Int. J. of Life Sciences, 2013, Vol.1 (2): 123-132 ISSN: 2320-7817 | e-ISSN: 2320-964X

Formation of Primordial Germ Cells (PGCS) in the *Labeo rohita* (Ham)

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Article Received: 12/04/2013 | Article revised: 17/04/2013 | Accepted: 03/06/2013

ABSTRACT

Labeo rohita is one of the Indian major carp present in all the freshwater ecosystems of India. Primordial germ cells (PGCs) are studied by collecting different stages of *L. rohita* and were identified on the basis of their shape and size. PGCs were elliptical to spherical in shape with clear cytoplasm from 24 hrs hatchling (ah), PGCs were identified and on 60 mm development stage they were located on gonadal ridge. PGCs were bigger than the somatic cells which are present in their vicinity. Migrating germ cells were generally elliptical in shape and produced cytoplasmic extensions which help them in migration and adhesion. PGCs were seem to originate from the gut endoderm and started descending along the wall of intestine. From 24 hrs onwards, mesonephric duct are pushed upwards due to the development of air sacs between kidney and alimentary canal. Gonads were seen suspended in the coelomic cavity from 10 mm stage onwards from the dorsal body wall by a mesentery.

KEY WORDS

Labeo rohita, PGCs, Developmental stages, Gonads

INTRODUCTION

Germs cells prior to sex differentiation are called primordial germ cells as appear in the early embryonic stages. Number of species from various vertebrate groups show that PGC are first recognizable in the gut endoderm and from here they migrate to the gonad region (Allen, 1907, 1909 and 1911). Others however disagreed to this theory and believed that first of definitive germ cells were derived from PGCs but that subsequent sets were derived from somatic cells (Gatenby, 1916). Thus, it appears that there was no unity of opinion regarding the origin of definitive germ cells in vertebrate. Later, considerable work was done on isolated periods in the history of germ cells of fishes, but still no consecutive account has appeared of the whole cycle (Hann, 1927). PGCs are biologically important as founders of the germ cell lineage, they may have many applications for bioengineering of fish because of their ability to be converted into individual fish Primordial germ cells: the blueprint for a piscine life (Yoshizaki *et al.*, 2002). Development of PGCs is a very classic phenomenon and therefore, investigators are curious to know at what development stage they will be irreversibly determined. In the present work, identification, migration and differentiation of primordial germ cells is determined during development in the Indian major carp *L. rohita*.

MATERIALS AND METHODS

Healthy spawn of *L. rohita* were collected from the Pench Fish Seed Centre (Maharashtra State Govt) located near Nagpur city. For the present study, the stages were selected include the hatchling of 24 hrs, 36 hrs, 48 hrs, 60 hrs and 72 hrs. Subsequent stages of development were collected taking into consideration the length of the hatchling. These included stages of 10mm, 20mm, 30mm, 40mm, 50mm and 60mm.

International Journal of Life Sciences
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All stages were fixed by immersing in aqueous Bouin's fixative for 24 hours. However, the fingerlings measuring beyond 30mm were fixed similarly, only after slitting the abdomen for proper fixation. Decalcification of these stages was carried out in 15% formic acid solution containing 5% formalin. 5% ammonium oxalate was used to assess the decalcification test for 5 minutes. Later the material was washed and transferred to 70% alcohol. Then it was dehydrated in graded series of alcohol, cleared in xylene and embedded in paraffin wax at 60°c- 62°c and blocks were prepared.

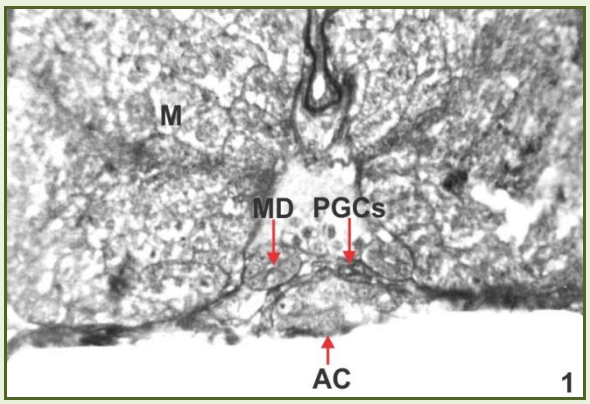
Both the transverse and saggital sections at 6-8µm on rocking microtome and were mounted serially. The sections were stained with Haematoxyline-Eosine (HE).

