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DIETARY SUPPLEMENTATION OF VITAMIN C, L-ASCORBATE 2-TRIPHOSPHATE CALCIUM (LATP-Ca) ON OOCYTE DEVELOPMENT OF MAJOR CARP ROHU (*LABEO ROHITA*) REARED IN A NATURAL CONCRETE POND

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ABSTRACT

The aim of this study was to evaluate the effect of vitamin C L-ascorbate 2-triphosphate (LATP-Ca) on Indian major carp rohu (*Labeo rohita*) (Hamilton) during intensive aquaculture. Rohu were fed with vitamin C (LATP-Ca) supplementation at doses of D_1 (control) and D_2 (500 mg/kg) diet for 10 weeks. The effects were assessed by comparing the treated groups of fish to that of control group. Vitamin C (LATP-Ca) supplementation exerted significant ($P<0.05$) effects on oocyte development of rohu (*Labeo rohita*). A number of distinct developmental stages of oocyte can be delineated and oocyte growth in major carp rohu (*Labeo rohita*) found in two distinct phases; **primary growth phase** (PGP) and the **secondary growth phase** (SGP). In PGP, oogonia, chromatin nucleolus, early and peri nucleolus stage oocyte with Balbiani's vitelline body were observed while two types of inclusions, lipid and protein, respectively were formed during vitellogenesis in the SGP. SGP development was found for a short period of time as compared to PGP. Similarly, development of oocyte was very poor in fish fed with controlled diet.

Key words: Major carp, *Labeo rohita*, vitamin C, histology, oocyte development.

INTRODUCTION

Morphology, biochemistry and physiology of gonads have been described by a large number of workers in different groups of fishes. Ovaries are not only responsible to produce eggs but also synthesize and secrete hormones of different kinds that have a far reaching effects on the reproductive biology and behavior of the fish. Oogenesis starts from simple proliferation of oogonia up to the formation of mature oocyte and consequently ovulation after final maturation. During this process, size of oocyte increases many folds and this is mainly due to accumulation of yolk granules which are formed in liver under the influence of a specific steroid hormone, 17β-estradiol (Wallace and Selman 1981) and migrate to oocyte through blood (Wallace 1978). Oocyte development in teleosts has been reviewed by Wallace and Selman (1981), de Vlaming (1983), Nagahama (1983), Guraya (1986), and West (1990). Yamamoto *et al*. (1965) divided the oocyte development of rainbow trout into eight stages which includes chromatin nucleolus stage, perinucleolus stage (subdivided into early and late stage), oil droplet stage, primary

yolk stage, secondary yolk stage, tertiary yolk stage, and maturation stages. Shrestha (1980) described the ovarian cycle of *Noemacheilus beavani* and divided it into seven distinct phases. Sen *et al*. (2002) divided the development stages of *Labeo rohita* (collected from rivers of West Bengal, India) into seven different stages as primary growth phase, perinucleolar stage, pre-vitellogenic or yolk vesicle stage, vitellogenic stage post-vitellogenic stage, germinal vesicle break down stage, and spawning stage.

Several criteria have been employed for staging the process of oocyte development. These criteria are size, amount and distribution of various cell inclusions (Nagahama 1983). However, it is now generally recognised that oocyte growth occurs in two distinct phases including all these oocyte development stages. These phases are the primary growth phase (PGP) and the secondary growth phase (SGP) (Tokarz 1978, Khoo 1979). The primary growth phase is variously named as previtellogenesis (Raven 1961), the first growth phase (Jorgensen 1974, Khoo 1979), or the primary growth phase (Wallace and Selman 1981). The PGP in teleosts involves the increase in size of the primary oocytes with some nuclear changes, and shows little variation between species (Tokarz 1978, Wallace and Selman 1981, de Vlaming 1983, Guraya 1986). The secondary growth phase (SGP) of oocyte is a gonadotrophin-dependent phase. The enlargement of the oocyte, attributable mainly to the accumulation of yolk (vitellogenesis) occurs in this phase (Nagahama 1983). Oocyte development might be species specific with the size, amount and distribution of various cell inclusions or formation order.

The oocytes are expelled into the ovarian cavity or peritoneal cavity, a process known as ovulation. The follicular layers that remain behind in the ovary are known as Post-ovulatory follicle (POF). The entire process or at least part of it is hormone dependent (Lone *et al*. 2001, 2008). However, final oocyte maturation and ovulation are not always associated because oocyte of most teleosts do not undergo ovulation following steroid maturation *in vitro* (Narimatsu *et al.* 2007).

