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Development of a Continuous Cell Line from Tilapia Liver

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Abstract: Tilapia is no longer a disease-resistant species. There is a number of bacterial and viral diseases affected tilapia and some of them are devastating. Intensive farming practices have led to increased disease incidence. Development of cell lines from various tissues of tilapia is desirable for developing cell models for *in vitro* study. In present study was established primary tissue culture from tilapia liver and accelerate its growth rate by using growth media containing 20% serum concentration. Rapid cells replication leads to uncontrolled cells proliferation. DNA fragmentation showed wide variation between the origin DNA of primary liver cells and replicated cells. Also, electron microscope detected morphological changes between the origin cells and replicated one. The primary cells were mutated and formed continuous cell line from hepatic tissue of tilapia fish can be used in tilapia viruses' investigation.

Keywords: Tilapia, cell line and mutation.

1 Introduction

Tilapia is native to warm, fresh and brackish waters of Africa, South and Central America and southern India. Approximately 150 species have been imported into Australia, primarily for use as aquarium fish. Out of the 70 species of tilapia, nine are used in farming and of these Nile tilapia (*Oreochromis niloticus*) is the main cultured species which responsible for the significant increase in global tilapia aquaculture production. The major producing countries are China, Egypt, Indonesia, Philippines, Mexico, Thailand, Taiwan and Brazil [1].

In the last 5 years, there has been an unprecedented development in the tilapia industry in terms of global production. International trade was increased by 26% per year, and industrialization of several integrated companies has emerged in recent years, each producing over 10,000 MT/year. So, tilapia has emerged from obscurity to become the number one commodity aquaculture species in the world [2].

Tilapia are farmed in different culture systems (extensive, semi-intensive, intensive, monoculture, polyculture, monosex culture, mixed sex culture) depending on the farmer's resources, site characteristics, environmental conditions, socio-economic factors, technological know-how and market demand. All the indications are that tilapia is no longer a disease-resistant species. There is a number of bacterial and viral diseases affected tilapia and some of them are devastating [3].

Also, adoption of intensive farming practices, unregulated use of inputs and inbreeding in hatcheries, has led to increased disease incidence in tilapia. There have been severalincidences of mass mortality of tilapia in culture systems, which are suspected to be caused by microbial diseases. Hence, development of a cell line from tilapia for identifying pathogenesis of viral diseases and for vaccine production against viral diseases is necessary [4].

Although viral infections in tilapia in Egypt are likely to exist, no virus has been isolated and characterized because the tilapian cell lines not be provided for researches. So, development of cell lines from various tissues of tilapia is desirable for developing cell models for *in vitro* study of their cellular physiology, molecular biology, genetics, immunology, endocrinology, nutrition, comparative biology and virology [5].

The present study reports new continuous cell line from tilapia liver.

2 Material and Methods

2.1 Primary Cell Culture

Healthy Nile tilapia with average body weight 50 - 60 gm were obtained from wet lab of health and management fish department of central laboratory for aquaculture research. All fish specimens were euthanized by

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keeping them on ice for 15 min and surface sterilized by dipping in ethanol for 5 min.

Primary cell cultures were initiated by aseptically collecting liver tissue. The tissues were transferred to phosphate buffered saline (PBS), containing 1000 IU penicillin, 1000 µg streptomycin and 25 µg amphotericin B per ml. Liver samples were stirred with trypsin versen solution. Two mm^3 of the fragment solution were individually explanted into 50 cm^2 tissue culture flasks with 20ml Dulbecco's Modified Eagle's Medium (DMEM). The flasks were incubated at 28°C and the medium was replaced every five days. The flasks were observed daily for attachment, spreading, proliferation and morphological details [6].

2.2 Subculture

When the culture has reached around 80% confluence, the spent culture media was aspirate from the culture vessel without disturbing the cell monolayer. When majority of the cells have detached, equal volume of complete media was added into the culture vessel. Cell suspension was centrifuged at 1500 rpm for 3 minutes and the supernatant was aspirated after checking. The cell pellet was re-suspended in pre-warmed fresh complete media and seeded in new culture vessels [7].

2.3 Accelerated Growth Rate

The cells were harvested with 0.25% trypsin–EDTA solution and seeded at 1:3 ratios and maintained in the complete DMEM medium with serum concentration 20% for enhanced growth [8].