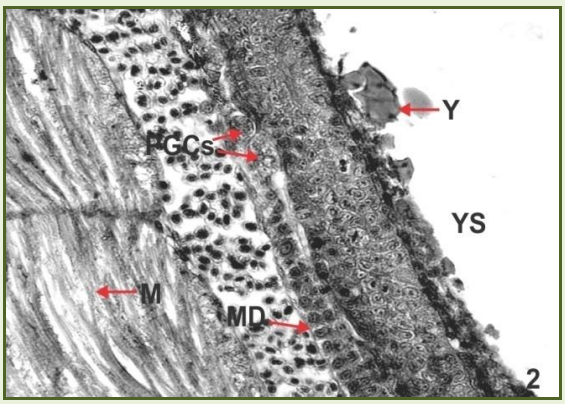
RESULTS

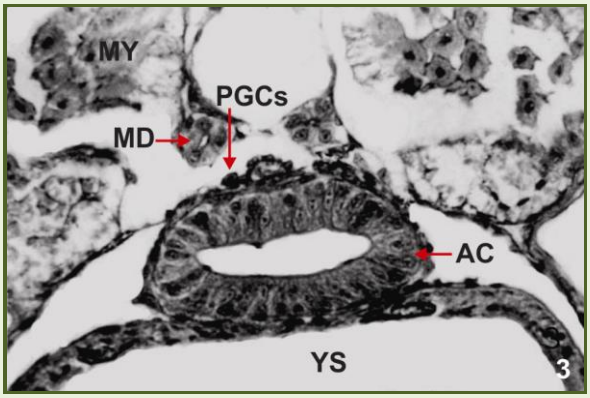
Development of PGCs was studied from 24 hrs upto 60 mm stage in *L. rohita*. Both saggital and transverse serial sections were considered for locating the PGCs. Identification was made on the basis of their characteristic shape and size.

24 hrs stage:

In transverse section, yolk sac appears bigger in size and alimentary canal is very small. Primordial germ cells (PGCs) are visible closely placed against the wall of alimentary canal on the dorsal side. Mesonephric ducts are closely pressed against the primordial germ cells and these are present in between the myotome blocks (Fig 1). Diameter of primordial germ cell is 4.2 $\pm$ 0.1 µm.







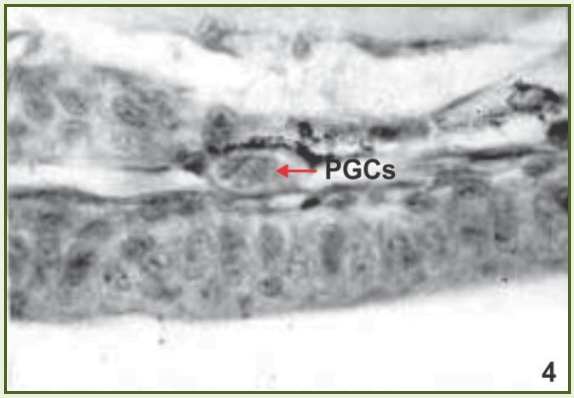


Fig. 1: Transverse Section (T. S) of 24 hrs hatchling of *Labeo rohita* showing the primordial germ cells (PGCs), mesonephric duct (MD), alimentary canal (AC) and yolk sac (YS) X200.

Fig. 2: Sagittal section of the 24 hrs hatchling showing elliptical shaped PGCs (arrow) with spherical nuclei X400.

Fig. 3: Transverse Section (T.S) of 36 hrs hatchling of *Labeo rohita* showing mesonephric duct (MD), alimentary canal (AC) and yolk sac (YS) X200.

Fig. 4: Sagittal section of the 36 hrs hatchling showing elliptical shaped PGCs (arrow) with spherical nuclei X400.

In sagittal section, the primordial germ cell appears above the alimentary canal in the form of evagination placed against the wall of alimentary canal. It is presumed that the germ cells are in the process of migration. The nuclei of all the germ cells are spherical in shape (Fig 2).

36 hrs stage:

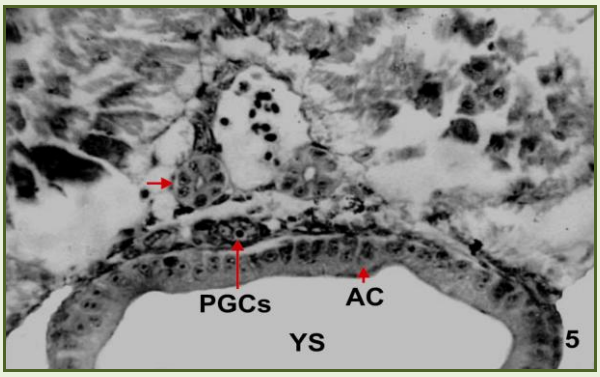
The yolk sac is reduced whereas the alimentary canal increases in size but folding of alimentary canal is still not prominent. Primordial germ cells are closely pressed against the wall of alimentary canal on the dorsal side. Mesonephric ducts move away from the dorsal wall of the intestine and placed in between the myotomes (Fig 3). Primordial germ cells increase slightly in size. Their number also increases. Diameter of primordial germ cells at this stage is 4.7 $\pm$ 0.7 µm.

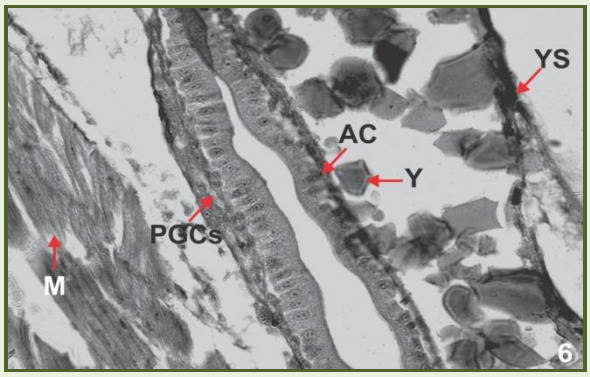
In sagittal sections, primordial germ cells are scattered among the somatic cells in the region which is posterior to the yolk sac. PGCs are elliptical in shape with spherical nuclei (Fig 4).

