

TRIALS FOR PREPARATION OF PRIMARY CELL CULTURES FROM SOME FRESHWATER FISHES

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Abstract

This work has been designed to establish and characterize primary tissue culture from freshwater fishes *Oreochromis niloticus* and *Clarias gariepinus*. Four organs of *O. niloticus*, namely, gonads, kidney, liver and spleen and three organs of *C. gariepinus*, namely, gonads, kidney and spleen were used. The cells multiplied well in MEM-essential medium, supplemented with 10% foetal calf serum. The primary cell culture became confluent sheets after 5 and 7 days, with temperature for optimal growth at 25 and 28°C for *Oreochromis niloticus* and *Clarias gariepinus*, respectively. The optimum pH was 7.2 for both species. These primary cultures were attempts to establish permanent fish cell lines to be used as tool for viral isolation in freshwater fishes.

INTRODUCTION

Cell cultures can be prepared from different tissues and organs of many fish species (Bols and Lee, 1991), Fryer and Lannan (1994). As with other animals, two types of cultures can be distinguished, primary cultures and cell lines. Primary cultures refer to the in-vitro maintenance of cells taken directly from fish, while, cell lines are cultures that arise after several sub culturing or passaging of primary cultures (Schaeffer, 1990).

The uses of fish cell cultures are many, varied and growing, one general use is the study of infectious viral diseases affecting fish.

Rainbow trout (*Oncorhynchus mykiss*) is the most commonly used species in fish research. Although a large number of fish cell lines (~ 159) have been established for isolating and identifying fish viruses (Fryer and Lannan, 1994), relatively, few marine fish cell line (~34) are available (Chen and Kou, 1987).

In Egypt, propagation of primary tissue cultures from gonads of tilapia (Habashi, 1980), and from ovaries of tilapia and catfish (EL Trabali *et al.*, 2000) after 7 and 9 days of incubation at 25°C and 30°C and the optimal pH 7.2-7.4, respectively, has been reported

The present study was planned to establish primary cell cultures from gonads, kidney, spleen and liver of two freshwater fishes (*Oreochromis niloticus*) and (*Clarias gariepinus*) as an aid for isolation and identification of fish viruses, as well as,

determination of the optimal temperatures and pH required for the optimal growth of these primary cell cultures.

MATERIALS AND METHODS

1-Fishes

The freshwater fish *Oreochromis niloticus* (*O. niloticus*) weighing 80-100 g were obtained from the Central Lab of Aquaculture Research, Abbassa, Sharkia Governorate. The *Clarias gariepinus* (*C. gariepinus*) weighing 100-150 g were collected alive from River Nile at Giza. They were held at Fish diseases research department, Animal Health Research Institute, Dokki, at 28°C. The fishes were fasted for one day before use, after which, they were sacrificed and allowed to bleed. The fishes were dipped in 5% chlorex for 5 min, and then, wiped with 70% alcohol.

2- Preparation of primary cell cultures (Lai *et al.*, 2000)

The organs (kidney, liver, spleen, ovaries and testis) were removed from the body and washed three times in MEM- essential medium containing antibiotics (penicillin 100 IU/ml and streptomycin 100 µg /ml) and 1% antimycotic.

The tissues were minced with scissor and transferred to a tissue culture flask containing 10 ml of 0.25% trypsin solution (0.25% trypsin and 0.2% EDTA). The solution was gently agitated with a magnetic stirrer for 1h.

By centrifugation of the supernatants, cells at 3000 r.p.m for 15 min, the sediment was transferred with an equal volume of MEM medium supplemented with 10% foetal calf serum (FCS) containing penicillin 100 IU/ml and streptomycin 100 µg /ml and mixed well to inhibit trypsin activity.

The sediment cells were seeded into 25 ml (cm³) tissue culture flasks and maintained at 25-28°C.

