Original magnification x100; B. During follicular phase, nuclear reaction in ciliated cells (Ci) and secretory blebs (S); a. magnified view of selected area showing nuclear reaction in secretory cell (S), stromal cells (arrow); B. During follicular phase, nuclear reaction lamina epitheliasis (LEP) with ciliated cells (Ci) and secretory blebs (S); b. magnified view of the selected area showing nuclear reaction in the lining epithelium and stromal cells (arrows); C. Nuclear reaction in the lining epithelium with ciliated cells (Ci) and secretory blebs (S); D. During the follicular phase, the nuclear reaction in the lining epithelium (LEP), glandular epithelium (G) and propria submucosa. Polymer HRP method. Original magnification of B, C and D. x400.

Strong nuclear reactivity for PR was recorded in the various tunics of the ampulla. During the follicular phase, PR staining was detected in the lining epithelium of mucosal folds, few stromal cells present in the connective tissue compartment, and endothelial cells of blood capillaries (Figure 2A). Comparable findings were documented during the luteal phase of the estrous cycle (Figure 2B). However, the staining strength was weaker as contrasted to that noted in the estrogen-dominant phase. The glandular epithelium and the muscle fibers in tunica muscularis also showed strong reactivity to the PR antibody (Figure 2C). Few connective tissue fibers and endothelial cells, as well as, tunica media layer cells of large blood vessels in tunica serosa were also immunoreactive to PR (Figure 2D).

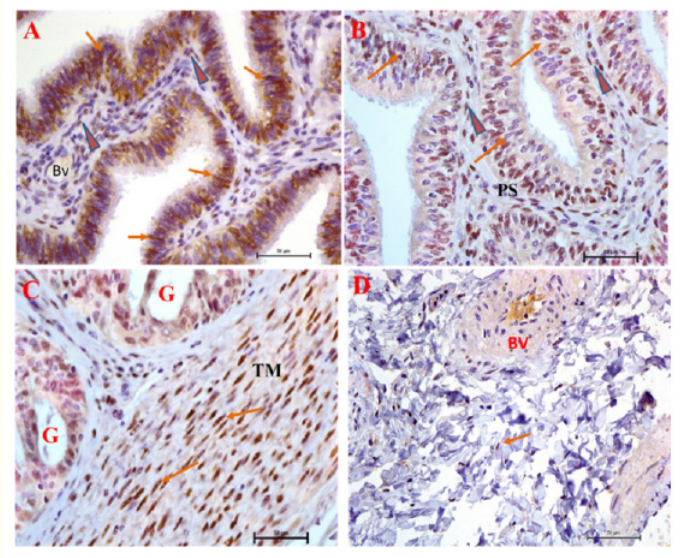

Figure 2: Immunostaining of ampulla of buffalo with anti-PR antibody. A. During follicular phase, nuclear reaction in lamina epithelialis (arrow), endothelial cells of blood vessels (Bv), propria submucosa (arrowhead); B. During luteal phase, nuclear reaction in lamina epithelialis (arrow), propria submucosa (arrowhead); C. Nuclear reaction in glandular epithelium (G) and muscle fibres (arrows) in Tunica muscularis; D. Nuclear reaction in connective tissue fibre (arrow), blood vessels in Tunica serosa. Polymer HRP method. Original magnification. x400.

The isthmic region of the buffalo oviduct also revealed immunoreactivity for PR. Variable degree of nuclear expression was observed in the cells of lining mucosa, connective tissue, the muscle layer, and serosal layer (Figure 3A). The strong nuclear staining was observed in mucosal cells and glandular epithelium, while stromal cells, in the propria submucosa, showed moderate to strong reactivity (Figure 3B). The muscle cells in the tunica muscularis showed a strong staining for PR (Figure 3C) while moderate reactions were observed in connective tissue fibers and endothelial cells of blood vessels present in tunica serosa (Figure 3D). During the progesterone dominance phase, the strong staining in the nucleus was noted in the lining mucosa and connective tissue (Figure 3E). The glandular epithelium also showed a strong reaction while most of the muscle fibers in tunica muscularis were intensely positive (Figure 3F).

