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Research Article

SPATIOTEMPORAL EXPRESSION OF PRLR IN MURINE OVIDUCT DURING PREIMPLANTATION PERIOD

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ABSTRACT. In mice, the oviduct is required for the timely accomplishment of gamete transport, fertilization, preimplantation embryo development, and to deliver a competent and healthy conceptus to the endometrium. Apart from the ovarian steroids, prolactin (PRL)from hypophysis acting through prolactin receptor (PRLR) plays a major role during early pregnancy. The present study is aimed to know the spatiotemporal localization of PRLR in the mouse oviduct during the pre- and peri-implantation phases of early pregnancy. Oviducts were collected from mice during different phases of early pregnancy, fixed in 4% paraformaldehyde, subjected to immunohistochemistry and western blotting. The intensities of immunohistochemical staining were measured in the oviductal subregions using ImageJ (Fiji) software. The expression of PRLR was principally found in the apical membrane and cytoplasm of oviductal cells, irrespective of its subregions (infundibulum, ampulla, and isthmus). Nevertheless, the intensity of PRLR varies throughout early pregnancy. The highest expression of PRLR was observed in the ciliated epithelial cells of the infundibulum and ampullary region of the oviduct on gestation day (GD) 1.5 and 2.5. However, downregulation of PRLR in the oviductal epithelial cells was noticed during the peri- and post-implantation periods. The diminished PRLR expression in the epithelial cells was maintained thereafter. Western blot analysis revealed a single protein band with an apparent molecular weight of 90 kDa. It is inferred that time specific expression pattern of PRLR along the oviductal subregions indicates the requirement of prolactin hormone and its receptors during the preimplantation period.

Keywords: *Prolactin receptor, mouse, oviduct, ampulla, ısthmus*

INTRODUCTION

The oviduct is a muscular tube, which is the first interface between the female genital tract and fertilized embryos [1-3]. The oviductal epithelium synthesizes and secretes various molecules into the lumen, such as growth factors, proteases, antioxidants, sex hormones, etc [4]. Besides, it plays an important role in gamete transport, fertilization, preimplantation embryo development, and embryo transport, thus, rendering a suitable microenvironment for preimplantation embryos. In rodents, the oviduct is coiled and composed of three subregions ordered from the ovary towards the uterus: infundibulum, ampulla, and isthmus. Further, mesosalpinx, myosalpinx, and endosalpinx are the histotrophic compartments of the oviduct. As multiple factors are required for *in vitro* fertilization (IVF) [5,6], understanding the oviductal microenvironment can improve IVF and preimplantation embryo development.

Prolactin (PRL), 23kDa protein, secreted primarily by lactotropes in the adenohypophysis [7], is closely related to growth hormone [8]. It plays a key role in the regulation of reproductive functions in mammals [9,10] and is crucial for physiological adaptations of pregnancy and lactation [11-13]. The diverse biological actions of PRL are mediated via its cognate prolactin receptor (PRLR). The PRLR is a single-pass transmembrane protein, a member of the cytokine receptor superfamily [14,15]. Multiple PRLR isoforms were identified in various animals [7,10,16]. Two major isoforms of PRLR identified in mice are long and short forms of PRLR [10,17]. Number of factors in the oviduct that influence preimplantation embryo development process may be affected by the PRLR mutation [18]. The expression of PRLR has been reported in mouse ovaries [19,20], oocytes and embryos [21,22], and the uterus [23]. Another report documents cell type-specific localization of PRLR isoforms in the oviduct of prepubertal female mice [17]. Despite extensive research on the expression of PRLR in reproductive tissues of women and laboratory rodents, the expression and function of this receptor in the oviduct during early pregnancy remained unclear. Currently, researchers consider the oviduct as the most suitable microenvironment for early embryonic development. Hence, the present study is aimed to characterize the expression pattern of PRLR protein in mouse oviducts during early pregnancy from preimplantation stage to gestation day (GD) 6.5 by immunohistochemical and western blot analysis.

