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Screening of Selected Chemotherapeutant by using Primary Liver cell Culture of *Labeo rohita* (Hamilton, 1822)

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Abstract

Development of primary cell culture from liver tissues of *Labeo rohita* was successfully completed by following explant culture method. The L-15 media supplemented with 20% foetal bovine serum (FBS) at 28ºC has resulted in good attachment and proliferation of primary culture. FBS is required for efficient attachment of liver explant but increased supplementation of FBS in media did not stimulate the proliferation of cells. Radiation from liver explant was first seen after 48 h of attached explant. The monolayer formation of liver cell culture was observed after twenty one days and it was subsequently sub cultured for more than 10 times. The *in vitro* cytotoxicity of a commonly used drugs (Oxytetracycline), chemotherapeutant (Benzalkonium chloride) and insecticide (Fipronil) was studied on primary liver cell culture of *L. rohita* by assessing cell viability using tetrazolium (MTT) assay which revealed that Fipronil is most toxic compared to OTC and BKC. Reduction in cell viability was observed with the increase in concentration of chemotherapeutants. The IC_{50} values of OTC, BKC and Fipronil calculated from dose-response curve were 76.43 μg ml^{-1}, 3.22 μg ml^{-1} and 0.014 μg ml^{-1} respectively. The study revealed that primary cell culture from liver tissue of *L. rohita* can be used as a potentially bio-indicator for the *in vitro* screening of the acute toxicities of Fipronil.

Keywords: *Labeo rohita,* primary cell culture, *in vitro* screening, Insecticide

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Introduction

Fish is an important resource and is being used for the development of vertebrate cell and tissue models for use in the biomedical sciences. The established fish cell lines and its applications have provided new information in several areas of fish biology (Ma et al., 2001), physiology (Hightower & Renfro, 1988), molecular genetics (Hong et al., 1998) and in transgenic applications (Chen et al., 2003). A number of cell lines from commercially important fishes are available and have been applied as experimental models for determination of heavy metal toxicity (Ryan & Hightower, 1994). Species-specific cell lines are important tools for studying toxicology, gene regulation and expression in fishes and are also essential for isolating and identifying fish viruses and viral pathogenesis (Babich et al., 1991; Ferrero et al., 1998; Huang et al., 2011; 2014). Primary culture of hepatocytes are generally used as an *in vitro* model to study the metabolism and toxicity of novel xenobiotics in the area of biomedical sciences for many years and it is also well accepted now that isolated fish hepatic culture can easily provide an ideal system for evaluating many aspects of hepatic metabolism, including the biochemical and cellular processes involved in the activation of toxic chemicals, environmental pollutants and assessment of herbicides (Rogiers et al., 1995; Guillouzo, 1998; Kelly et al., 1998; Salvo et al., 2015; Franco et al., 2018).

Cells are a key level of organization for understanding mechanisms of toxicity and cultured cells have been applied as alternative models in mechanistic studies and toxicity identification in ecotoxicology. Considering the importance of having an *in vitro* tool from a vital organ of Indian major carp, *L. rohita* to carry out cytotoxicity studies, the present study was undertaken for developing the primary cell culture of liver tissue from *L. rohita* fingerling and for evaluating the effects of selected chemotherapeutant, antibiotic and insecticide at cellular level.

Materials and Methods

Indian major carp, *L. rohita* was used as experimental animal for which 200 numbers of fingerlings (mean weight: 10 ± 0.52 g) were procured from Aarey fish farm, Mumbai, India and acclimatized in 1000 l circular tank acclimatized for 15 days prior to the experiment. Fishes were given commercially available pelleted feed containing approximately 30% protein at 5% of body weight daily. Aeration was maintained round the clock, debris was siphoned out at alternate day with 50% of water was exchanged.