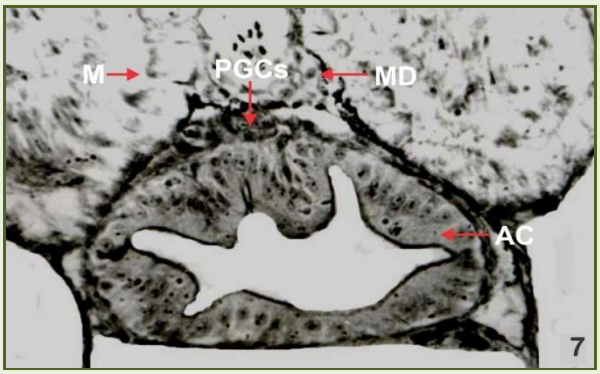
48 hrs stage:

In 48 hrs stage, yolk sac decreases in size, while the size of alimentary canal gradually increases. The alimentary canal shows some folds. At this stage, gonadal ridge is located above the alimentary canal over which PGCs are located. Mesonephric ducts move away from PGCs (Fig 5). The diameter of PGCs at this stage is 4.95 $\pm$ 0.49 µm.

In sagittal section, the PGCs are scattered among the somatic cells in the region which is posterior to the yolk sac. These cells are larger than in the earlier stage. Cytoplasm is faintly stained while the nucleus is deeply stained (Fig 6).







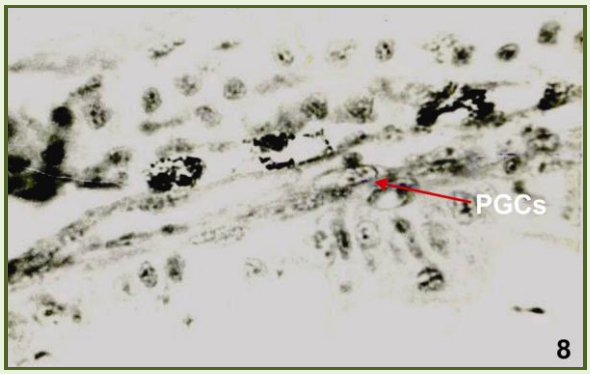


Fig. 5: T. S. of 48 hrs hatchling showing folded alimentary canal with PGCs and Mesonephric duct (MD) X200.

Fig. 6: Sagittal section of 48 hrs hatchling showing muscles (M), alimentary canal (AC) and yolk sac (YS) X400.

Fig. 7: T. S. of 60 hrs hatchling showing folded alimentary canal with PGCs and mesonephric duct X200.

Fig. 8: Sagittal section of 60 hrs hatchling showing the PGCs (arrow) with nuclei X400.

60 hrs stage:

In 60 hrs, the yolk sac is very much reduced in size. The size of alimentary canal increases with prominent internal folding. Above the alimentary canal, gonadal ridge is distinctly formed. PGCs are situated just above the gonadal ridge on the dorsal side of the alimentary canal in groups. Mesonephric ducts appear in between the myotomes (Fig 7). The diameter of PGCs in this stage is about 5.65 ± 0.64 µm.

In sagittal section, large number of PGCs is present just above the alimentary canal on the gonadal ridge. The movement of PGCs starts from posterior side and they move towards the anterior direction (Fig 8).

72 hrs stage:

In transverse section, the yolk sac appears to have further reduced in size. The alimentary canal increases in size with prominent internal folding. PGCs are located above the dorsal side of the alimentary canal over the gonadal ridge. Mesonephric ducts are somewhat triangular in shapes which are located in between the myotomes (Fig 9). The diameter of PGCs at this stage is 6.08 ± 0.31µm.

In sagittal section, the pancreatic cells are very prominent which are darkly stained. At the anterior end of the pancreatic cells, PGCs are present. Certain variations in their shape and size are observed. Some are oblong while others appear elliptical (Fig 10).

10 mm stage:

At this stage, the yolk sac is completely absorbed. Air bladder is visible. Liver and Kidney are well developed. Alimentary canal with prominent internal folding is observed at this stage. The alimentary canal is surrounded by a gonadal ridge. PGCs form a string of bead like structures. Both large and small sized PGCs are present. Pancreatic cells are very well developed. The diameter of PGCs increases to 7.7 ± 0.62 µm (Fig 11).

In sagittal section, the germ cells are seen in the posterior region of the abdominal cavity. The large cells are predominant towards rostral part while the small cells are more prevalent in caudal region. All these cells are spherical in shape. Along the margin of gonadal tissue there occurs black pigment on each side called as cyst cells (Fig 12).