3 - Sub culture and maintenance

When a complete confluent monolayer sheet had been formed in primary culture, cells were washed with MEM. Subsequently, 0.25% trypsin solution was added and incubated until cells were dislodged from flask surface. The cells were counted using haemocytometer and transferred into a new flask with fresh MEM supplemented with penicillin 100 IU/ml and streptomycin 100 µg /ml and 10%FCS medium, at a density of 10⁶ /ml as described by *Nicholson and Byrne (1973)*.

4- Effect of temperature and pH on tissue culture

Three experiments were performed to study the effect of temperature and pH.

In the first experiment, the cell cultures were incubated at 25, 28, 30, 32°C. with a pH 7.2

In the second experiment, the pH of MEM solution was adjusted at pH 6, 7.2, 7.7, 8 and incubated at 25°C.

In the third experiment, alternative temperature and pH were used to determine the optimal temperature and pH required for each type of organ cell line.

All tissue culture flasks were examined daily with an inverted microscope for growth and confluence.

RESULTS

A- Establishment and characterization of the primary cell culture

After 24 hours post-incubation at 25°C, the cells were attached to the culture flask forming small aggregates, while, the majority were completed within 3-5 days. After one week, the attached cell aggregates became confluent monolayer sheet composed of fibroblasts and epithelial cells.

The growth and establishment of the primary cells differed according to fish species and type of organs .It was found that ovary and kidney cells grew faster and established a confluent sheet than spleen, liver and testicular cells.

The primary cell culture became a confluent with temperature for optimum growth at 25 and 28°C for *O. niloticus* and *C. gariepinus* respectively, and the pH was 7.2 for both species, as shown in Tables 1&2. The combination of pH and temperature adjustment are very essential for optimum fish cell culture growth, as shown in Table 3.

The shape of *O. niloticus* cells was mainly spindle, while, *C. gariepinus* was rounded and some cells appeared clumping.

The following tables represented the degree of growth established in five types of cells in fish species *O. niloticus* and *C. gariepinus*.

Table 1. Degree of growth of different *O. niloticus* and *C. gariepinus* cells in primary cell culture at different temperatures and 7.2 pH.

Type of cells	<i>O. niloticus</i>				<i>C. gariepinus</i>			
	25°C	28°C	30°C	32°C	25°C	28°C	30°C	32°C
Ovary	+++	++	+	-	++	+++	+	-
Kidney	+++	++	+	-	++	+++	+	-
Spleen	++	+	+	-	+	++	+	-
Testis	++	+	+	-	+	++	+	-
Liver	++	+	-	-	+	-	-	-

+++ Confluent monolayer sheet ++ Slow confluent monolayer sheet growth.

+ Faint monolayer sheet growth - No growth

Table 2. Degree of growth of different *O. niloticus* and *C. gariepinus* cells in primary cell culture at different pH and incubated at 25°C.

Type of cells	<i>O. niloticus</i>				<i>C. gariepinus</i>			
	6	7.2	7.7	8	6	7.2	7.7	8
Ovary	-	+++	+	-	-	+++	+	-
Kidney	-	+++	+	-	-	+++	+	-
Spleen	-	++	+	-	-	++	+	-
Testis	-	++	-	-	-	++	+	-
Liver	-	++	-	-	-	-	-	-

+++ Confluent monolayer sheet ++ Slow confluent monolayer sheet growth.

+ Faint monolayer sheet growth - No growth

Table 3. The optimum temperature and pH values for confluent monolayer growth for *O. niloticus* and *C. gariepinus*.

Temperature (°C)	<i>O. niloticus</i> pH				<i>C. gariepinus</i> pH			
	6	7.2	7.7	8	6	7.2	7.7	8
25	-	+++	+	-	-	++	-	-
28	-	++	-	-	-	+++	-	-
30	-	+	-	-	-	+	-	-
32	-	-	-	-	-	-	-	-

+++ Confluent monolayer sheet ++ Slow confluent monolayer sheet growth.

+ Faint monolayer sheet growth - No growth

B- Sub culture

The cell cultures have been sub cultured for 8 passages in approximately 2 months. During sub cultures 1-8, a combination of 50% new and 50% old medium was used, at 6-7 days interval in MEM-10% FCS. The cells in the primary fish cell cultures were able to be seen in Figures 1-10.