Preparation of liver explant was done by following the method described by Rathore et al. (2001) with partial modification. Briefly, rohu fingerlings starved for two days to reduce the bacterial load of gut and was maintained in sterile water before explant culture. Fish was anaesthetized by putting MS 222 @ 100 mg l^{-1} water, cleaned and wiped with 70% ethanol. Thereafter fish was taken to laminar hood and was cut open with sterile scissors, liver tissues were taken out aseptically and washed thoroughly three times with PBS containing 500 IU ml^{-1} penicillin, 500 μg ml^{-1} streptomycin and 2.5 mg ml^{-1} amphotericin B in petri dish. The tissues were cut into small fragments of approximately 1-2 cubic mm with sterile scissors, washed twice with chilled PBS and seeded into 25 cm^2 cell culture flasks with about 50 μl foetal bovine serum (FBS) and allowed to attach to the surface of the flask overnight. The flasks were thereafter incubated at 28°C in L-15 medium supplemented with 20% FBS.

The primary cell cultures were maintained at 28°C using L-15 medium supplemented with 20% FBS in 25 cm^2 tissue culture flasks. Subculture of cells was done when primary explant culture reached 85-90% confluence and it was carried out using TPVG solution (0.1% trypsin, 0.2% ethylenediaminetetraacetic acid-EDTA and 2% glucose in 1X PBS). The sub cultured cells were grown in fresh L-15 media supplemented with 20% FBS. In the initial subcultures, half of the growth medium was changed after an interval of five days.

The flasks were observed daily for morphological details *viz.,* explant attachment, cell to cell contact, spreading, degree of confluence, and general appearance using an inverted microscope (Axio observer-A1, Zeiss, Belgium).

To determine the optimum temperature and serum concentration, the cells were grown at different temperature range and FBS concentrations. The effect of temperature was determined by seeding cells in 1×10^5 number and incubated at 28°C for 24 h and subsequently at selected temperatures of 24, 28 and 32°C in triplicates. Trypsinization of triplicate flasks at each temperature was carried out every day and cell density was measured with haemocytometer for total of seven days. The growth response to various concentrations of FBS (5, 10, 15, 20 and 25%) was carried out using the same procedure as mentioned above, at 28°C.

The cytotoxicity of commonly used drug i.e. Oxytetracycline (OTC), chemotherapeutant i.e. Benzalkonium Chloride (BKC) and insecticide i.e. Fipronil was studied on primary cell culture of rohu liver by exposing the selected drugs in different dilutions and subsequently assessing the cell viability using the 3-(4,5-dimethyl-2thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay following the method described by Borenfreund et al. (1988) which is a colorimetric assay based on the ability of NAD(P)H-dependent cellular oxidoreductase enzymes, reducing the tetrazolium dye MTT to its insoluble purple coloured formazan form. The assay was carried out using MTT kit (Bigenuix, Medsystems Pvt. Ltd., New Delhi, India). Different dilutions of OTC (Himedia, India) *viz.,* 20, 40, 60 and 80 μg ml^{-1}, BKC (Lysotan: 10% BKC, Schuke and Mayr GmbH, Germany) *viz.,* 1.0, 2.0, 3.0 and 4.0 μg ml^{-1} and Fipronil (RFCL Limited, New Delhi, India) *viz.,* 0.001, 0.01, 0.1 and 1.0 μg ml^{-1} were prepared for determining IC_{50} value at cellular level. To assess the cytotoxicity, fish liver cells at exponential proliferation phase were harvested and diluted at a concentration of $1x10^6$ cells ml^{-1} in L-15 medium with 10% FBS (v/v). About 200 ml aliquots of $1X10^6$ cells ml^{-1} were seeded in 96-well flat bottomed tissue culture plates (Tarsons, India) and incubated for 48 h at 28°C and the next day the medium was removed and some wells were re-fed with fresh medium used as control whereas, other wells were amended with varied concentrations of above mentioned chemicals. The toxic endpoints were determined after an exposure period of 48 h for which the test medium was replaced by 20 μl MTT and incubated for 4 h at 28°C. After incubation the solution was removed carefully and 150 μl dimethyl sulphoxide (DMSO) was added per well to solubilize the purple formazan crystals. Each sample was assayed in triplicate wells. Absorbance of each well was measured at 490 nm and viability percentage was calculated using the formula given below. The IC_{50} value was calculated from dose response curve.