MATERIALS AND METHODS

The study was conducted in the year 2019 at the Department of Zoology, Karnatak University, Dharwad. Thirty sexually mature female Swiss albino mice, *Mus musculus* (25 to 30 gm), exhibiting regular estrous cycle, were purchased from Geniron Biolabs, Bangalore. In the animal house, settings had been established for two weeks at the requisite laboratory conditions. All mice were housed in polypropylene cages at ambient temperature (27±1˚C) and supplied food and water *ad libitum*. The animal rooms were maintained on a 12-hour light:dark cycle with 40-50% relative humidity.

Isolation of oviducts from pregnant mice

Adult female mice in proestrus or early estrus were cohabited with fertile male mice in a 1:2 ratio in the evening, and copulation was confirmed by the presence of copulatory plug in the next morning. The vaginal plug positive day was designated as gestation day 0.5 (GD 0.5). Pregnant female mice were sacrificed on GD 1.5 (n=5), 2.5 (n=5), 3.5 (n=5), 4.5 (n=5), and 6.5 (n=5), respectively. The oviducts were isolated and fixed in 4% paraformaldehyde (4% PFA).

Immunohistochemical analysis of PRLR in mouse oviduct during early pregnancy

PFA fixed oviduct samples were dehydrated in a series of increasing concentrations of ethanol (10% to 100%), clarified in benzene, and embedded in paraffin (Fisher Scientific, New York). Molded blocks of oviducts were sectioned at 5µm on a rotary microtome (Leica RM2025, Germany). Serial sections were collected on albumin-coated slides and subjected to immunohistochemistry. After deparaffinization and rehydration, the sections were placed in trisodium citrate buffer (pH=6.0, C3674 Sigma-Aldrich, St. Louis, MO, USA) for antigen retrieval. Endogenous peroxidase activity was quenched by incubating tissue sections in 3% hydrogen peroxide (H_2O_2). Non-specific staining was blocked by incubating slides with 5% Bovine Serum Albumin (BSA, Sigma-Aldrich, St. Louis, MO, USA) in PBS. Sections were incubated with a polyclonal antibody specific for PRLR (host: rabbit; 1:200; M-170, cat. No. sc-30225, Santa Cruz Biotechnology, USA) at 4˚C overnight. After 3 washes in PBS and PBS-T, the sections were incubated with goat anti-rabbit IgG conjugated to HRP (1:400, sc-2004, Santa Cruz Biotechnology, USA). Staining was visualized with 3,3'-diaminobenzidine (DAB; D5637, Sigma-Aldrich) with H_2O_2 as peroxidase chromogen. Negative control of the immunohistochemical reaction was performed by replacing the primary antibody with antibody diluents. Following counterstaining with Harris-Haematoxylin (39411, Fisher Scientific), slides were mounted permanently with DPX. Sections were observed and photographed under a Nikon Eclipse 80imicroscope with ACT-2U software (Nikon Corporation, Tokyo, Japan).

Quantitative evaluation of immunoexpression of PRLR

The immunohistochemical staining intensity of PRLR in oviductal subregions was evaluated by measuring the optical densities (ODs). Briefly, the images were captured with Nikon Eclipse 80i with ACT-2U software at 200X magnification. The OD was evaluated using ImageJ (Fiji) software. Each image was first deconvoluted using the H/DAB vector into three different coloured images (i.e., green, brown, and blue). The brown-DAB image was converted to grayscale, and its intensity was recorded by measuring the mean integrated intensities (mean gray value). At least five fields of each histological region were recorded in each section, and five sections per animal were evaluated. The intensity numbers were converted into the OD using the following equation:

$$\mathrm{OD} = \log\left(\frac{\text{maximum intensity}}{\text{mean intensity}}\right)$$

where the max intensity = 250 and the mean intensity = mean gray value. The results were exported to a Microsoft Excel spreadsheet.