20 mm stage:

In transverse section, kidney and liver appear fully matured. From the wall of alimentary canal an extension is formed on which numerous PGCs are present. PGCs are larger in size and more in number in this stage. They are compactly arranged. Their nucleus is darkly stained and cytoplasm is lightly stained. The diameter of PGCs increases slightly to 8.32 ± 0.31 µm (Fig 13).

In sagittal section, the gonadal ridge is seen towards the kidney. Large and small sized PGCs are present along the gonadal ridge. Dark pigment granules are present along the margin of the gonadal ridge (Fig 14). The nucleus is eccentric in position.

30 mm stage:-

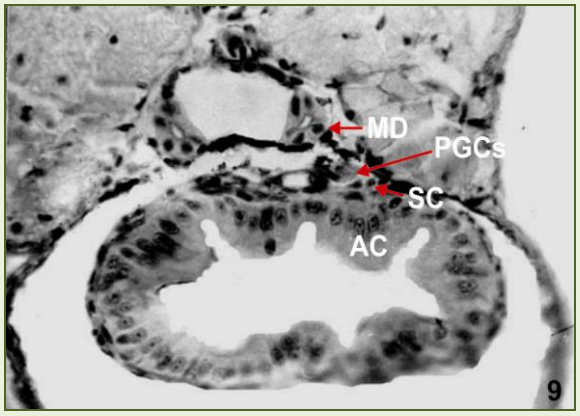
In transverse section, liver and kidney is very prominently observed. Above the margin of alimentary canal, darkly stained pancreatic cells are present. By the side of pancreatic cells, PGCs are located (Fig 15). Their nucleus is darkly stained with lightly stained cytoplasm. The cells vary in size and their diameter is about 8.5 ± 0.37 µm.

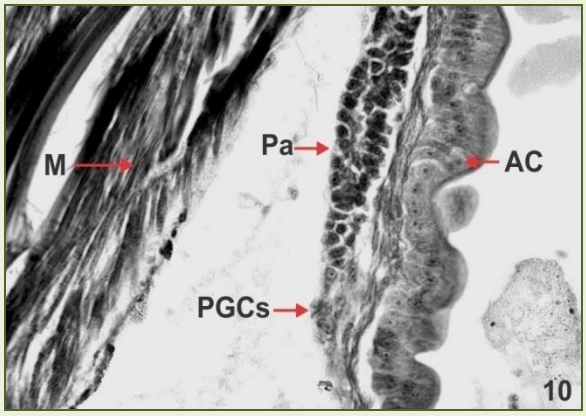
In sagittal section, the germ cells at this stage appear spherical in shape. The nucleus is eccentric in position. The PGCs are lying in close opposition to the kidney (Fig 16). The cytoplasm is faintly stained and nuclei are moderately stained.

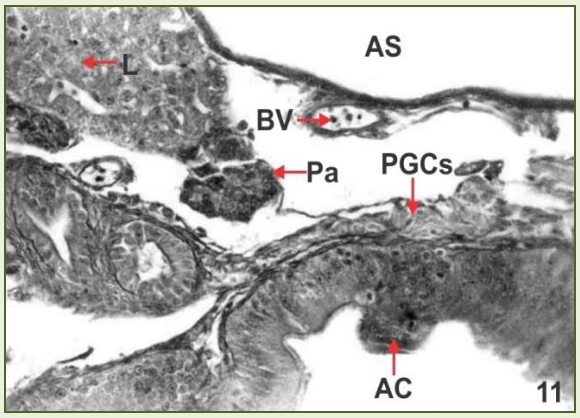
40 mm stage:-

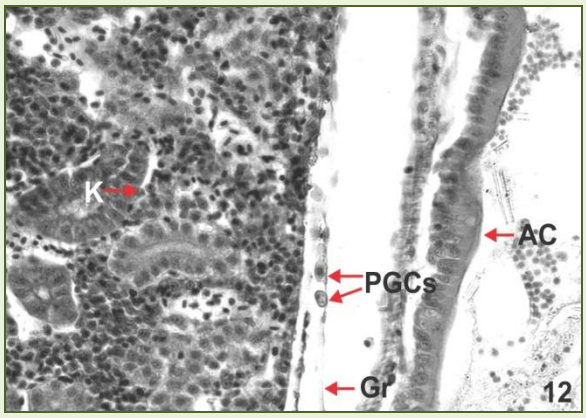
In transverse section, air sac is seen between kidney and alimentary canal. Liver is very well developed. The alimentary canal undergoes numerous foldings. Pancreatic cells are darkly stained. From the wall of alimentary canal, a string of extension is formed called as gonadal ridge in which numerous PGCs of both large and small sizes are held. PGCs are present between the darkly stained pancreatic cells and alimentary canal (Fig 17). The diameter of PGCs in this stage is 9.92 ± 0.32 µm.