2.4 DNA Fragmentation

Samples of normal hepatic tissues, after 10th and 25th passage were lysed in NTE buffer (100 mM NaCI, 40 mM Tris-HCI, 20 told EDTA, pH 7.4) containing 0.5% SDS and 0.2 mg/ml proteinase K. Samples were vigorously, grinded, centrifuged and collected in new tubes. After overnight incubation at 37ºC, DNA was extracted twice with phenol-chloroform and precipitated by ethanol. The samples were dissolved in TE buffer and digested for 2 h with 0.1 mg/ml RNAse A. DNA fragmentation was analyzed on a 1.8% Agarose gel in the presence of 0.5 µg/ml ethidium bromide by using electrophoresis [9].

2.5 Morphological Studied by Transmission Electron Microscopy

The cells were collected from the prescription tubes and fixed with glutaraldehyde solution. Tissue is dehydrated at -90 degree centigrade. Samples are infiltrated and embedded in resins that are cured with heat. Ultramicrotome was used to section samples at thicknesses of between 50 and 200 nm. Sections are placed onto grids for support in the microscope. Further staining with uranyl acetate was used for enhances contrast. Normal hepatic tissues also used [10].

2.6 Cell Preservative

The subcultures were stored in the freezing medium, which consisted of DMEM plus 50% FBS and 10% dimethyl sulphoxide (DMSO). The cells with 80% confluency were harvested. These cells were resuspended to 1×10^6 cells/ml and stored in a cryovial (1.5 ml cell suspension per vial). The cryovial were kept at −20 ◦C for 2 h, −70 ◦C overnight and then transferred to liquid nitrogen containers (−196 ◦C) [7].

2.7Revival Preservative Cell

For revival, cryovial was thawed quickly in water bath at 37 ◦C and centrifuged at 200 × g for 5 min at room temperature. The cells were resuspended with DMEM medium supplemented with 10% FBS, 100 IU/ml penicillin, 100 µg/ml streptomycin and 0.25µg/ml amphotericin B and seeded in a 50 cm2 tissue culture flask. The viability of the revived cells was estimated by trypan blue staining and the cells were counted on a hemocytometer [6].

3 Results and Discussion

3.1 Primary Cell Culture

The radiation of cells explants started after 2 to 3 days in 50 cm^2 tissue culture flasks with presence of some small liver fragments formed aggregated groups. Complete cell monolayer was formed approximately within 10 days after the implantation (Figure 1).

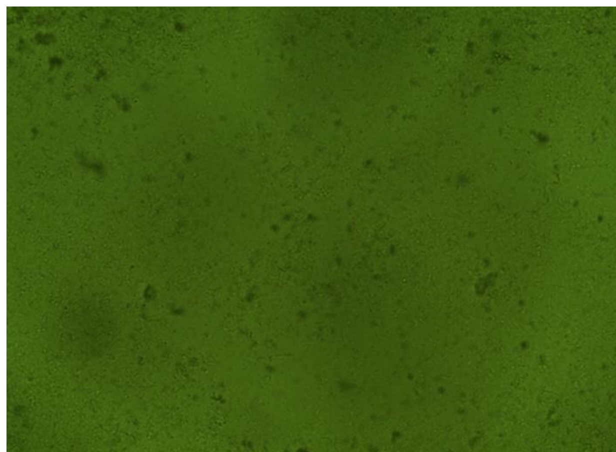

Fig.1. Complete cell monolayer was formed with presence liver fragments. X (100).

Cell lines is an important biological tool can be obtained for

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carrying out researches in physiology, virology, pharmacology, toxicology, carcinogenesis and transgenic fields. Several fish cell lines are processed by which cells are grown under controlled conditions, developed from kidney, heart tissues caudal fin and ovary [11]. The established cell lines were done in many countries to fix their native problems affecting cultured species while in Egypt, the work with fish cell lines still scanty and need more developed specially in tilapia which facing storm of summer mortality and needing established tilapian cell lines used for studying the disease problem. The present study reports new continuous cell line from tilapia liver can be used for this aims.

In primary culture the radiation of cells started after 2 to 3 days and the cells take round shape. The cells proliferation becomes more systematic after formation complete sheet. The obtained cell shape after its primary cultures depended on the origin of the tissue from which the cells were established then fin and heart of common carp primary cultures reported from fish in India were comprised of fibroblastic shape when development primary cell cultures from kidney of freshwater fish *Heteropneustes fossilis* [12]. Also, epithelioid cell shape in cell cultures from fish has been reported by several workers [13].

3.2 Subculture

The cells were subcultured on 7 days intervals in DMEM medium which given chance for purification the cultured cells from the liver fragments and increased the cultured yield.