Western blot and dot-blot assay

Proteins from ovarian tissues were extracted using RIPA buffer (Sigma Aldrich, USA), and the determination of protein concentration was performed by following the Bradford assay. Thirty micrograms of total protein per sample were separated by 8% sodium dodecyl sulphate (SDS)- polyacrylamide gel electrophoresis (PAGE). After electrophoresis, resolved proteins were transferred to a polyvinylidene fluoride membrane(Immobilon Millopore, Bedford, MA). The complete transfer was assessed using protein standards (Fermantas Life Sciences). The membrane was treated with blocking buffer (5% non-fat dried milk in tris buffered saline [TBS] containing 0.1% Tween 20) for 1 h at room temperature. The membrane was incubated overnight at 4˚Cwith a polyclonal antibody specific for PRLR (host-rabbit, 1:1000, M-170, cat. no sc-30225, Santa Cruz Biotechnology, USA) or β actin (Host-rabbit, 1:1000, N-21, cat. No. sc-130656, Santa Cruz Biotechnology, USA)as a loading control. The membranes were washed three times with TBS and then incubated withgoat anti-rabbit IgG conjugated to HRP (1:400, sc-2004, Santa Cruz Biotechnology, USA) for 2 hr at RT. After washing, immunoblotting signals were visualized by staining with TMB/H2O2 (SFE33128, Genei) at room temperature for 5-10 m.

For the dot-blot assay, small circles were drawn on the PVDF membrane, and two µl of total protein extracts were spotted at the center of each circle. Once the membrane dried, it was blocked with 5% non-fat dried milk in TBST for 1 h at room temperature. The membrane was then incubated with polyclonal antibody specific for PRLR (1:1000, M-170, Santa Cruz Biotechnology, USA) overnight at 4˚C. After washing with TBST three times, membrane was incubated withgoat anti-rabbit IgG conjugated to HRP (1:400, sc-2004, Santa Cruz Biotechnology, USA) for 1 hr at RT. The membranes were washed three times with TBS; protein signals were visualized by DAB staining (Sigma-Aldrich) at room temperature for 1-2 minutes.

Statistical analysis

Data were analyzed by statistical analysis software SPSS version 26 (SPSS Inc, Chicago, USA). All data were tested, and the normality of the data distribution was established. The differences in the expression of PRLR among different regions of the oviduct during early pregnancy were assessed by one-way analysis of variances (ANOVA) followed by Tukey's post-hoc test. Differences in the intensity betweenthe apical cell membrane and cytoplasm was assessed by independent sampleT-test. Data were presented as mean ± SEM. $P<0.05$ and $P<0.01$ were set as the limit of statistical significance.

RESULTS AND DISCUSSION

Immunolocalization of PRLR in different regions of oviduct during pre, peri and postimplantation period

In the present investigation, attempts were made to document the spatiotemporal immnulocalization of PRLR in the mouse oviduct during various days of early pregnancy by immunohistochemistry and western blot analysis. The results reveal that irrespective of gestation days, PRLR positive staining was observed in the apical surface and subapical cytoplasm of epithelial cells of the oviductal mucosa (endosalpinx)throughout the oviductal segments (Fig. 1-3). The highest expression of PRLR was observed on GD 1.5 and 2.5 in ciliated cells of infundibulum and ampulla (Fig. 1A-B, D-E). Whereas, connective tissue located under the epithelium (lamina propria) exhibited weak immunoreactivity for PRLR (Fig. 1). The myosalpinx exhibited weak positive immunoreactivity for PRLR, except on GD 1.5 where its immunoreactivity was intense (Fig. 1 A-C). On GD2.5 highest expression of PRLR was found in the ciliated epithelial cells of the infundibulum and ampulla and non-ciliated cells of the isthmus (Fig. 1D-F, Fig. 4A).Moreover, the expression of PRLR is abundant in the apical cell membrane than in the cytoplasm irrespective of oviductal subregions (Fig. 4 B-C). Since PRLR is a single transmembrane protein and acts through JAK/STAT pathway and tyrosine phosphorylation [24,25], its immunoreactivity in the apical cell membrane is expected.