Vitamin C is an essential vitamin for normal physiological functions in animals including fish (Lim and Lovell 1978). Most teleosts are unable to synthesize ascorbic acid due to the lack of l-gulonolactone oxidase that is responsible for synthesis of vitamin C de novo (Wilson 1973, Fracalossi *et al*. 2001). Therefore; an exogenous source of vitamin C is required in fish diets. Inadequate supply of dietary vitamin C usually results in a number of deficiency signs such as spinal deformation, impaired collagen formation, internal haemorrhaging and retarded growth (Halver *et al.* 1969, Al-Amoudi *et al.* 1992, Gouillou-Coustans *et al.* 1998). The requirement of vitamin C varies to some degree, with fish species, size, diet and experimental conditions. Thus, the role of vitamin C on oocyte development is much important but, information on carp is very limited and *Labeo rohita*, rohu is one of the Indian major carp. Its high commercial value makes it a promising aquaculture species in the future. Therefore, the purpose of the present study was to determine oocyte development using histological techniques of major carp rohu *(Labeo rohita*) during intensive aquaculture.

MATERIALS AND METHODS

Fish collection and experimentation

Labeo rohita (rohu) is a major carp and an annual breeder. Based on observations of wild fish, it attains maturity in the end of second year of life (Jhingran and Pullin 1985). Thus, one year old (mean weight 39.56±0.25 g/fish) rohu *Labeo rohita* (H) used in the present study were obtained to the Aqua Research Lab, University of Delhi, India from a commercially well managed Jahangirpuri fish farm, located some 14 km from the research lab. Rohu were kept in outdoor

conditions, acclimatized for 7 days and cultured 34 fishes/tank in rectangular cemented tanks (500 l) under two feeding regimes. Two practical (artificial) diets (protein 40%) containing two doses 0 and 500 mg kg^{-1} of vitamin C, L-ascorbate 2- triphosphate Calcium (*HiMedia*, India) were formulated along with other ingredients *viz.*, fish meal, wheat flour and cod liver oil (Labh and Chakrabarti 2011). In the first group, fish were fed without incorporation of vitamin C (LATP Ca) in the artificial diet served as control (D_1) and the second group of fish were fed with incorporation of vitamin C (LATP Ca) in the artificial diet, which served as high level (D_2). Fish were fed twice daily at 9.00 am and 6.00 pm at the rate of 3% of body weight. Three replicates were used for each feeding scheme. Water temperature, pH and dissolved oxygen level were recorded weekly throughout the study period. The duration of experiment was 10 weeks and then the fishes were harvested.

Experimental sampling

At the time of sampling, fish were removed from the cemented tank one by one with a scoop net and immediately placed in a water tank containing clove oil (5 ppm) dissolved in absolute alcohol (Merck, Germany) in a ratio of 2:5 (Berka 1986) and allowed to stay for 3-5 minutes. The time period was changed according to weight and length of fish.

Morphological and biochemical parameters

Before dissection and removal of gonads total body weight to the nearest gram (g) and total body length, standard body length and body depth (at the start of the dorsal fin level) to the nearest mm were recorded. The specific growth rate (SGR) was calculated using the formula: SGR = 100 × (InWt - InWi)/t, where Wi and Wt were the initial and final body weights and t time in days. Vitamin C contents in oogonia were assayed (Dabrowski and Hinterleitner 1989).

Dissection and removal of gonads

After measurements, an incision was made on ventral side of the abdomen from posterior to interior most tip of the fish with a sharp scalpel and a bone cutter. Ovaries were found attached on the lower side of the swim bladder in the abdominal cavity. Features like colour, position of gonadal ducts and any abnormality, if present, were noted. Photographs of the ovaries were taken *in situ* and then were separated carefully, then after their size and weight were recorded.