The utero-tubal junction showed transitional histology between the oviduct and uterus and is known to separate viable embryos and unfertilized oocytes. There were comparatively broader mucosal folds and a higher number of oviductal glands than other segments of the oviduct. The PR immunoreactivity was observed in the nuclei of cells lining the luminal surface, connective tissue, glandular epithelium, muscle layer, and a serosal layer of the utero-tubal junction of the buffalo oviduct (Figure 4A). During the follicular phase, the strong expression for PR was observed in the nucleus of cells of tunica mucosa and oviductal glands while a modest reaction was detected in the stromal cells in propria submucosa (Figure 4B). During the luteal phase, strong reactivity for PR was observed in the luminal epithelium while a weak reaction was observed in stromal cells of the connective tissue layer (Figure 4C). Strong immunoreactivity was observed in the epithelium lining of the oviductal glands (Figure 4D). In the sow utero-tubal junction, prominent staining for PR was observed in the tunica muscularis after artificial insemination and progesterone hormone might influence sperm transportation and the fertilization process [8].

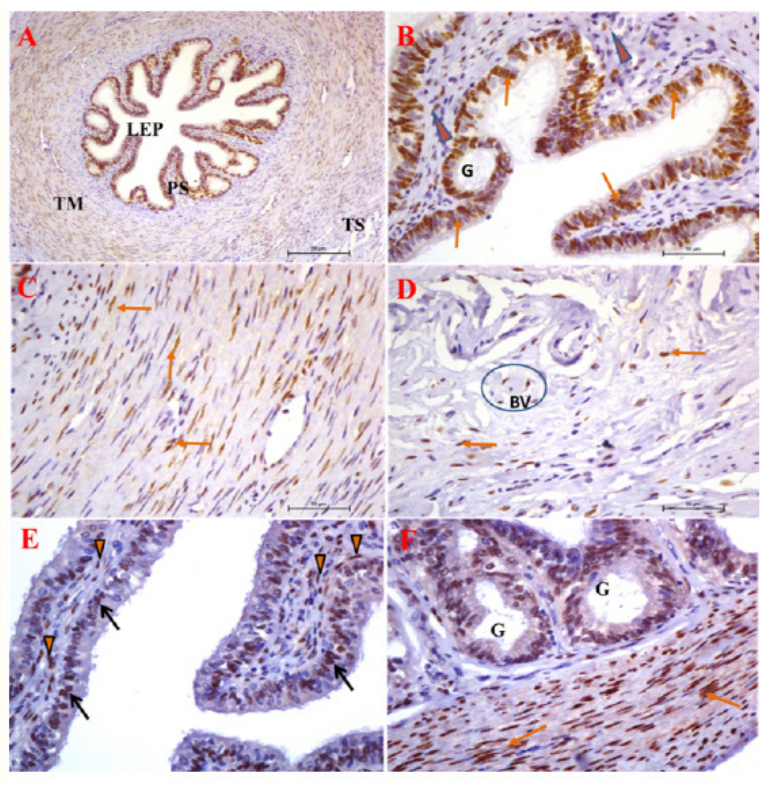

Figure 3: Immunostaining of isthmus of buffalo with anti-PR antibody. A. During the follicular phase, the nuclear reaction in lamina epithelialis (arrow), endothelial cells of blood vessels (Bv), propria submucosa (arrowhead); B. During the follicular phase, the nuclear reaction in lamina epithelialis (arrow), propria submucosa (arrowhead); C. Nuclear reaction in the glandular epithelium (G) and muscle fibers (arrows) in Tunica muscularis; D. Nuclear reaction in connective tissue fiber (arrow) and blood vessels in Tunica serosa; E. During the luteal phase, the nuclear reaction in lamina epithelialis (arrow), propria submucosa (arrowhead); F. Nuclear reaction in the glandular epithelium (G) and muscle fibres (arrows) in Tunica muscularis. Polymer HRP method. Original magnification. x400.