C-The effect of temperature on growth of primary cell culture

The results showed that the optimum temperature for *O. niloticus* cells to form confluent monolayer sheet was 25°C for 5 days incubation at pH 7.2. The cells were faintly grown at 30°C with no growth at 32°C or more, while, the optimum temperature for *C. gariepinus* cells to form confluent monolayer sheet was 28°C for 7 days incubation at pH 7.2. The cells were faintly grown at 30°C with no growth at 32°C or more as in case of *O. niloticus*, Table (1).

D- Effect of pH values on growth of primary cell culture

The results showed that the optimum pH values for confluent monolayer sheet was 7.2 for both *O. niloticus* and *C. gariepinus* at temperatures 25 and 28°C for 5- 7 days, respectively. The cells growth was faint at pH 7.7, cells did not attach at pH 6 and 8 (Table 2).



Fig. 1. Confluent monolayer cells from ovary of *O. niloticus* at (4th passage) at 25°C and 7.2 pH (X40).



Fig. 2. Confluent monolayer cells from kidney of *O. niloticus* at (4th passage) at 25°C and 7.2 pH (X40).



Fig. 3. Confluent monolayer cells from spleen of *O. niloticus* at 4th passage at 25°C and 7.2 pH (X40).

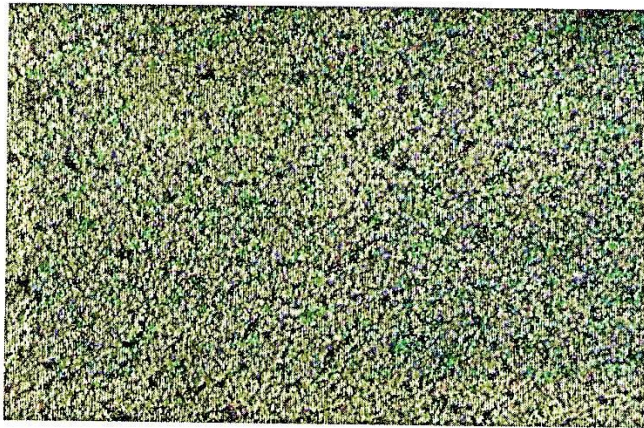


Fig. 4. Confluent monolayer cells from testes of *O. niloticus* at 4th passage at 25°C and 7.2 pH (X40).

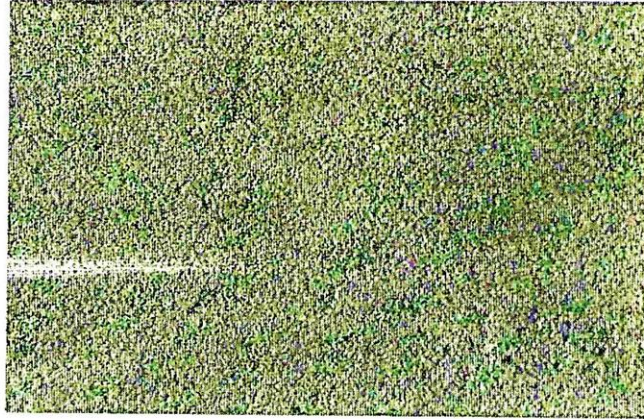


Fig. 5. Confluent monolayer cells from liver of *O. niloticus* at 4th passage at 25°C and 7.2 pH (X40).

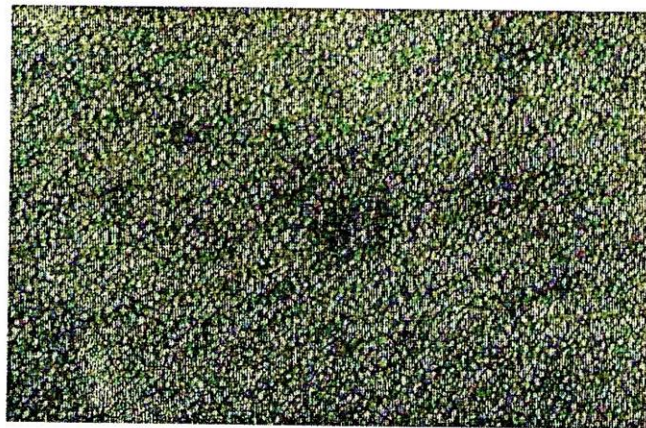


Fig. 6. Confluent monolayer cells from ovary of *C. gariepinus* at 4th passage at 28°C and 7.2 pH (X40).



