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# Immuno histochemical localization and expression of MIS receptor alpha protein in the oocyte of *Labeo rohita*

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**Abstract**
Steroid progesterone induces the resumption of maturation in oocytes via a nongenomic pathway through binding to a novel membrane progestin receptor (mPR). Moreover, the membrane progestin receptors (mPRs) that act as intermediaries in these nongenomic steroid actions have been identified biochemically on plasma membranes of fish and amphibian oocytes, as well as on spotted seatrout and mammalian sperm. However, the expression and localization of MIS receptor α gene/protein during ovarian development of the Indian major carp, *Labeo rohita* have not been reported. We analyzed MIS receptor α transcript abundance during hCG-induced oocyte maturation, *in vitro* by immunohistochemistry. However, the present study focused to identify the immunohistochemical localization of the mPRα protein in the oocyte of *Labeo rohita*. The oocyte sections were incubated with specific antibody for mPRα protein immunoreactions. Present results provide an idea of expression of mPRα protein in the oocyte membrane of *L. rohita.*

**Keywords:** Immunohistochemical, mPR alpha, *Labeo rohita*, Oocyte, Maturation.

## 1. Introduction
Vertebrate gametes, which have little or no transcriptional activity, have proven to be valuable models for elucidating nonclassical mechanisms of steroid action (Revelli *et al*., 1998 and Luconi *et al*., 2004) [12, 8]. Membrane progestin receptors (mPRs) that act as intermediaries in these nongenomic steroid actions have been identified biochemically on plasma membranes of fish and amphibian oocytes, as well as on spotted seatrout and mammalian sperm (Blackmore and Lattanzio, 1991; Thomas *et al*., 1997; Baldi *et al*., 1999) [3, 2, 16]. Progesterone acts as a maturation-inducing hormone (MIH) resulting in meiotic resumption of oocytes form prophase-I arrest in *Xenopus* (Kishimoto, 2003) [7]. The maturation inducing steroids induce the oocyte maturation and ovulation by the action of non-genomic and genomic mechanism. Zhu *et al*., (2003b) [23] have reported the endogenous expression of mPRα and mPRβ protein in the oocyte plasma membrane. Hanna and Zhu (2008) [5], have first reported the immunolocalization of mPR proteins in the pituitary, cortical granules in the oocyte, spermatogonia/spermatocytes of zebrafish. Immunohistochemical studies of Hanna and Zhu (2008) [5] reported the co-localization of proteins and mRNAs of both mPRα and mPRβ in the major reproductive organs, including ovary, testis and pituitary of zebrafish. They reported the expression of mPRα and mPRβ in denude oocytes and follicular layer cells. They further stated that mPRα and mPRβ proteins were localized at or near the oocyte membrane of maturationally competent stage IV oocytes of *D. rerio*. Zhu *et al*., (2008) [24] have reported the expression of mPRα and mPRβ proteins in the late vitellogenic oocytes of zebrafish. They conducted immunohistochemical studies using mPR specific antibodies and pre-immune serum. mPRα and mPRβ proteins were localized at or near the oocyte membrane and in the follicular layers of oocyte. The distinct family of membrane progestin receptors mPRα, mPRβ and mPRγ that mediate rapid, nongenomic actions of progestins has been identified and characterized in several fish species, frogs, rats and humans. Not much work has been reported in the immunolocalization of mPRα proteins in the oocyte sample of *L. rohita*. This is the first study to examine the tissue specific immunohistochemical localization of mPRα protein in the oocyte sample of *L. rohita*. Milla *et al*., (2008) [9] have studied the immunolocalization of mineralocorticoid protein in the testis of rainbow trout.

Reports from various studies indicate that mPRα and mPRβ have important role in the regulation of meiotic maturation of oocyte and motility of sperm in both fish and humans. Studies of Thomas *et al*., (2008) [19] have reported the mPRα mRNA expression in the human sperm by the RT-PCR analysis and the localization of mPRα protein by western blotting method. Furthermore, they have conducted immunocytochemical studies in whole human sperm confirmed the mPRα protein expression in the plasma membrane of sperm and it is localized to the midpiece of sperm. It indicates the role of mPRα in the motility of sperm by regulating progestin. The fully grown oocytes of *D. rerio* were defolliculated and were subjected to immune of fluorescent staining using anti-mPRα and mPRβ antibodies. The expression of mPRα and mPRβ on the oocyte membrane was confirmed by Tan *et al*., 2009 [15]. Immunohistochemical localization of the Pgmrc1 protein in the ovaries of wild type *P. monodon* was observed by Preechaphol *et al*., 2010 [11]. They identified and characterized Pgmrc1 the reproduction-related genes that are differentially expressed during ovarian development.