Histological studies

Whole ovary samples of immature and mature fishes for histological studies were taken and fixed in 10% buffered formalin (4% formaldehyde in phosphate buffer) until processed for histological examinations. Samples were trimmed to approximately 1 cm^3 and loaded to plastic tissue cassettes. The sample loaded cassettes were then processed for routine histology. The samples were first passed through different grades of ethyl alcohol ranging from 30-100% for dehydration and then cleared in two changes of xylene. The tissues were finally taken to embedding centre, transferred to molten wax and xylene in the ratio of 70:30 and in the last changed to molten wax for 4 h for impregnation of wax. The wax embedded tissues, attached to cassettes, were finally trimmed for microtomy. The sections were floated in warm water 37-40°C for stretching. The stretched sections were placed on glass slides, and put on a hot plate overnight for drying. Gill' haematoxylin and eosin staining method was used for this study (Gerrits 1990). The sections were stained with Harris hematoxylin and eosin and mounted with DPX. The photomicrography was done on LEICA PM-5000 microscope with digital camera.

Statistical Analyses of the data

To understand the significant difference between diet, the data obtained were analyzed statistically with One-way ANOVA followed by

Duncan's Multiple Range Test at $P<0.05$ significance level using SPSS version 15.

RESULTS

Cent percent survival of carp was recorded in all the treatments. The final average weight of fish was significantly ($P< 0.05$) higher in the carp fed with diet D_2 as compared to diet D_1. The final average weight was 13.6% higher in the fish fed with D_2 diet compared to control (D_1) diet fed fish. Vitamin C level was significantly ($P< 0.05$) higher in fish fed with D_2 compared to others regardless of tissues (Table 1). Significant changes were observed in the oocyte of treated fish compared to the control one. Water temperature ranged from 27.8 to 31.3°C, pH ranged from 7.4 to 7.8 and dissolved oxygen level ranged from 6.7 to 7.3 mg/l throughout the study period.

Gonad weight, length and width

The two lobes of the ovary are separate in the beginning, however, as they grow, the lobes come together and an oviduct is formed. The weight of gonad (33.11±2.06 g) was the lowest in the stocking fish at the beginning of the experiment, 97.38±5.14 g in the D_1 diet fed fish, while the highest value (187.8±23.02 g) was recorded at the end of the experiment in gonad of D_2 diet fed fish (Figs. 1, 2 and 3). The ovarian width followed ovarian length and weight and was minimum in D_1 (5.702 ± 0.09 cm) while maximum values (9.45 ± 0.15 cm) were in D_2 diet fed fish.

Table 1. Average weight (g), specific growth rate (SGR), Vitamin C concentrations in oogonia (µg mg^{-1}) of rohu (*Labeo rohita*) cultured under two different feeding regimes.

Parameters	D_1 (Control diet)	D_2 (Vitamin C added diet)
Average Weight	42.27±0.17	49.32±0.13
SGR	0.069±0.012	0.236±0.08
Vitamin C in Oogonia	159.81±24.2	375.64±21.52

Histological studies of the gonads

In major carp rohu (*Labeo rohita*) abundant oogonia were observed in the different stage of ovary development (Fig. 4). They occur in nests of small rounded cells (6.02±2.55 µ) with a very high nucleus (Fig. 5) in the D_1 diet fed fish while in the fish fed with D_2 diet oogonial proliferation in newly formed primary oocytes (23.33±6.05 µ) and early perinucleolus stage of oocyte (39±3.39 µ) developed. Zona radiata and protein droplets observed in fully grown treated fish (Fig. 6).

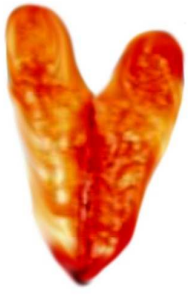


Fig. 1. Virgin/immature ovary present in the stocking fish.

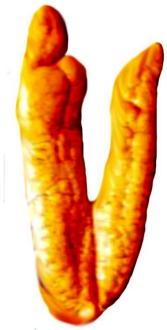
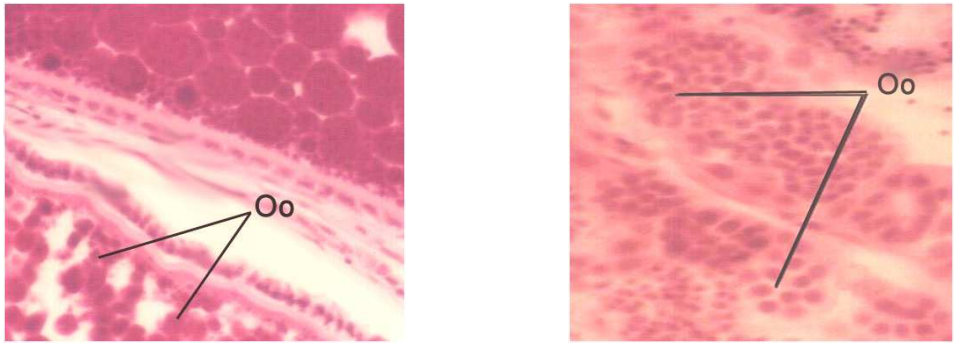