The highest expression of PRLR in epithelial cells on GD 1.5 and 2.5 suggests that surge of PRL may have a stimulatory effect on oviductal epithelial cells' expression of PRLR to execute its function. The results suggest the involvement of endocrine or paracrine PRL signalling pathways in the oviduct during the preimplantation period. As far as our knowledge is concerned, there is a single recent report on the cell-specific expression of PRLR isoforms in mouse and human fallopian tubes [17]. It reports that in the mouse fallopian tube, the expression of long-form of PRLR (~97 kDa) decreased after PRL treatment and increased after blockage of endogenous PRL secretion in a time-dependent manner. Additionally, the null mutation of the prolactin receptor gene produces multiple reproductive defects, and the authors suggest that fertilized eggs develop poorly to the blastocyst stage in PRLR -/- mice due to the deficient environment of the embryo in the oviduct [18]. Another study reports that surges of PRL appear with semi-circadian rhythmicity until day 8 of pregnancy in mice [26]. Further, osmoregulatory function of the prolactin is required for the adaptability of embryos to the various liquid environments during the course of its transfer from the ovary to the endometrium [27]. The PRL directly stimulates transcellular active Ca^{2+} transport and influences Ca^{2+} absorption in the intestinal epithelial cells in female rats [28]. The results of the present study indicate the possible role of PRL induced PRLR in the regulation of Ca^{2+} -dependent ciliary beats for gamete and embryo transport in the fallopian tube, as suggested by Shao et al. 2008 [17]. In our study, the highest expression of PRLR was seen in the ampullary region of the oviduct during the preimplantation period where ciliary cells dominate. Hence, the highest expression of PRLR during GD 1.5 and 2.5 indicates the requirement of PRL and PRLR in the ampullary region of the oviduct for the transport of gametes and preimplantation embryos.

Immunostaining for PRLR was also detected mainly in the epithelial cells of oviduct on GD 3.5 (Fig.2A-C). However, the intensity of immunoexpression of PRLR diminishes considerably in the epithelial cells of the infundibulum and ampullary region. The observed results suggest the downregulation of PRLR. On GD 3.5, compacted morula stage embryos enter the isthmus region of the oviduct by the ciliary action of the ampulla. Because the embryos move from the ampulla to the isthmus on GD 3.5, ciliary action is no more needed at this stage. Hence, the downregulation of PRLR in ciliary cells of the ampulla can be associated with the absence of embryos in this region. Further, both expression patterns and intensity of PRLR in the oviductal epithelial cells on GD 4.5 were similar to those of GD 3.5 (Fig. 2D-F, Fig 4A). Besides, the immunoexpression of PRLR drastically decreased in the epithelial cells of all three regions of the oviduct on GD 6.5 (Fig. 3A-C, Fig. 4A), and the same was maintained thereafter.

It is well established that the ovarian steroids, estradiol (E_2) and progesterone (P_4) are known to modulate the oviductal functions [29-32]. P_4 secretion does not begin until around 48 h pc and there was a significant rise in the plasma P_4 level on day 3 of pregnancy [33]. Studies have shown downregulation of PRLR in various tissues when plasma progesterone level rises [17, 34, 35]. Thus, the downregulation of PRLR in the oviduct from GD 3.5 onwards was expected, as it could be due to enhanced progesterone levels on these gestation days. The above-mentioned studies and results from the current study reveal the essentiality of PRLR in the epithelial cells and it can be concluded that during the preimplantation period, PRL acting through PRLR regulates numerous oviductal functions.

