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Establishment of a cell culture system and characterization of the primary cultures from different organs of the Nile tilapia *Oreochromis niloticus*

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ABSTRACT

In addition to toxicology, carcinogenesis, functional genomics studies, and lacking *in vivo* complexities, fish cell culture considered necessary to study virus-host interactions and isolate viral pathogens in fishes. Therefore, the present study aimed to establish a cell culture system and characterize the primary culture from different organs of Nile tilapia *Oreochromis niloticus* fish. Leibovitz-15 (L-15) medium containing L. glutamine and supplemented by 15% of Foetal Bovine Serum (FBS), 200 IU/ml penicillin, 200 µg/ml streptomycin and 2.5 µg/ml amphotericin were used for culturing the cells. In this study, Primary cultures from different organs (brain, testis, and ovaries) from *Oreochromis niloticus* were established. The results of the present investigations showed that brain primary cultures grew slowly. Active growth was observed in ovary primary culture on the 7th day with fibroblast-like growth. Testis primary culture showed active growth on the 2nd day and was capable of maintaining organ function.

INTRODUCTION

Cell culture from fish is an excellent tool for many advanced applications. The cell lines derived from a particular fish species are more appropriate for studying the viruses reported from that species and studying virus-host interactions (**Crane and Hyatt, 2011 and Pandey, 2013**). It is an excellent alternative to obtain good quality chromosome preparations in cytogenetic studies of small and large species. With the establishment of a cell bank available at any time and in case of a repetition of cytogenetic methodologies, it is not necessary to go back to the field for new individuals (**Paim *et al.*, 2018**). Fish cell lines are appropriate for *in vitro* assays, since they are believed to retain fish-specific traits in their metabolism of chemicals (**Schenel et *al.*, 2015**). Cell cultures lack *in vivo* complexities such as bioaccumulation and depuration. Finally, experiments with cell

cultures satisfy a societal desire to reduce the use of animals in toxicology testing (**Bols *et al*., 2005**).

So far, a good few cell lines from freshwater and marine fish species have been reported from a broad range of species and tissues (**Ou-Yang *et al*., 2010; Wang *et al*., 2010; Babu *et al*., 2011; Collet and Lester, 2011; Lakra *et al*., 2011 and Yan *et al*., 2011**). The investigators in the tropics regions are lacking cell lines of their choice, and which may be the reason for the limited number of studies on viruses from warm water fish species (**Sunil *et al*., 2001 and khamiss and Hashem**, **2012**).

On the other hand, Nile tilapia, *Oreochromis niloticus* is the six most cultured species in the world (**Reantaso, 2017 and Hashem *et al*., 2019 and 2020**). The global production of tilapia in 2015 was estimated at 6.4 million metric tons (MMT), (**FAO, 2017**).

Concerning the great share of tilapia on economic, environmental, nutritional and biological aspects, the present study interested in the establishment and characterization of primary cell culture from different organs (brain, testis, and ovaries) of *Oreochromis niloticus* fish.

MATERIALS AND METHODS

Healthy *Oreochromis niloticus*, weighing 30-50 g, from fish breading lab, GEBRI were used. All procedures were performed under sterile conditions in a laminar flow hood (Edge GARD Hood, U.S.). All prepared buffers, solutions, and media were sterilized propriety by autoclaving or by filtration through sterile syringe filter (0.2 µm). All containers and equipments were sterilized by autoclaving (**Chris and Part, 1997**; **Kumar *et al*., 2001 and khamiss and Hashem, 2012**).

Before sacrificing, the fish was kept for 15 min in well-aerated and clean water to flush loosely bound bacteria from fish skin. Fish was washed with sterile water at room temperature and swabbed with 70% ethanol, then fish was decapitated and the ventral side of the body was cut open aseptically as describe by **Khamiss and Hashem, (2012).** Three organs (brain, testis, and ovaries) were obtained for the preparation of primary culture. All organs were obtained with sterile instruments. Each organ was rinsed twice for 5 min in 5 ml of Leibovitz-15 (L-15) medium with L. glutamine (Biowest, France) containing 500 IU/ml penicillin, 500 µg/ml streptomycin (**Wen *et al*., 2008 and Wei *et al*., 2014**) and 2.5 µg/ml amphotericin (**Stanbridge, 1971; Loir, 1999 and Hasoon *et al*., 2011**). Each organ was transferred to a sterilized Petri dish and cut into pin head sized pieces. By using a pipette, sterile pieces of tissues were transferred to tissue culture T-flasks 25 cm^2. Five ml of L-15 medium supplemented by 15% of FBS (Biowest, France) (**Ganassin *et al*., 1999; Wen *et al*., 2008 and Hasoon *et al*., 2011**), 200 IU/ml penicillin, 200 µg/ml streptomycin (**Leal *et al*., 2009**) and 2.5 µg/ml amphotericin (Biowest, France) (**Stanbridge, 1971; Loir, 1999 and Hasoon*et al*., 2011**) were added. Then T-flasks were incubated at 25^oC. The culture medium was changed every two days.