Adherent cell lines were grew until they have covered the surface area available in the tissue culture flask or the medium is depleted of nutrients. At this point the cell lines were subcultured to prevent the culture dying. To subculture the cells were brought into suspension into a small volume of medium mechanically with the aid of cell scrapers without using of trypsin for keeping cell health as that recorded by [7] who cited, the degree of adhesion varies from cell line to cell line but in the majority of cases proteases are used to release the cells from the flask. However, this may not be appropriate for some lines where exposure to proteases is harmful or where the enzymes used to remove membrane markers/receptors of interest.

3.3 Accelerated Growth Rate

Cell number was rapid proliferated and increased in number in cultures with FBS as tested at 20%. At lower cell densities cells attached with similar efficiencies, however at higher cell densities more cells were able to attach in the presence of 20% FBS and liver fragments was disappeared (Figure 2).

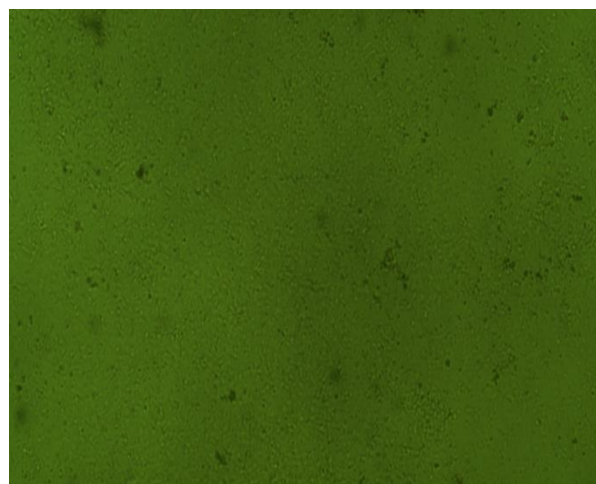

Fig. 2: Purified cells cultured with complete monolayer sheet. X (100).

Most additives to cells media are in serum-free or reduced media replacing substances that serum would normally provide however that does not entirely discount the possibility of using further additives to media already supplemented with serum [14]. The growth of cell cultures required DMEM supplemented with FBS for both cell attachment and proliferation influenced. In present study, it was used growth medium containing 20 % FBS at which showed rapid proliferated and increased in cells number. Using of serum at this concentration aimed to enhanced cell growth and not more than this concentration as recorded by [8] serum concentration can also have an effect on primary cultures. The concentration varies from 5% to as high as 20%. Serum concentrations are not usually much higher than this, as there is evidence that high serum concentrations may inhibit cell growth.

3.4 DNA Fragmentation

The large proportion of the tested DNA at samples No. 2 (after 10th passage) and 3 (after 25th passage) appeared as small fragment with tailing (head-to-tail fork collisions) of rest degenerated or fragmented DNA up to 200 base pairs (3) and 500 base pairs. Round of replication appeared with normal samples (No. 1) (Figure 3).

The main aim of culturing of primary hepatic tissue culture in growth media contain 20% serum was induction of cells mutation to obtain uncontrolled cell growth which was cleared after 10th passage. DNA fragmentation technique was used to illustrate presence changes at nucleic acid level. DNA of normal hepatic tissues, after 10th and 25th passage were tested with DNA fragments technique that is cleared DNA damage occurred during cells replication depends on uncontrolled multiplication correlates with the appearance of DNA fragments. The large proportion of the tested DNA at samples No. 2 (after 10th passage) and 3 (after 25th passage) appeared as small fragment with tailing (head-to-tail fork collisions) of rest degenerated or fragmented DNA (Figure 3).

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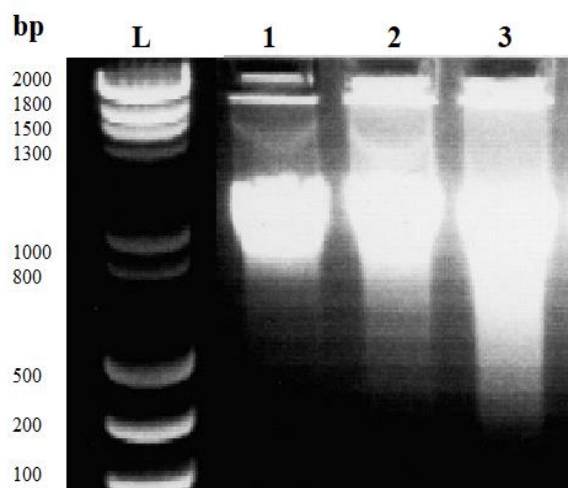

Fig. 3: Large proportion of the tested DNA at samples No. 2 and 3 as small head to tail fork collisions and round of replication in normal sample No.1.