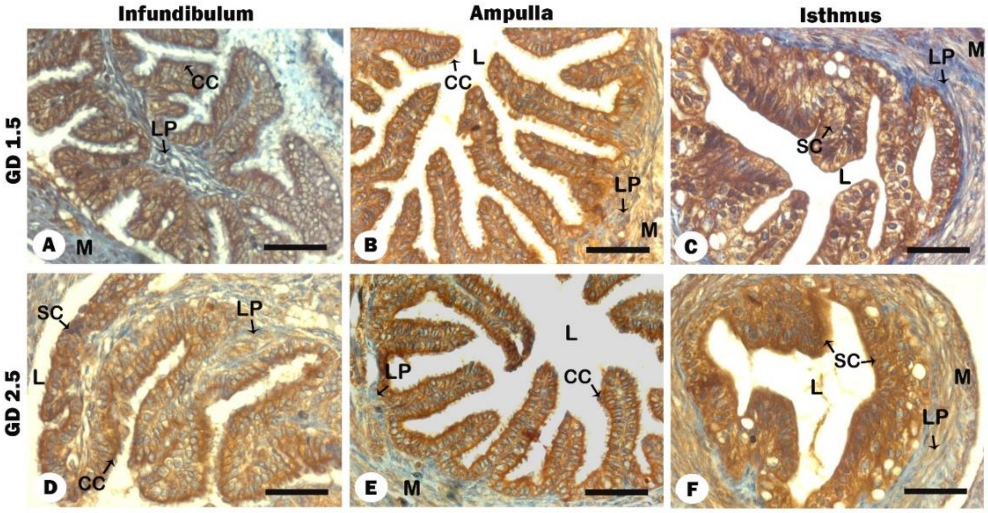

Fig. 1. *Immunohistochemical localization of PRLR in mouse oviductal subregions during preimplantation period. Highest expression was found in the epithelial cells of infundibulum and ampullary region during GD 1.5 and 2.5. CC-ciliated cells; L- lumen; LP- lamina propria; M- muscle layer; SC- secretary cells. Scale bar – 30 µm.*

In addition to immunohistochemistry, we have carried out western blot and dot blot analysis for oviduct samples to confirm the antibody specificity. Results of the dot-blot analysis further confirm these findings (Fig. 5A, D). Results of the western blot analysis supported our observations of immunohistochemistry The molecular weight of the PRLR band was approximately 90 kDa, that is related to the long-form of PRLR (Fig. 5B), which is in agreement with an earlier report on the predominant existence of long form of PRLR in the oviduct [17].

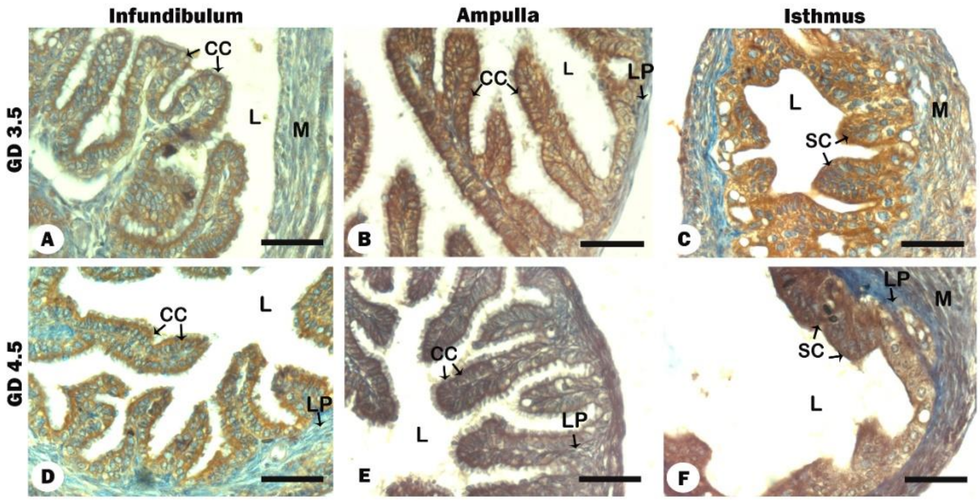

Fig. 2. *Immunodetection of PRLR protein in the various subregions of the mouse oviduct during peri-implantation period of pregnancy. CC-ciliated cells; L- lumen; LP- lamina propria; M- muscle layer; SC- secretary cells. Scale bar – 30 µm.*