Viability (%) = [Mean Absorbance of Sample/ Mean Absorbance of control] x 100

Results and Discussion

Since the development of first fish cell line from rainbow trout gonad (Wolf & Quimby, 1962), about 283 fish cell lines have been established from fish and has been used in life sciences (Lakra et al., 2011). Though cell lines derived from different organs of *L. rohita* have been documented in literature (Lakra & Bhonde, 1996; Rao et al., 1997; Sathe et al., 1997; Joseph et al., 1998; Ahmed et al., 2009), establishment of primary cell culture and subsequent passaging from *L. rohita* liver has not been reported so far. The main purpose of this study was to develop primary liver cell culture from *L. rohita* and use it as an *in vitro* screening tool to study cytotoxicity of selected drug, chemotherapeutant and insecticide such as Oxytetracycline (OTC), Benzalkonium chloride (BKC) and Fipronil respectively.

Liver tissue explant culture under aseptic condition from fingerlings of *L. rohita* was implanted in L-15 medium, supplemented with 20% FBS and incubated at 28°C. Radiation from liver explant was first seen after 48 h of attached explant. Spindle shaped cells were observed to attach and spread on the bottom surface of tissue culture flask (25 cm^2) from 3rd day onwards (Fig. 1A). Similar observation has been reported in trout hepatocyte culture where cells grew in spindle like form until reaching 50% confluency after which they formed uniform monolayer (Ostrander et al., 1995). The cells were found to multiply and formed confluent monolayer within three weeks with 80-85% confluence (Fig. 1B). The cell growth was observed to be slow but was ready for subsequent passaging in another fifteen days. During sub-culture the new growth of the cells exhibited fibroblast like shape (Fig. 1C). According to some of the earlier studies it has been reported that, primary cultures from different tissues of fish mostly comprised of fibroblastic cells (Singh et al., 1995; Lakra & Bhonde, 1996; Lai et al., 2003). Explant cell culture from tissues/organs is an easier and established method for primary cell culture than the single cell suspension culture method. The monolayer formation of cell culture usually takes place when the fibroblastic cells spread in the tissue culture flasks. The monolayer formation took 8 days from heart explant of catla, 10 days in rohu and 20 days in mrigal (Rao et al., 1997). Fin explant culture from *Tor putitora* has taken more than 8 days for monolayer formation (Prassana et al., 2000).

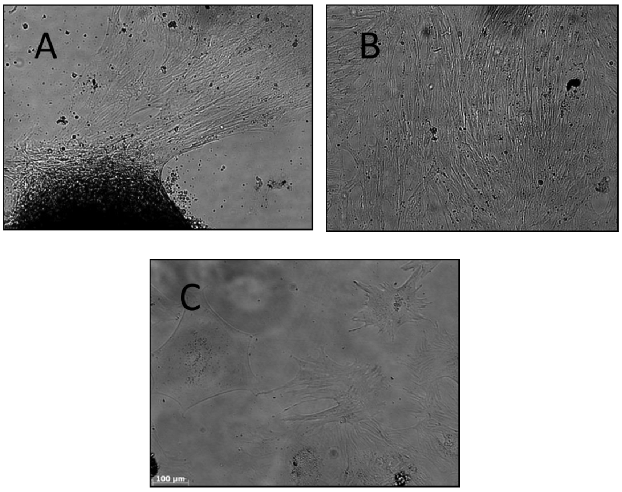

Fig. 1 A. Spindle shaped cells migrating from attached liver tissue explant after 48 h of culture.
1. B. Formation of confluent monolayer within three weeks and ready for sub-culture
1. C. Fibroblast shaped cell attached and spread on cell culture flask after sub-culture

Primary liver cell culture showed good growth in L-15 media containing 10-20% FBS has shown highest confluence when media supplemented with concentration of 20% FBS (Fig. 2 B), but considerably slower in 5 and 25% FBS. Similarly, significantly higher cell confluence was observed when the primary liver cell was cultured at an incubation temperature of 28ºC (Fig. 2 A). These results are in accordance with the earlier reports in different cell cultures developed from rohu (Lakra & Bhonde, 1996; Rao et al., 1997; Sathe et al., 1997; Joseph et al., 1998; Ahmed et al., 2009). In addition, suitability of L-15 medium in supporting fish cell cultures compared to other media has also been frequently documented in the literature (Chen et al., 2005; Ahmed et al., 2008).