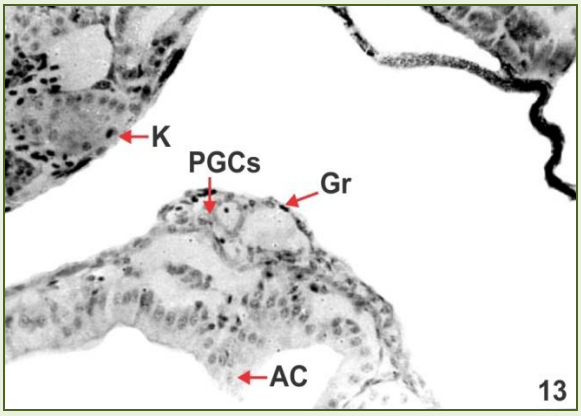
In sagittal section, PGCs further appear to have increased in size and the nucleus of these cells is eccentric in position. The nucleus is darkly stained while cytoplasm is lightly stained. These cells are arranged in a linear fashion (Fig 18).











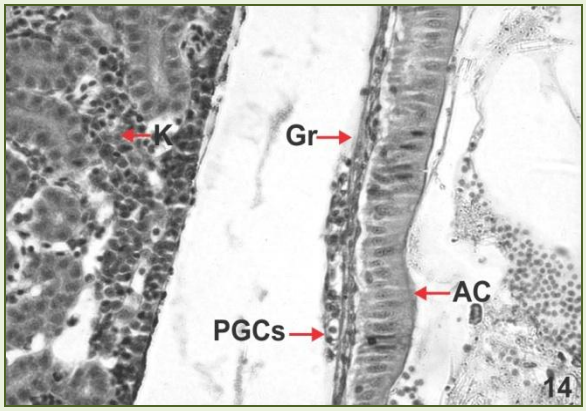


Fig. 9: T. S. of 72 hrs hatchling showing folded alimentary canal (AC) with PGCs, supporting cell (SC) mesonephric duct (MD) X200.

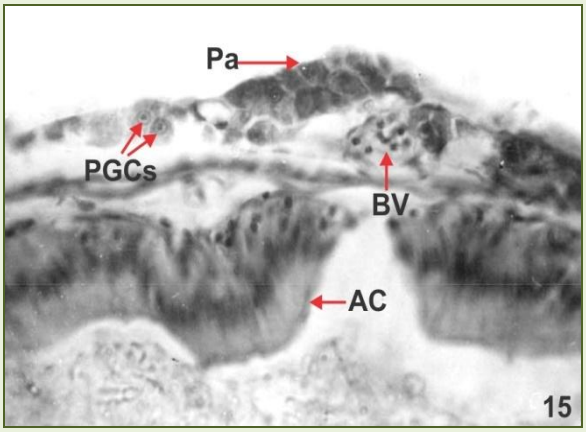
Fig. 10: Sagittal section of 72 hrs hatchling showing PGCs along muscles (M), alimentary canal (AC) and pancreatic tissue (Pa) X400.

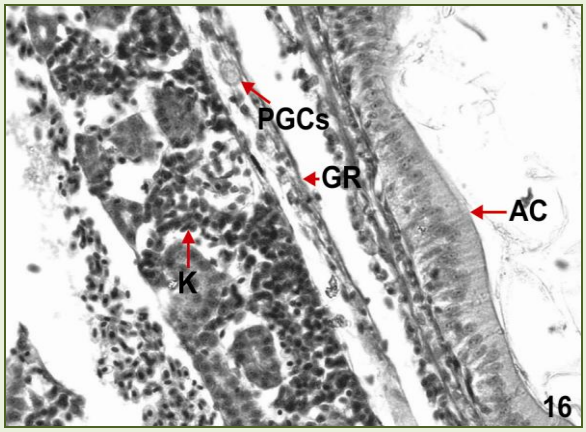
Fig. 11: T. S of 10 mm stage of *L. rohita* showing gonadal ridge (Gr) along with PGCs (arrow), liver (L), pancreas (Pa), alimentary canal (AC) and air sac (AS) X200.

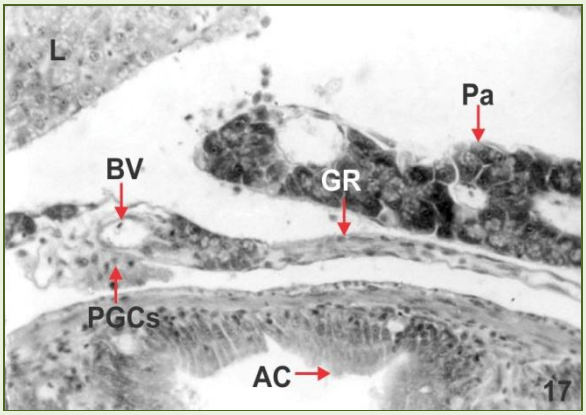
Fig. 12: Sagittal section of 10 mm stage of *L. rohita* showing gonadal ridge with spherical shape PGCs (arrow) along with alimentary canal (AC), and kidney (K) X400

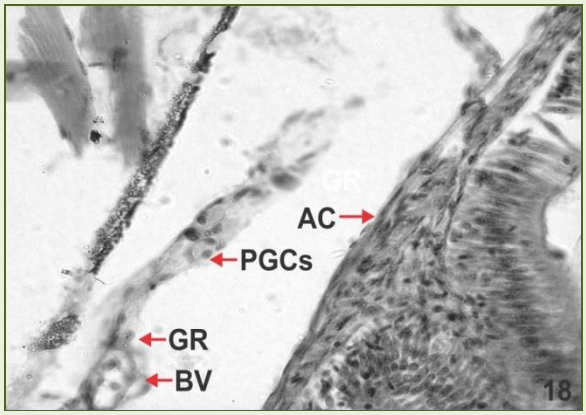
Fig. 13: T.S of 20 mm stage showing large and small sized PGCs (arrow) with darkly stained nuclei X200.