## 2. Materials and Methods

Immunohistochemical analysis was performed following the method of Stephenson *et al.,* (2009) [14] with minor modifications.

### 2.1 Principle

Immunohistochemistry is a wide used biological technique that combines anatomy, physiology, immunology and biochemistry. Developed from the antigen-antibody binding reaction, it can be considered as methods that visualize distribution and localization of specific antigen or cellular components in separated tissues, or tissue sections.

### 2.2 Formalin-Fixed, Paraffin-Embedded Tissue Sections

Ovaries were dissected out from *L. rohita* broodstock. Sections were fixed in formalin and embeded in paraffin blocks according to standard procedures. Glass slides were cleaned with 95% ethanol; treated with subbing solution and air dried. A section was taken to 4–6 micron thickness and applied to slides. Deparaffinized in xylene three times for 5 minutes each. Sections were gradually hydrated through graded alcohols: washed in 100% ethanol twice for 10 minutes each, then 95% ethanol twice for 10 minutes each. Washed in deionized $H_2O$ for 1 minute with stirring. Excess liquid was aspirated from slides.

Heat treatment (recommended method): Slides were placed in a container and covered with 10 mM sodium citrate buffer, pH 6.0; or with 50 mM glycine-HCl buffer (glycine: sc-29096), pH 3.5, with 0.01% (w/v) EDTA (EDTA: sc-29092). The slides were heated at 95º C for 5 minutes and heated at 95º C for 5 minutes. Slides were allowed to cool in the buffer approximately for 20 minutes. Washed in deionized $H_2O$ thrice for 2 minutes each. Excess liquid was aspirated from slides.

Optional: Slides were incubated for 5–10 minutes in 0.1–1% hydrogen peroxide and in deionized $H_2O$ to quench endogenous peroxidase activity. Washed in PBS twice for 5 minutes.

### 2.3 ABC Staining Systems (ImmunoCruz™)

Specimens were incubated for 1 hour in 1.5% normal blocking serum in PBS (Buffers and General Solutions). Blocking serum (Normal Sera for Immunohistochemistry) ideally should be derived from the same species in which the secondary antibody is raised. After treatment in a blocking solution, the sections were incubated with mPRα (N-15) primary antibody (Santa Cruz, Biotechnology) for 30 minutes at room temperature or overnight at 4º C. Optimal antibody concentration should be determined by titration; recommended range is 0.5–5.0 µg/ml diluted in PBS with 1.5% normal blocking serum. Tissue sections were rinsed with PBS for 5 minutes. Further the slides were incubated for 30 minutes with biotin-conjugated secondary antibody approximately at 1 µg/ml diluted in PBS with 1.5% normal blocking serum. After administration of secondary antibody the slides were washed with PBS three times for 5 minutes each. Incubation was carried out with avidin biotin enzyme reagent for 30 minutes. Wash with PBS three times for 5 minutes each. Sections were then incubated with peroxidase substrate as provided for 30 seconds–10 minutes, or until desired intensity develops. Individual slides should be monitored to determine the proper development time. Sections were wash in deionized $H_2O$ for 5 minutes. If desired, counter-stain in Gill´s formulation hematoxylin (sc-24973) for 5–10 seconds. Slides were immediately washed several times with deionized $H_2O$. Sections were dehydrated through alcohol and xylene as follows: Soaked in 95% ethanol twice for 10 seconds each, then 100% ethanol twice for 10 seconds each, then xylene three times for 10 seconds each. Excess xylene was wiped out. 1–2 drops of permanent mounting medium (Clarion sc-24942), was added immediately and covered with a glass coverslip and observed under light microscopy.

## 3. Results

Localization and binding of mPRα mRNA encoding protein in late vitellogenic oocytes of *Labeo rohita* were confirmed by immunohistochemistry. The section of 5micron thick ovarian tissues was taken and stained with suitable antigenicity properties corresponding to the N-terminal regions of the mPRα receptors was identified using immunostaining of primary and secondary antibody along with counter stain and the slides were observed under light microscopy. (Fig. 1A) show the immunostaining of late vitellogenic oocytes of *L. rohita* without specific mPRα primary antibody and using with preimmune serum retrieved from same species in which the secondary antibody is raised against, it react as a negative control. Another slide was stained with specific mPRα primary antibody along with secondary antibody administration in suitable time incubation. Strong immunoreactivity was observed only on the oocyte surface layer containing the plasma membrane of a representative fully grown *L. rohita* oocyte under high power magnification (Fig. 1B). The slide captured under light microscopy counterstained with Gill´s formulation hematoxylin for 5–10 seconds was shown (Fig. 1C). A clear region specific immunoreactive staining was not yet detected in follicle cells or in the cytoplasm of the oocyte *L. rohita*. These results clearly demonstrate the localization of the MIS receptor in the oocyte plasma membrane of *L. rohita*. This localization of mPRα is consistent with its proposed role in mediating the 17,20β-P induces to increase the oocyte maturation in *L. rohita*.