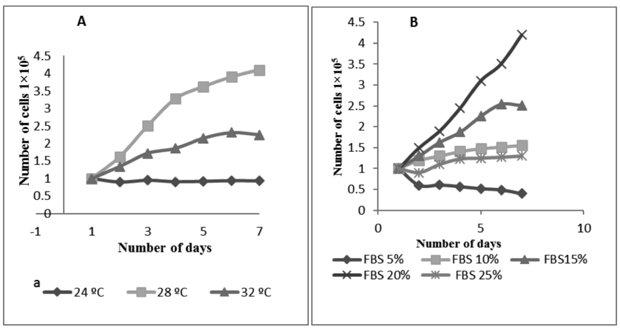

Fig. 2A. Effect of different incubation temperature (°C) on cell growth
2B. Effect of different FBS concentration in L-15 media (% v/v) on cell growth

Though media composition is important for the success of cell culture, other factors *viz.*, supplements, pH, incubation temperature have a profound effect on cell growth. The foetal bovine serum (FBS) has widespread use in fish cell culture because of its lack of toxicity and growth stimulation properties. Moreover, it is also having virus neutralizing property and thus reduces chances of contamination during the initial phases of culture. In the present study, the confluence increased as the FBS concentration increased and highest confluence was observed when the media was supplemented with FBS at 20% for primary culture and 10% FBS inclusion in the media for subculture of cells. Similar observations were made in the earlier studies where growth rate of SICE cells increased as the FBS concentration in the media was increased from 2-20% at 28°C (Parameswaran et al., 2006; Ahmed et al., 2008). However, poor growth was noticed when FBS concentration exceeded 20%. In contrast to this, according to Ganassin & Bols (1998), RTS11 cells, a macrophage cell line developed from spleen of rainbow trout needed high concentrations of FBS (30%) to maintain proliferation.

In the present study, the confluence increased as the incubation temperature increased showing highest confluence at a temperature of 28°C beyond which it decreased. This is found to be in agreement with the earlier observation where optimal temperature for cell lines from eye of rohu and brain of catla ranged between 28 to 32°C with maximum growth at 28°C (Ahmed et al., 2009). In addition, a number of other researchers have supported this finding that fish cell cultures grow at optimum temperature of 28°C with good proliferation of cells (Sathe et al., 1995; Joseph et al., 1998; Lai et al., 2000; Kang et al., 2003; Lai et al., 2003). Further, a temperature of 35-37°C was often found lethal to many of fish cells (Tong et al., 1997).

Cell culture has been used for toxicological assessment of chemicals, environmental samples, genotoxicity and oxidative stress (Saito et al., 1991; Kammann et al., 2001; Teju et al., 2017). Fish cell lines have been proven to be valuable, rapid and cost-effective *in vitro* tools in the preliminary ecotoxicological evaluation of xenobiotics (Fent, 2001). Interactions between chemical contaminants and biological systems initially take place at the cellular level. The use of *in vitro* cell cultures for ecotoxicological assessment can therefore be an important tool for the early and sensitive detection