Distinct PR localization was detected in different elements of oviduct in the mucosal lining, connective tissue layer, muscle layer, and outermost serosal layer during both the phases of the estrous cycle. Therefore, it is clear from the study that all the compartments were responsive to the progesterone hormone, and thus physiologically active. Similarly, PR was demonstrated by IHC in the different histological layers of all the parts of the oviduct of heifers under untreated and superovulated estrous cycle, irrespective of the cycle phase and the flowing strengths of steroid hormones in the blood [20]. The presence of PR in ciliated cells and secretory cells indicated that ciliary beating and secretory activities of the cells were under the control of the steroid hormones. However, in Thai Swamp buffalo, PR immunostaining was detected in the secretory cells in all segments of the oviduct [22]. Steroid hormones are known to affect the beating of the cilia which are mostly located in the ampulla [23]. The secretory blebs protruding out of the apical surface of the cells also showed a positive reaction suggesting that the secretory blebs consisted of nuclear fragments. Similar observations have been recorded using the transmission electron microscope in a buffalo oviduct where nuclear fragments were observed in secretory blebs [24].

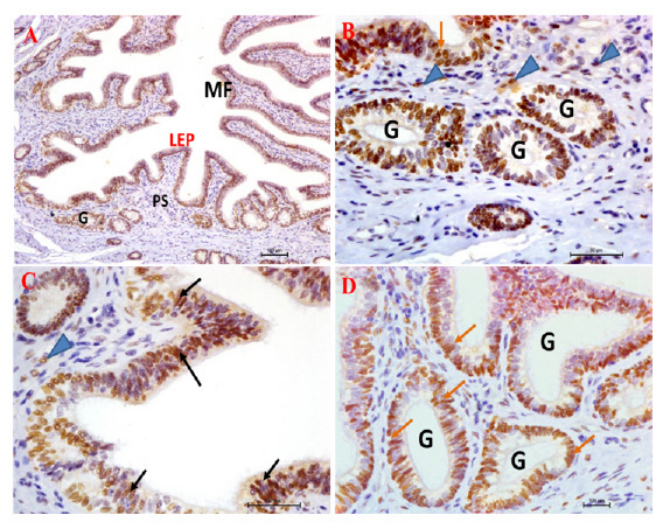

Figure 4: Immunostaining of utero-tubal junction of buffalo with anti-PR antibody. A. During the follicular phase, the nuclear reaction in mucosal folds (MF), lamina epithelialis (LEP), the glandular epithelium (G), propria submucosa (PS); B. During the follicular phase, the nuclear reaction in lamina epithelialis (arrow), propria submucosa (arrowhead) and glandular epithelium (G); C. During luteal phase nuclear reaction in lamina epithelialis (arrow), propria submucosa (arrowhead); D. During luteal phase Nuclear reaction in the glandular epithelium (arrows) in oviductal glands (G). Polymer HRP method. Original magnification. x400.

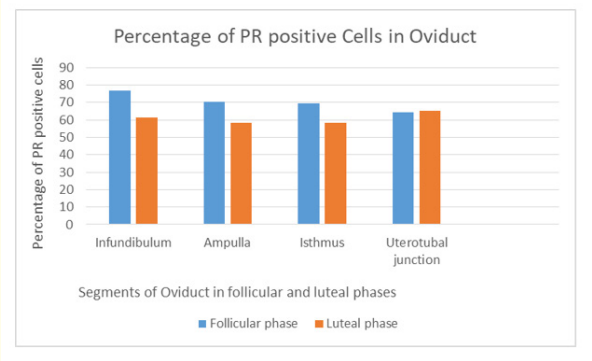

Figure 5: Graph showing percentage of PR positive cells in oviduct during follicular and luteal phases of the estrous cycle.

The measurable evaluation of the proportion of PR stained cells in various portions of the oviduct of buffalo during distinct stages of the estrous cycle has been shown in Figure 5. As per the data assessment, the infundibulum contained the highest proportion of PR immunopositive cells, and the utero-tubal junction was the least. The infundibulum and ampulla had a significantly higher

number of PR-positive cells, as compared to the uterotubal junction ($p < 0.05$). The first three segments of the buffalo oviduct demonstrated a higher proportion of PR positive cells during the estrogen-dominant phase of the estrous cycle ($p < 0.05$) while no significant difference was observed in the immunoexpression of PR in the last segment.

During the follicular phase, intense nuclear PR reactivity was observed in the cells forming mucosa, few stromal cells were present in the connective tissue layer and lining tissue layer of blood capillaries of ampulla while during the progesterone hormone dominant phase, the strength of reactivity was reduced as compared to that of the estrogen hormone dominant phase. The cells of the oviductal glands and the muscle cells in the muscular tunic also showed immunoreactivity. Similar to our findings, specific nuclear staining and faint cytoplasmic staining were observed in the lamina epithelialis, the stromal cells, and the tunica muscular layer in the ampulla of a cow [25]. PR staining in rat ampulla was detected in the nucleus of cells in glands, pseudo glands, and cells of connective tissue in the estrogen-dominant phase. PR localized fairly during the progesterone dominant phase and reached a maximum after depot-medroxyprogesterone acetate administration [26].