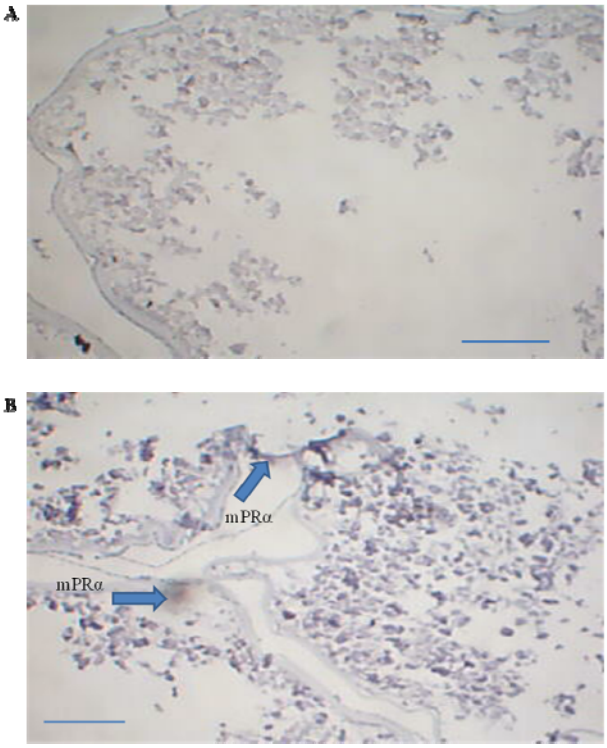


**Fig 1:** Immunohistochemical localization of mPRα protein in late vitellogenic oocytes of *Labeo rohita* brood stock using hematoxylin staining by immunohistochemistry. Picture (A) shows immunostaining with preimmune serum as a negative control. Picture (B) shows immunostaining of late vitellogenic stage of oocytes with mPRα antibody. Pictures (A and B) at a larger magnification, respectively. Scale bar: 50 µm.

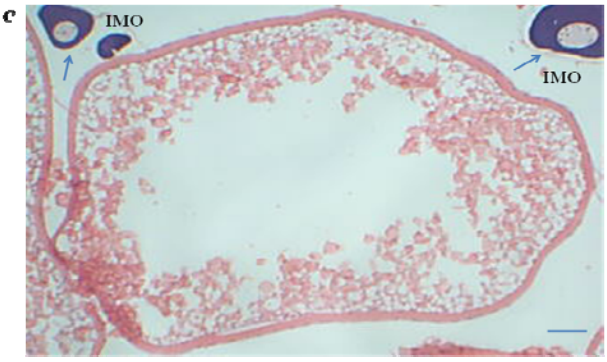


**Fig 1:** Histochemical localization of MIS receptor α protein in the oocyte of *Labeo rohita* hematoxylin staining. The blue arrow marks indicating the immature oocytes of *L. rohita*. Picture (C) at a lesser magnification respectively. Scale bar: 5 µm.

## 4. Discussion

This is the first study to examine ovarian tissue specific immunohistochemical localization of mPRα protein in the reproductive organs of *L. rohita*. Our results confirm the expression of mPRα in the late vitellogenic oocytes sample of *L. rohita*. They have reported and suggested that the mPRs, which mediate a number of nongenomic actions of progestin, are localized in the major reproductive tissues of zebrafish including the ovary, testis and pituitary. The localization of mPRα and mPRβ in these reproductive tissues is indicative of their specific roles in mediating nongenomic progestin signaling in the pituitary, spermatocytes, sperm, and oocytes (Hanna and Zhu, 2009) [6]. Sandberg and Borg (2007) [13] have suggested the receptor proteins which are not at the membrane, particularly for the mPRα, appear to be restricted to cortical granules just under the membrane. Activation by the MIS may cause release of mPR in these granules and be recruited to the oocyte membrane. The immunocytochemical studies and western blot analysis of Thomas (2008) [19], in the ovarian follicles of seatrout tissues showed that the mPRα protein is localized peripherally in the region of the oocyte plasma membrane, with no staining intracellularly and lower expression in the follicle cells surrounding the oocytes. The localization of mPRα on the oocyte plasma membrane and sperm of seatrout is consistent with previous biochemical binding and functional studies which demonstrate the presence of membrane progestin receptors on these germ cells mediating progestin induction of maturation of oocyte and sperm hypermotility. Hanna and Zhu, (2008) [5] have stated that mRNA of mPRα of *D. rerio* had been increasing in the follicle enclosed intact oocytes from the immature stage to the maturationally competent stage. In contrast mPRβ were expressed similarly in all stages of the intact oocytes. *D. rerio* mPRα and mPRβ proteins were observed by western blotting using mPRα or mPRβ antibodies. They further reported that the mPRα and mPRβ protein levels were higher in the total ovary, testis and other gonadal tissues, pituitary and those in the gills, intestine and muscle of *D. rerio*. They observed the receptor proteins of both mPRα and mPRβ in all the stages of oocytes and the level of proteins were increased as oocytes developed from early stage to late stage.