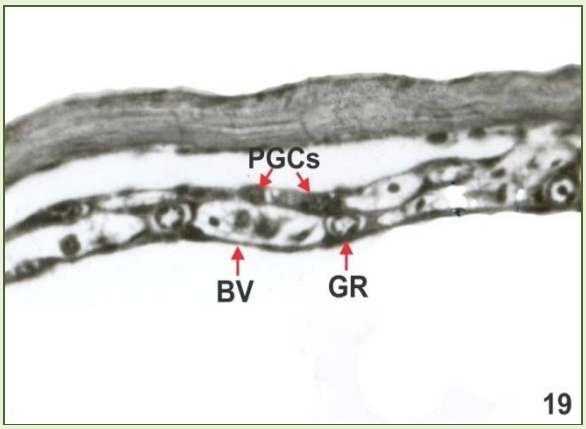
Fig. 14: Sagittal section of 20 mm stage showing gonadal ridge (Gr) with PGCs, alimentary canal (AC) and kidney (K) X400.











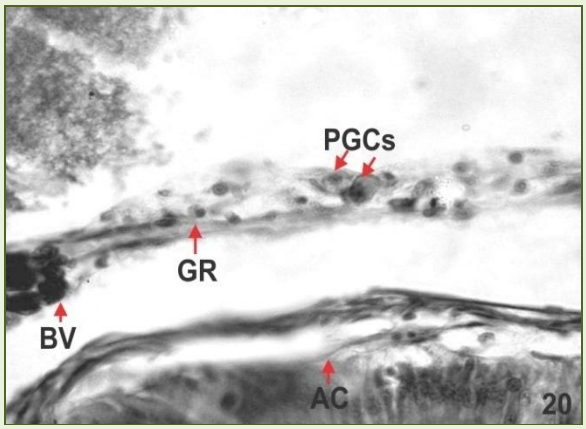


Fig. 15: T. S of 30 mm stage showing PGCs with darkly stained Pancreatic Cells (Pa) X200

Fig. 16: Sagittal section of 30 mm stage showing PGCs lying in between the alimentary canal (AC) and kidney (K) X400.

Fig. 17: T. S of 40 mm stage showing gonadal ridge (Gr) in between the alimentary canal (AC) and pancreatic cells (Pa) X200.

Fig. 18: Sagittal section of 40 mm stage showing gonadal ridge (Gr) along with dividing PGCs (arrow) with prominent nuclei X400.

Fig. 19: T. S of 50 mm stage showing PGCs with blood vessel (BV) X200.

Fig. 20: Sagittal section of 50 mm stage with gonadal ridge (Gr) along with PGCs (arrow) with darkly stained nuclei and pancreatic cells (Pa) close to the wall of alimentary canal X400.

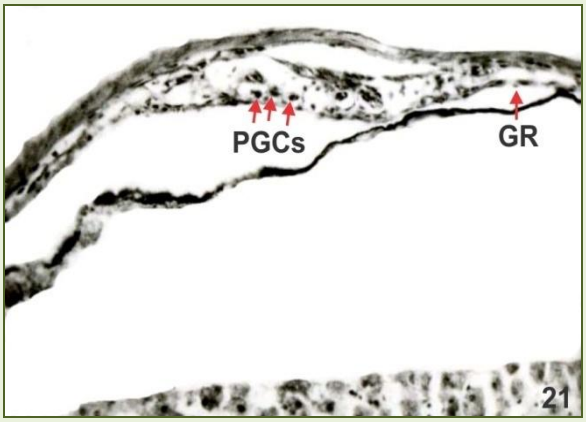


Fig. 21: T. S of 60 mm stage showing gonadal ridge protruding along the wall of air sac with large and small sized PGCs X200.

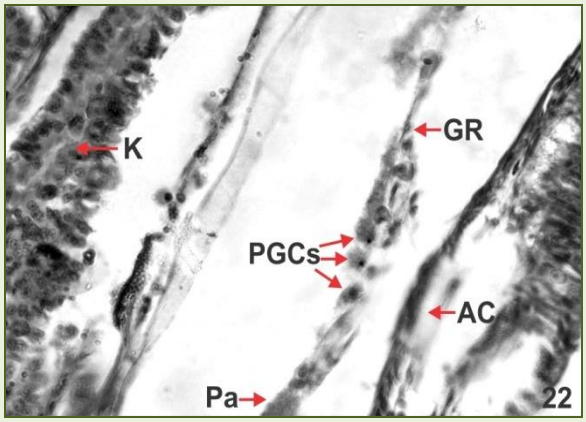


Fig. 22: Sagittal section of 60 mm stage showing spherical and oblong PGCs (arrow) with eccentric nuclei X400.