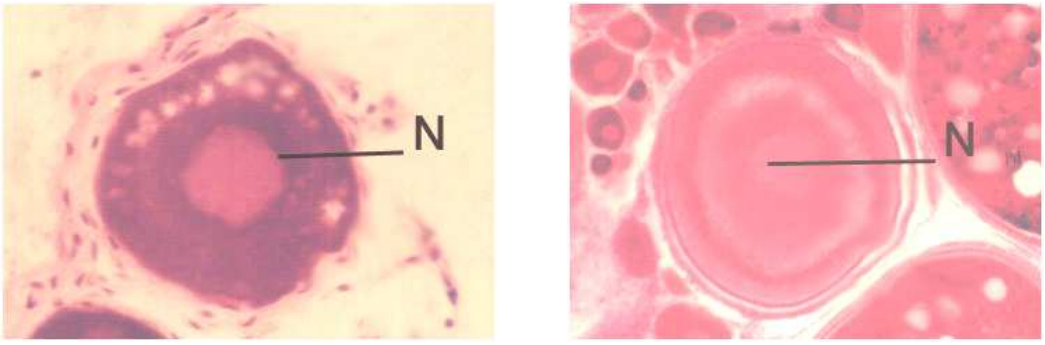
Fig. 2. Ovary present in the control (D_1) diet fed fish.

Fig. 3. Ovary present in the treated (D_2) diet fed fish.



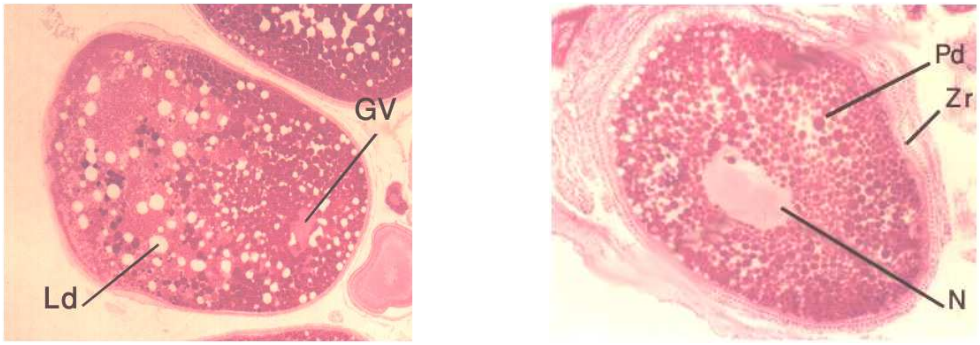
(a) (b)

Fig. 4. Micrograph showing transverse sections of growing oocytes observed in the D_2 diet (b) fed fish as compared to the control (a) diet fed Labeo rohita; Oo=Oocytes H&E.



(c) (d)

Fig. 5. Micrograph showing transverse sections of developed nucleus (d) observed in the oocyte of D_2 diet fed fish as compared to the developing nucleus (c) found in the oocyte of control diet fed fish; N=Nucleus H&E.



(e) (f)

Fig. 6. Micrograph shows zona radiata (Zr) and protein droplets (Pd) found well developed in the secondary growth phase of D_2 diet fed fish (f) as compared to the D_1 diet (control) fed fishin which Granular vesicle (Gv) and various lipid droplets (Ld) can be observed (e) H&E.