Immunohistochemistry studies of Preechaphol *et al*., 2010 [11] have revealed positive signals of the Pgmrc1 protein in the follicular layers and cell membrane of follicular cells and in the various stages of oocyte. No positive immunoreactive signals were observed by them in the oocytes and other locations of ovaries of *P. monodon*. Our studies have been proved the receptors immunoreactive bindings were observed in the membrane of the oocytes of *L. rohita*. Tan *et al*., 2009 [15] have detected the strong immunoreactivity of mPRα and mPRβ on fully grown oocyte surfaces using confocal microscopy. Immunocytochemical staining conducted by Tan *et al*., 2009 [15] have reported the mPRα localization in both the germ and interstitial cells of zebrafish. However, further studies are required to make clear the specific signaling roles of mPRα in the gonads and other tissues of *L. rohita*.

In contrast, the steroid specificities and binding kinetics be at variance noticeably from those of the nuclear progestin receptor which has been characterized in the ovary and testes of spotted seatrout, suggesting that the mPR is structurally unrelated to the nuclear progestin receptor (Pinter ant Thomas, 1997) [10]. Hanna and Zhu, (2009) [6] have reported and suggested that the mPRα and mPRβ showed distinctive expression in the testis of zebrafish. They conducted immunostaining for mPRα which is consistent with the previous finding of mPR localization in sperm. They also noted the migration of mPRα and mPRβ to the oocyte membranes as they become maturationally competent. Results of Tubbs *et al*., (2010) [22] have reported the mPRα expression in all the testicular germ cell stages in atlantic croaker. They first reported the characterization of mPRα protein distribution in many species and demonstrated the expression of mPRα protein in the reproductive tissues and brain of atlantic croaker. Luconi *et al*., (2004) [8] have reported that progesterone stimulates sperm hypermotility and acrosome reaction in several mammalian species. Studies of Amanze and Iyengar, 1990; Cosson *et al*., (2008) [1, 4] have revealed that in

fish success of fertilization depend upon the ability of sperm to enter a specialized pore in the egg called micropyle, which in turn is related to their motility. mPR α protein in the testis serve as a biomarker of sperm motility as well as fertilization capability in teleosts. Thomas *et al*., (2005) [18] have conducted western blot analysis and immunocytochemical studies in the testis of atlantic croaker. They observed immunoreactive band of mPRα with the membrane fractions of sperm and testis of atlantic croaker. These results suggested the localization of mPRα in these tissues. In croaker mPRα is localized to the sperm midpiece region in which hypermotility is regulated. Evidence was also obtained and suggests that multiple mPR subtypes participate in the well-characterized nongenomic actions of progestins to initiate oocyte maturation in fish (Thomas *et al*., 2004) [17].

Hanna and Zhu, (2008) [5] have revealed that the mPRα and mPRβ showed distinct expression in the zebrafish testis. They further stated that mPRα protein is localized to the spermatozoa; mPRβ is expressed in spermatogonia and spermatocytes. Immunofluorescence analysis conducted by Thomas *et al*., 2008 [20] in human sperm shows the localization of mPR antibody with the mPR receptor in the midpiece of sperm but it is not detected in the acrosome, low motility of human sperm show lower mPRα protein concentrations than the normal motile sperm. These reports suggested the relationship of mPR protein on croaker and seatrout sperm and their motilities, results revealed the immunocytochemical localization of mPRα proteins in the sperm midpieces of croaker, seatrout and flounder (Tubbs and Thomas 2008) [21]. Studies of Thomas *et al*., (2008) [20] have reported the lower expression of mPRα on the human sperm membrane showed low motility. Our result shows the positive immunohistochemical localization of mPRα protein in the oocyte of *L. rohita*. The present result suggests a clear idea of binding of mPRα protein localized in the oocyte plasma membrane of *L. rohita*. From our studies it is confirmed the feature of 17, 20β-P action is that maturational steroids act through receptors associated with the plasma membrane of the oocyte. This localization of mPRα is consistent with its proposed role in mediating 17,20β-P induced to increases the oocyte maturation in *L. rohita*. Further research is needed to determine whether progestin plays a role in the release of mPR types in these follicular cells layers, granules, and regulation of maturation signaling within oocytes and other than the gonadal tissues of *L. rohita*.

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