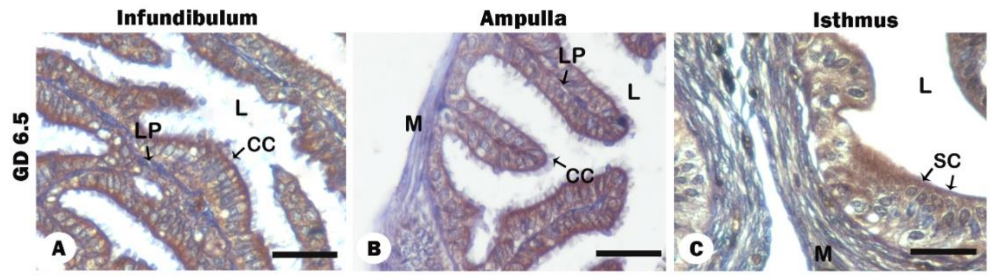

Fig. 3. *Immunoexpression of PRLR in the various subregions of the mouse oviduct during postimplantation period of pregnancy. Note the downregulation of PRLR protein during postimplantation period. CC-ciliated cells; L- lumen; LP- lamina propria; M- muscle layer; SC- secretary cells. Scale bar – 30 µm.*

To summarize, the highest expression of PRLR noticed on GD 1.5 and 2.5, coincides with the launching of theembryo into the oviduct, and the downregulation of PRLR follows the progression of the embryo from the oviduct to the uterus on GD 4.0. Our results suggest that PRLR expression in mouse oviduct is persisted during early pregnancy and its intensity varies as the pregnancy progresses.

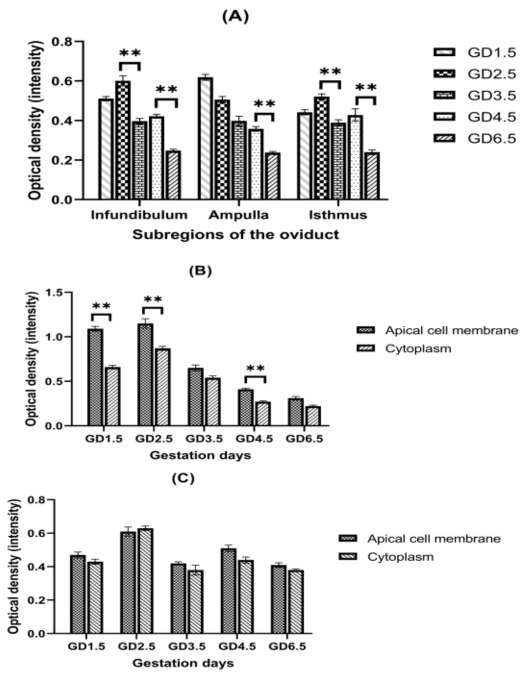

Fig. 4. A) *Immunoexpression intensity of PRLR in the epithelial cells of oviductal subregions during early pregnancy.* ***B)*** *Densitometric analysis of PRLR in the apical cell membrane and cytoplasm of the ciliated cells (ampulla) among various days of early pregnancy.* ***C)*** *Intensity of immunoexpression of PRLRin the apical cell membrane and cytoplasm of the nonciliated cells (isthmus) among various days of early pregnancy. Quantitative immunostaining was performed with ImageJ [Fiji] software. Graphs were plotted with the help of Graphpad Prism. The value of optical density (OD) was used as the level of expression. Data were presented as mean ± SE. (One way ANOVA). *=P<0.05, **=P<0.01.*