DISCUSSIONS

Vitamin C is an indispensable nutrient required to maintain the physiological processes of different animals including fishes (Tolbert 1979). However, fish depend upon an exogenous source of vitamin C as they cannot synthesize it due to the absence of the enzyme L-gulonolactone oxidase (Wilson 1973). Diets devoid of vitamin C result in

prominent deficiency signs and most notable are spinal deformities, particularly scoliosis and lordosis. Often abnormalities of the cartilage of the eye, gill, gill opercula, and fins are observed in ascorbic acid deficient fish, as well as reduced appetite, slow growth, internal and external haemorrhage, fin erosion, and anaemia. In the present study, the average weight and specific growth rate of major carp *Labeo rohita* increased as the dietary inclusion of vitamin C increased. The increased growth rates in several fish species fed diets sufficient in vitamin C are well documented elsewhere (Dabrowski *et al.* 1990, 1996, Lee *et al.* 2001). Navarre and Halver (1989) reported that a higher weight gain was observed in rainbow trout fed high dietary AA (500 to 2000 mg/kg diet). Tewary and Patra (2008) reported that in *Labeo rohita* maximum growth (50.88±0.18) was observed in fish fed with 1000 mg AA/Kg diet, while the lowest growth (30.83±0.12) was observed in control diet fed fish.

Oogenesis is a fundamental phase in the reproductive process of organisms. Its comprehension is essential to understand the reproductive biology of species. Moreover, it provides a detailed picture of the reproductive state of females, which permits us to design and improve economimanagement proposals for species of economic importance. Histological studies on oogenesis provide some information, but they present several limitations when applied to vertebrates. *Labeo rohita* is heterosexual. Its reproductive system consists of an ovary with two lobes, present on each side of the air bladder in body cavity. They were attached with the swim bladder and the length of two lobes was not equal (Figs. 1, 2 and 3). In the immature fish the two lobes are separate, however, as they grow, they unite posteriorly and form a short oviduct that opens to the exterior through gonopore lying above the anus. Externally each ovary was covered with a thin peritoneal membrane and beneath the membrane lies the tunica albugenia which becomes thinner and thinner as the ovary reaches to full maturity.

Histologically, gonadal development in *Labeo rohita* is group synchronous type. This type, which is found in fish that spawn annually or once in a spawning season, breeders will develop a cluster of vitellogenic oocytes and advance synchronously through further stages of development (oogenesis), whereas the rest of the oocyte population remains arrested and is used for next year's cycle. In *Labeo rohita* the ova starts development in earnest in March and increase to full and mature size in June. Its spawning, in wild, probably takes place in late June and July, during the local monsoon season. Sex can be recognized in breeding season when abdomen is rounded (called "*chhalli*" in local language) and vent bulged out and becomes reddish in colour. In the present study also, it seems that ovaries of rohu started increasing in weight and showed histological advancement. The annual reproductive cycle of *Labeo rohita* female was studied on the basis of gross appearance, and weight of ovaries. Histologically, this was based on oogenesis, size of oocytes, size and behaviour of nucleus, nuclear membrane, number and location of nucleoli, appearance and distribution of yolk vesicles, yolk granules, appearance of oil droplets (if any), final maturation of oocytes in the treated fish as compared to the control diet fed fish. It is clear from the Figs. 1, 2 and 3 that ovaries present in the fish of without treated with vitamin C were small in size while the size of ovary was comparatively larger in their weight and length. This has been shown for many fish like major carps (Sen *et al.* 2002, Day *et al.* 2004, 2005, Bhattacharyya and Maitra 2006). It is known that major carps spawn during the monsoon season when rainfall is at maximum of the year and a casual relation existed between these parameters as was reported by earlier workers (Jhingran 1986, Jhingran and Pullin 1985, Pillay and Kutty 2005).

However, it has also been shown many times that the size of the oocyte depends on the size of the fish and the larger fish tend to have bigger and higher number of eggs (Fernandez-Delgado and Herrera 1995, Olivia-Paterna *et al.* 2002, Plaza *et al.* 2007).

In conclusion, vitamin C is not only essential for fish growth but is also for the growth of the gonad. Well developed growth of ovary observed in treated diet fed fish as compared to the controlled diet fed fish. The content of vitamin C in oogonia was one and half fold high in treated (D_2) as compared to the control (D_1) diet fed fish. Morphological study reveals that the two lobes of the ovary are separate in the beginning however as they grow, the lobes come together and an oviduct is formed. Well developed nucleus, protein droplets and oocytes were observed in the micrographs of both treated and controlled diet fed fish. Zona radiate observed in fully grown treated fish which plays a role in the vitellogenic process before fertilization, i.e., the microvilli and pore canals and also helps in easier transportation of yolk materials into oocyte. Thus, from these studies it can be said that vitamin C is essential for oocyte development and overall growth of the fish. Further studies are needed in this connection from various culture systems used in the country.

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