Fig. 7. Confluent monolayer cells from kidney of *C. gariepinus* at 4th passage at 28°C and 7.2 pH (X40).



Fig. 8. Confluent monolayer cells from spleen of *C. gariepinus* at 4th passage at 28°C and 7.2 pH (X40).



Fig. 9. Confluent monolayer cells from testes of *C. gariepinus* at 4th passage at 28°C and 7.2 pH (X40).



Fig. 10. Faint monolayer cells from liver of *C. gariepinus* at 28°C and 7.2 pH (X40).

DISCUSSION

In Egypt, the diagnosis of viral diseases in fish had taken a minor interest in the past due to the low significance of fish viruses in warm water environment, and also due to most complicated required diagnostic techniques, and shortage in specific cell line necessary for such virus isolation and identification.

In the present study, cell cultures were prepared from freshwater fishes, and the optimum temperature and pH for optimum cell growth, were adjusted.

The primary cell cultures were prepared in this study from gonads, kidney, spleen and liver of *O. niloticus* and *C. gariepinus* and the results showed that the aggregates of cells grew after 24 h, forming a confluent sheet within 5 days in *O. niloticus* and 7 days in *C. gariepinus* as shown in Figures 1-10. These results corresponded with those of other investigators who prepared cell cultures from ovaries (Habashi, 1980 and Talaat, 2005), liver (Visoottviseth and Nantawan, 2001), kidney, spleen and gonads (Chen. and Kou, 1987). The trial of propagation of liver cells in *C. gariepinus* failed, while, growth in *O. niloticus* was slow. This result referred to various parameters which had impacts on growth of cell culture, namely, seeding volume and types of culture supplements, types of culture medium, incubation temperature, serum concentration, and carbon dioxide requirement, which agreed with Visoottviseth and Nantawan (2001).

The optimum temperatures for the growth of primary cell culture of *O. niloticus* and *C. gariepinus* were 25 and 28°C, respectively. Similar results were reported for *O. niloticus* by Browser and Palm (1980a, b) for *C. gariepinus*.

Concerning to pH which is necessary for cell growth, the optimum pH for performing a confluent sheet was 7.2 for both *O. niloticus* and *C. gariepinus*. Similar results were observed by Habashi (1980), Zhang and Congleten (1994) and Talaat, (2005).

Nowadays, fish tissue culture has been used as an important tool in research in the area of toxicology (Visoottviseth and Nantawan, 2001) besides isolation and identification of many viruses infecting freshwater fish.

Thus, it can be concluded that primary cell cultures from *O. niloticus* and *C. gariepinus* can be successfully grown under certain conditions from temperature and pH.

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محاولات لتحضير زرع نسيجي أولى من بعض أسماك المياه العذبة

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تم اجراء هذا البحث بهدف إقامة وتوصيف زرع نسيجي أولى من - الكلى - الطحال - الكبد لبعض اسماك المياه العذبة من نوع البلطى النيلي و القرموط الإفريقي. وقد تم إنماء الخلايا باستخدام الميم منيا والتي تحتوى على ١٠% سيرم. بقرى وقد تكونت خلايا الزرع النسيجي كامله بعد ٧,٥ أيام لكل من اسماك البلطى و القراميط على التوالي. وقد أثبتت الدراسة أن درجة الحرارة المثلى للنمو هي ٢٥ و ٢٨م لخلايا الزرع النسيجي لأسماك البلطى والقراميط على الترتيب بينما كانت درجة الأس الهيدروجيني ٧,٢ لكل منهما .