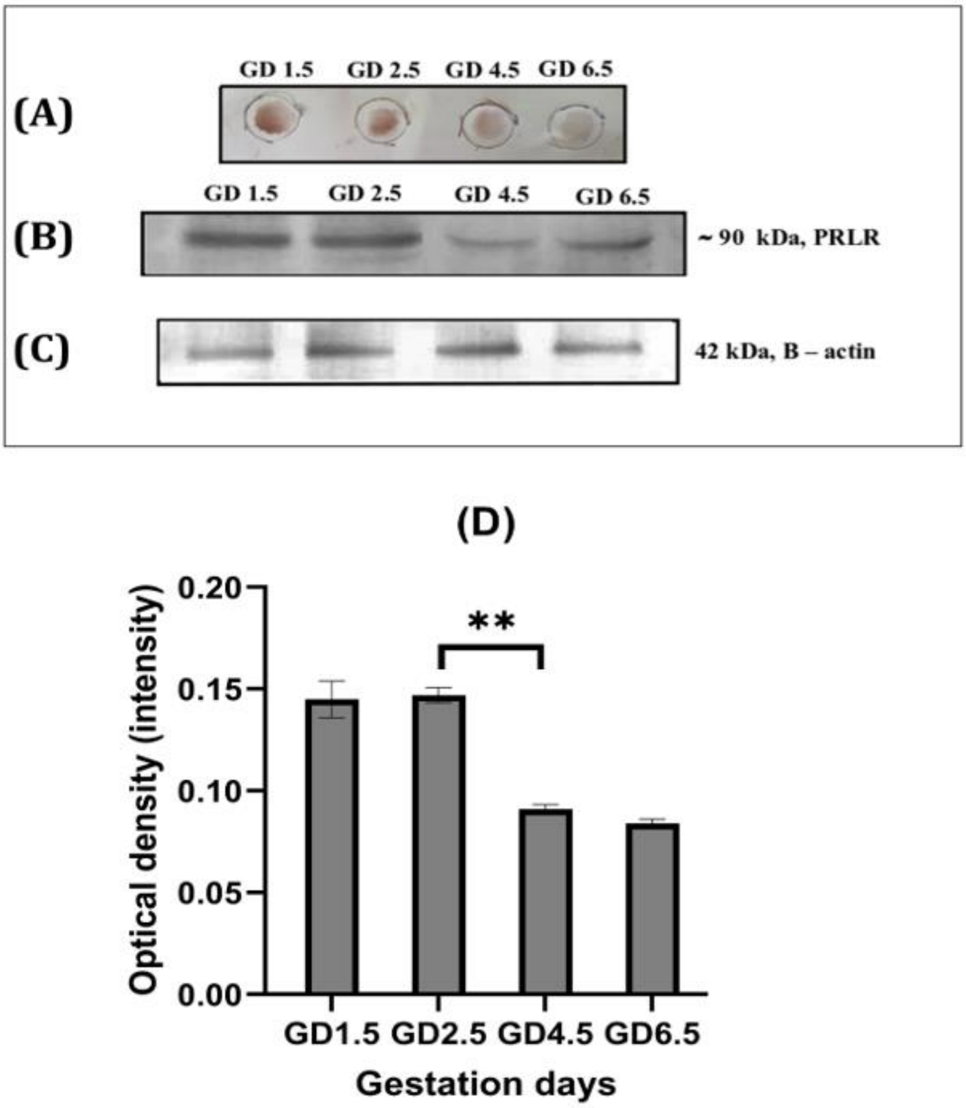

Fig. 5. (A) *Dot blot analysis and* ***(B-C)*** *Western blot analysis of PRLR protein in the mouse oviduct during different phases of preimplantation period.* ***(D)*** *Intensity of dot-blot assay of the oviduct protein extracts one arly pregnancy. Quantitative immunostaining was performed with ImageJ [Fiji] software. The value of optical density (OD) was used as the level of expression. Data were presented as mean ± SE. (One way ANOVA) ** =P<0.01.*

CONCLUSION

In conclusion, our results suggest that elevated PRLR expression in mouse oviduct persists during first 4.5 days of pregnancy and its intensity varies as the pregnancy progresses. Its time-specific expression pattern along various regions of the oviduct indicates its requirement for embryonic development, especially during the preimplantation period. Taken together, our results suggest that *in vivo* oviductal PRLR signaling has a role during preimplantation embryo development.

Acknowledgement. All protocols used in this experiment adhered to the CPCSEA guidelines for the Care and Use of Laboratory Animals approved by the Institutional Animal Ethical Committee No.639/GO/02/a/CPCSEA at Department of Zoology, Karnatak University, Dharwad.

Conflict of Interest. The authors declared that there is no conflict of interest.

Authorship Contributions. Concept: C.S., L.I., Design: C.S., L.I., Data Collection or Processing: C.S., P.K., L.I., Analysis or Interpretation: C.S., L.I., Literature Search: C.S., P.K., L.I., Writing: C.S., L.I.

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