50 mm stage:-

In transverse section, alimentary canal shows numerous coiling. Air sac, liver and kidney are well developed at this stage. PGCs form a strip like structure along the side of the alimentary canal. Some blood capillaries are visible among the PGCs. Prominent dark pigment is observed along the margins of the PGCs. Pancreatic cells are darkly stained. The diameter of PGCs at this stage is 10.28 ± 0.41µm. (Fig 19 and 20).

60 mm stage:-

In transverse section of 60 mm stage, air sac is very much prominent. Kidney, liver and alimentary canal are well developed. From the wall of air bladder, a strip of PGCs is observed. The germ cells at this stage are found scattered, but testis and ovary of the fish cannot be recognized at this stage though the fish reaches the fingerling stage. Some cells are large and the remaining cells are small. The cells are faintly stained. Their nuclei are eccentric in position. Some blood capillaries are visible among the PGCs. Prominent dark pigment is observed along the margin of PGCs (Fig.21 and 22). The diameter of PGCs at this stage is 10.98 ± 0.49 µm.

DISCUSSION

Germ cells provide continuity of life between generations. In *L. rohita* the primordial germ cells can be distinguished 24 hrs after hatching both in transverse and sagittal sections. These are placed above the yolk sac firmly sandwitched between the wall of alimentary canal and mesonephric ducts. Similar to our findings in freshwater teleost, the common carp *Cyprinus carpio,* the PGCs are found to be present at the sites of "gonadal ridges" in the newly hatched larva on day 3 after fertilization (Parmentier and Timmermans, 1985). In *L. rohita*, the mesonephric ducts are slowly pushed upwards from 36 hrs the yolk sac which is quite large initially in a newly hatched larva, slowly gets absorbed and by 72 hrs it is very much reduced. In 10mm stage it is not visible but air sac is seen. The diameter of PGCs in 24 hrs stage is 4.2± 0.1µm which gradually increases and reaches to about 10.98± 0.49µm in 60mm stage (fingerling).

In *L. rohita* these start to increase in diameter from 4.2 ± 0.1 µm at 24 hrs reaching upto 10.98 ± 0.49 µm at 60mm by fingerling stage. PGCs are first found before the gut is formed and later along the ventral and lateral margins of the gut in *Cottus bairdii* (Hann, 1927). In *L. rohita* these descend along the dorsal and lateral margins of gut from 36 hrs onward. In Zebra fish, *Danio rerio* at age 2 weeks post fertilization, PGCs are found in a dorsocaudal position in the body cavity, where they are arranged in groups of 10-20 cells (Maack and Segner, 2003). In *L. rohita* PGCs are located mostly in posterior half of the body and are identified on the basis their elliptical shape, faintly stained cytoplasm and distinct nucleus.

In *Salmoides salmoides*, early germ cells are characterized by yolk granules and attraction spheres in their cytoplasm and cellular membranes,

by their darkly staining nuclei and hyaline cytoplasm and by their ability to migrate independently (Johnston, 1951). In *Channa punctatus*, from the time of hatching, when the history of germ cells is traced, PGCs are identified on the basis of their larger size, faintly stained cytoplasm and distinct nucleus. Some PGCs show amoeboid movement (Belsare, 1966). In sagittal sections in *L .rohita*, such cells are observed clearly which may be migratory as they appear elliptical in shape from 24 hrs onward and in 10 mm stage they appear suspended from the dorsal body wall by mesentery. Same behaviour is described for *Xiphophorus maculates* (Wolf, 1931) *Micropterus salmoides* (Johnston, 1951) and *Lophius* (Dodd, 1910) where the germ cells are round in *Channa punctatus*. In the amphibian *Xenopus laevis* PGCs are found in dorsal mesentery, clumped at the root of the mesentery between stages 43-45 mm. At this stage the PGCs bulge outward from the dorsal mesentery at or near its junction with posterior body wall (Wylie and Heasman, 1975).

In *L. rohita* supporting cells are visible from 36 hrs onwards. These are differentiated from PGCs by their dark staining. In *Cottus bairdii PGCs* are surrounded by small flattened mesodermal cells which have the appearance of lateral peritoneal cells from which somatic portion of the gonads is destined to develop in. In 4.7mm embryo, the germ cells are arranged bilaterally in the genital ridges, one ridge lying on each side of the dorsal mesentery (Hann, 1927) in the *Cottus bairdii*.

Gonadal ridge formation is observed in *L. rohita* 36 hrs after hatching. The cells linning the alimentary canal are distinctly separated. PGCs start clumping at 48 hrs stage. Coelom formation is apparent from 36 hrs and by 72 hrs coelomic cavity becomes quite prominent. PGCs in *L. rohita* are present at the site of gonadal ridges before gonadal analage originated. Similar condition is found in goldfish (Stromsten, 1931), and in common carp, *C. carpio* (Parametier and Timmermans, 1985).