This suggests that the fragmented DNA mainly represents DNA undergoing replication and does not contain a significant amount of DNA generated by the normal (samples No. 1) round of replication. Consistent with this idea, almost all the total DNA still migrated at the exclusion limit of the gel, with only a small proportion appearing as fragments. This specific release of small fragments of replicated DNA. That was accepted by [15] who mentioned the form of mutation through the DNA fragment.

3.5 Morphological Studied by Transmission Electron Microscopy

The morphology of cells obtained directly from tilapia liver cells was revealed a strong compartmentalization of organelles consisting of restricted perinuclear cytoplasm containing rough endoplasmic reticulum (RER) and mitochondria. Most of the rest of the cell's cytoplasm volume was filled with β-glycogen particles (Figure 4).

During cell growth and subculturing with 20% FBS, cell was changed to be appeared differ mainly in a qualitative, fashion, and in the distribution of organelles from normal hepatocytes. Cells were not compartmentalized their organelles with large nucleus and small layer of cytoplasm in addition the cells become smaller in size (Figure 5).

Electron microscopes are very powerful tools for visualizing biological samples. They enable to view cells, tissues and small organisms in very great detail. TEM is a microscopy technique whereby a beam of electrons is transmitted through an ultrathin specimen, interacting with the specimen as it passes through it. An image is formed from the electrons transmitted through the specimen

Fig: 4. Normal tilapia liver cell. Transmission electron microscope. 5000X

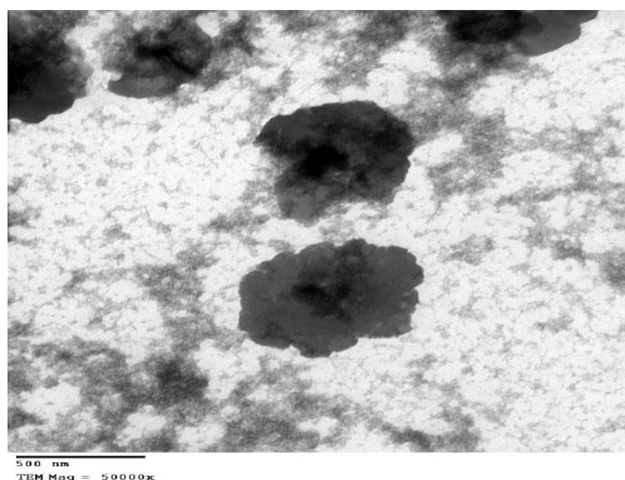

Fig: 5.Subcultured tilapia liver cell. Transmission electron microscope. 50000X

magnified and focused by an objective lens. It was used in the present study to compare between normal tilapian hepatic cell and the mutant one. Normal hepatocytes contained typical organelles with their intracellular distribution similar to that described for other teleosts. These cells revealed a strong compartmentalization of organelles consisting of restricted perinuclear cytoplasm containing rough endoplasmic reticulum (RER) and mitochondria. Most of the rest of the cell's cytoplasm volume was filled with β-glycogen particles. While, mutant cells appeared to differ mainly in a quantitative, rather than in a qualitative fashion, and in the distribution of organelles from normal hepatocytes. Cells were not compartmentalized their organelles and only small amounts of glycogen remained diffusely distributed or in small masses or foci. [16] Recorded the same feature in studying light and electron microscopic comparisons of normal hepatocytes and neoplastic hepatocytes of well-differentiated hepatocellular carcinomas in a teleost fish and mention mitochondrial intermembrane myelin bodies

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(MMB's) were found frequently in the mutant cells but less frequently in the normal hepatocytes. Some cells at the edge of the mutant appeared to be intermediate between inner mutant cells and normal hepatocytes in terms of abundance and distribution of organelles and glycogen. The possible significance of the MMB's and intermediate, edge cells is discussed, as well as the need to better characterize features of well-differentiated hepatic neoplasms in order to assure their proper inclusion in neoplasm incidence/prevalence data in fish carcinogenesis assays and field studies.

3.6 Cell Preservative

The produced cells after 25th passage were collected every 5 days and preserved in freezing medium.

The cultured cells were harvested every 5 days freezed in freezing buffer and revealed every month interval which showed more than 90% of preservative cells remained viable after the storage and retained the ability to attach and grow at 28°C.

3.7 Revival Preservative Cell

More than 90% of preservative cells remained viable after the storage and retained the ability to attach and grow at 28°C. Following storage, no obvious alterations in morphology or growth pattern were observed for cells.

4 Conclusions

It can be concluded that there is obtained continuous cell line from hepatic tissue of tilapia fish. It was more stable and can be used in tilapia viruses' investigation.

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