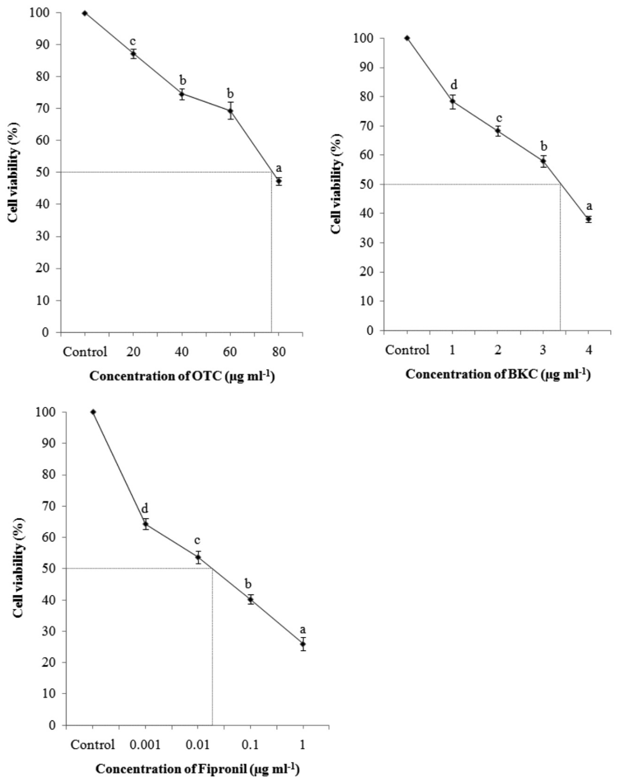

Fig. 3A. *In vitro* cytotoxicity of OTC to primary liver cell culture of *L. rohita* by MTT assay

3B. *In vitro* cytotoxicity of BKC to primary liver cell culture of *L. rohita* by MTT assay

3C. *In vitro* cytotoxicity of Fipronil to primary liver cell culture of *L. rohita* by MTT assay

of chemical exposure (Segner & Braunbeck, 1998). The *in vitro* cytotoxicity of any chemical is characterized by IC_{50} value, i.e. concentration which induces 50% loss of viable cells. The cytotoxic effect of OTC, BKC and Fipronil on primary liver culture is shown in (Fig. 3A, 3B and 3C) which shows that toxicity increased as the concentration of chemotherapeutants was increased. The IC_{50} values of OTC, BKC and Fipronil calculated from dose-response curve were 76.43 µg ml^{-1} (Fig. 3A), 3.22 µg ml^{-1} (Fig. 3B) and 0.014 µg ml^{-1} (Fig. 3C) respectively. Among OTC, BKC and Fipronil, Fipronil was found to be the most toxic to the primary liver tissue culture. In the present study, the lowest concentration of the OTC (20 µg ml^{-1}) tested on primary cell culture reduced viability of cells by about 13% and IC_{50} value was obtained as 76.43 µg ml^{-1}. According to earlier studies, IC_{50} values obtained for OTC were about 200 mg l^{-1} for *Salvelinus namaycush* (Marking et al., 1988) and 116 mg l^{-1} for *Oncorhynchus mykiss* (USEPA, 2001). This shows that toxicity of OTC varies from species to species and rohu primary liver tissue culture were found to be more sensitive to the chemical. Antimicrobials such as tetracyclines, oxytetracyclines and several sulfamides are frequently used in aquaculture and are usually considered to be less toxic for fish (Holten, 2000; Boxall et al., 2004). In the present study, the lowest concentration of the BKC (1.0 μg ml^{-1}) reduced viability of cells by about 22% and IC_{50} value was found to be 3.22 μg ml^{-1}. The result proved that the BKC, a frequently used antibacterial compound in aquaculture can be toxic to the cells. According to earlier study, the LD_{50} value of BKC for *Macrobrachium rosenbergii* (PL20) was reported as 2 mg l^{-1} in 24 h (Liao & Guo, 1990). The lowest concentration of the BKC (0.001 μg ml^{-1}) reduced viability of cells by about 37% and IC_{50} value was found to be 0.124 μg ml^{-1}. The information on the *in vitro* toxicity of Fipronil to fish is unavailable to compare the present result. However, according to earlier studies, LC_{50} values obtained for Fipronil were 0.430 ppm for European carp (USEPA, 1996), 0.042 mg l^{-1} for African tilapia (Diallo et al., 1998), 0.246 mg l^{-1} for rainbow trout, 0.083 mg l^{-1} for bluegill sunfish and 0.13 mg l^{-1} for sheep head minnows (Tomlin, 2006). This shows that the LC_{50} value varies from species to species and is highly toxic to the rohu primary liver tissue culture. This suggests that primary cell culture from liver tissue of rohu is potentially a suitable bio-indicator for the screening of the acute toxicities of Fipronil.

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