In embryo of 24 days in *Cottus bairdii*, genital ridge is surrounded by a distinct layer of peritoneal cells and is gradually constricted off from the region above and by 36 days secondary division of the germ cells is observed for the first time. Germ cells cluster is found which is the indication of recent division (Hann, 1927). In *L. rohita,* at 40 mm stage some division could be observed in some of the primordial germ cells.

In mouse embryos, on the basis of morphology in sectioned material (Clark and Eddy, 1975) and their behaviour in culture (Donovan *et al*., 1986), the PGCs seem to actively migrate from the hindgut to the genital ridge. After their immigration from the gut, PGCs extend long processes which are used to associate with one another and by aggregation they arrive at the genital ridges (Gompert *et al*., 1994). In *L. rohita*, however they are already present at the gonadal ridge from the beginning from which they descend in coelomic cavity. Coelomic cavity in *L. rohita* becomes distinct in 36 hrs which subsequently increases and in 10mm stage, the gonads, which are still undifferentiated are suspended from the dorsal body wall closely placed against the air bladder. Pancreatic cells are intensely stained with haematoxyline. These are distinctly seen in close association with PGCs above the alimentary canal from 72 hr stage onwards. The PGCs do not divide until they reach the region of gonad primordia in Medeka (Gamo, 1961). Division of PGCs is observed in *L. rohita* at 40 mm stage which is designated as semifingerling stage.

In *C. carpio* sex could not be differentiated even at 25-28 mm and only 17 to 20 weeks after fertilization when embryo became 52-82 mm in length, male and female could be clearly distinguished. In this carp, the indifferent gonads gradually change into immature female or male gonads; which can be detected by light microscopy from about week 10. The process of oogenesis starts at week 16 and spermatogenesis at week 19 (Paramentier and Timmermans, 1985). In *C. carpio,* sex differentiation could not be observed at 7 to 9 weeks, when germ cells number was increasing, however meiosis is reported to have occurred earlier in female than in the male carp (Parmentier and Timmermans, 1985).

In the present study, gonad remains undifferentiated even at 60 mm stage i.e. upto fingerling stage. With light microscopic studies, differentiation could not be seen. Distinction between spermatogonia and oogonia in fish is reported to generally rest on the somatic gonadal characteristics (Reinboth, 1980).

In several studies on early germ cells of fish, a similarity of ultrastructural features of PGCs,

spermatogonia and oogonia have been reported (Satoh, 1974; Brusle and Bursle, 1978a, b). In *C. carpio* there was no difference in the morphology of PGCs in both the sexes using light microscopy. However in *Micropterus salmoides* (Black Bass) sex is first microsopically distinguishable in the gonads of fingerlings of 3 cm length. 11 mm fry of Black Bass shows that gonad consists of gonia covered by two layers of epithelial cells; inner layer can be a true epithelial layer. In 11-13 mm fry, gonad is not a continuous strand of germ cells but is composed of discontinuous aggregations with intervening spaces. Gonad resembles a chain of beads. Such chain bead in *L. rohita* is observed at 10 mm stage. Actually, single isolated primordial germ cells are seen placed on the ridge in this stage. In Black bass, between 20 mm to 3 cm stage, gonads pass through an indifferent period, marked increase in size by an increase in stroma and by multiplication of germ cells (Johnston, 1951).

In 9.5 mm (52 days old) *Cottus bairdii*, female sex is distinguished from the male by an oviducal groove on the ventral surface of the ovary and also by the beginning of maturity phases among the germ cells. For males, in 9.7 mm (52 days old), sex can be distinguished by the presence of sperm ducts within the testes (Hann, 1927). Sex differentiation takes place about seven weeks after spawning season in female germ cells. In Japanese Eel (*Anguilla japonica*), sex differentiation occurs during late juvenile period i.e. at the time of pigmentation and upstream migration (Chiba *et al.,* 1999). In *Channa punctatus,* sex is differentiated suddenly between 5.4 mm to 12 mm stage (Belsare, 1966). In the *L. rohita* sex cannot be recognized in fingerling stage and even in adult during non breeding period, morphological identification of sex cannot be done but testes and ovaries are found in separate individuals. On the basis of morphological and histological, details five different phases in annual cycle could be marked.

PGC development consists of several processes including initiation, survival, proliferation, migration and homing of PGCs (Abe *et al.,* 2003). In *L. rohita* PGCs appear to have originated from the gut endoderm, which is clearly a visiblin sagittal section in various stages. In *Xenopus laevis*, the somatic cells differentiate themselves without any influence from PGCs and they come to lie beneath them (Wylie *et al.,* 1976).

Germ cells in the gonads are directly derived from the germ plasma containing cells. Consequently, the gonads form the area in which the primordial germ cells will be differentiated into male or female gametes (Timmermans, 1996).

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Cite this article as: Gadekar GP and Baile VV (2013) Formation of Primordial Germ Cells (PGCs) in the *Labeo rohita* (Ham). *Int. J. of Life Sciences,* 1(2): 123-132

Source of Support: Nil, **Conflict of Interest:** None declared