Nuclear reactions of ER and PR have been detected in the lamina epithelialis, stromal cells, and tunica muscular layers of the canine oviduct, similar to our findings, but marginal differences in staining patterns were observed between the ampulla and fimbriae [13].

Similar staining patterns were also recorded in the isthmus. The strong staining in the nucleus was detected in the covering cells of mucosa and glands, while the cells in the connective tissue layer showed fair to intense reactions. The intense reaction in tunica muscularis as compared to that in the follicular phase is indicative of its role in the contraction of smooth muscle fibers. In the isthmus of the cow, specific nuclear staining and faint cytoplasmic staining were detected in the lining mucosal cells, the connective tissue cells, and tunica muscularis layers in the isthmus of a cow [25]. In the pig oviduct, during the follicular phase, immediately before ovulation, no staining was recorded either in the ampulla or in the isthmus stroma, however, a slight expression of PR was observed in the lamina epithelialis of the ampulla [9].

Infundibulum and ampulla had a statistically significantly higher number of PR positive cells as compared to utero tubal junction. The variations observed in the percentage PR positive cells between the infundibulum, ampulla, isthmus, and uterotubal junction might reflect the different responses of these regions of the oviduct to estrogen and progesterone. The variation between segments of the oviduct has also been reported in swine [9]. Variation in the microcirculation of the respective regions might be responsible for this differential response.

The average strengths of blood estradiol in the follicular and luteal periods of the research buffaloes were 28.82 ± 1.13 pg/ml and 13.13 ± 1.18 pg/ml respectively. The average blood progesterone strength in the research buffaloes was 0.13 ± 0.42 ng/ml during the follicular and 2.63 ± 0.62 ng/ml during the luteal phases, respectively. The hormonal levels, obtained in the present study, were in the range described in buffaloes [27]. The first three parts of the oviduct showed a greater proportion of PR-staining cells during the estrogen-dominant phase while no substantial variation in the proportion of PR-staining cells during the two stages in the last segment.

The statistics on the proportion of PR stained cells and hormone strengths indicated that the PR representation was greater during the estrogen hormone supremacy and lesser in the progesterone hormone control. Thus it was not related to its hormone level. Therefore, it's likely that estrogen influenced PR localization in the oviduct in a stimulatory way. Similar to our observations, elevated PR mRNA transcripts were observed in the cow oviduct during the follicular phase [19]. However, no significant effects of estrogen or progesterone were observed on the PR levels in the oviduct of female lambs [14]. Contrary to our findings, the data recorded on PR expression at the gene level suggested that the serum progesterone concentrations probably exerted a direct control on the PR expression in the bitch oviduct [12]. Similar to our findings, in the canine oviduct, the reactions for PR showed changes throughout the estrous cycle and these changes were found to be related to the changing concentrations of estrogen and progesterone hormones. High immunostaining for PR was found during proestrus and low immunostaining during early metestrus. Stromal cells are generally stained with a higher intensity for PR than epithelial cells. Thus, some functions of steroid hormone on the epithelium may be mediated through stromal cells [13].

Conclusion

From the current research, it was established that the progesterone receptor was distributed in cells of mucosa, connective tissue compartment, glandular epithelium, the muscle layer, and serosal layer in different sections of the buffalo oviduct. In the lining mucosa, PR staining was observed in both cell types. The first two compartments had a higher proportion of PR-stained cells in comparison to that in the last segment ($P < 0.05$). The first three compartments revealed a greater proportion of PR-stained cells during the estrogen hormone dominant phase of the estrous cycle as compared to the progesterone hormone dominant phase of the estrous cycle ($P < 0.05$). The statistics on the proportion of PR-stained cells and hormone strengths indicated that the PR representation was greater during the estrogen hormone supremacy and lesser in the progesterone hormone control. Different portions of the buffalo oviduct responded in different ways to hormones.

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Conflict of Interest

All authors declare that they have no conflict of interest.

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