RESULTS

Brain primary culture:

Most of the brain tissues showed difficulties in the attachment to the surface of the flask. The clusters of cells left from detached tissues often showed well fixation to the surface of the flask on the 3rd day (Fig. 1A). There was varied morphology of cells. Cells

appeared epithelial-like cells (Fig. 1B), while other cells were fibroblast-like (Fig. 1C) were long and extended. Both epithelial-like and fibroblast-like cells were observed (Fig. 1D). Substantial outgrowth was observed with both fibroblasts-like and epithelial-like cells in the 9th day (Fig. 2A), with epithelial-like cells on the 10th day (Fig. 2B) and with both fibroblast-like and epithelial-like cells connecting between brain tissues on the 11th day (Fig. 2C and D). Outgrowth was maintained on the 12th day and 15th day (Fig. 3A and B). In addition to multipolar cells (Fig. 3C and D). Noticeable epithelial-like cell extension was observed on the 15th day (Fig. 4B). Noticeable fibroblast extension was observed from the 12th till 39th day (Fig. 4D, Fig. 5A and B).

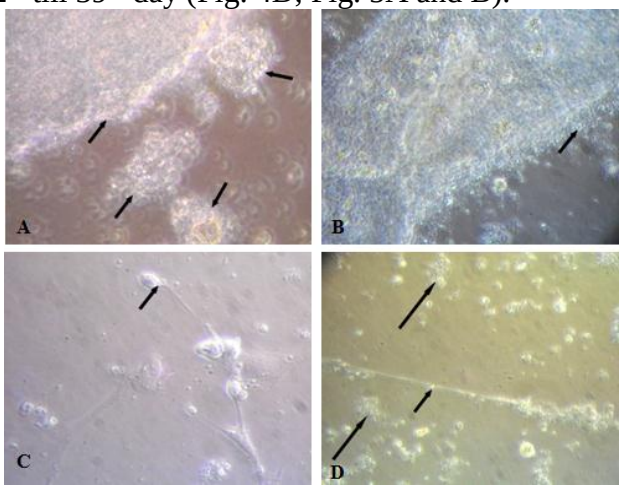

Fig. (1): *O. niloticus* primary brain culture. A., cell clusters attachment to the surface of the flask (3rd day), B., epithelial-like cells grow in the periphery of cell clusters (9th day). C., uni-polar fibroblast-like cell (9th day). D, epithelial-like cells (long arrow) grow in groups and fibroblastic-like cells (short arrow) (9th day).

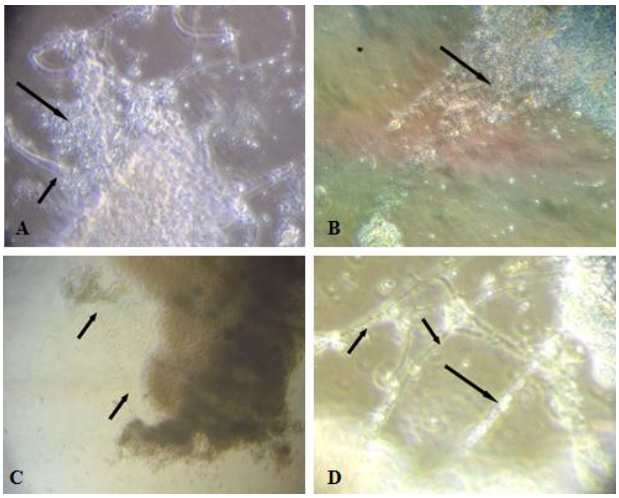

Fig. (2): -*O. niloticus* primary brain culture. A., substantial outgrowth with both fibroblast-like (short arrow) and epithelial-like cells(long arrow) (9th day). B., substantial outgrowth with epithelial-like cells (10th day), C., and D., substantial outgrowth with both fibroblast-like(short arrow) and epithelial-like cells(long arrow) connecting between brain tissues (11th day).

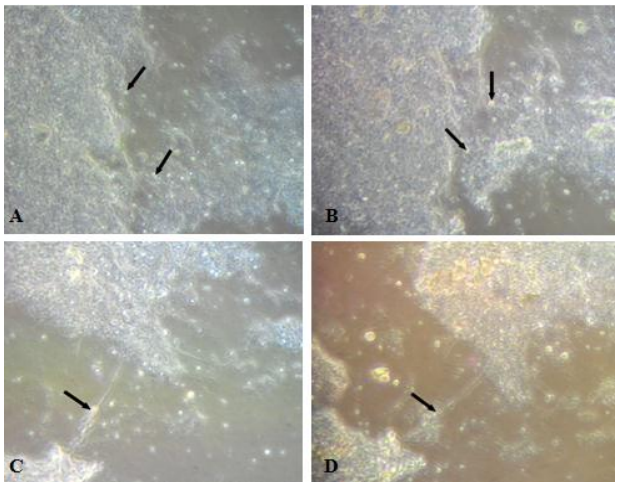

Fig. (3): *O. niloticus* primary brain culture. A., outgrowth with epithelial-like cells (12th day). B., outgrowth with epithelial-like cells (15th day). C., multipolar cell (12th day). D., multipolar cell (15th day).

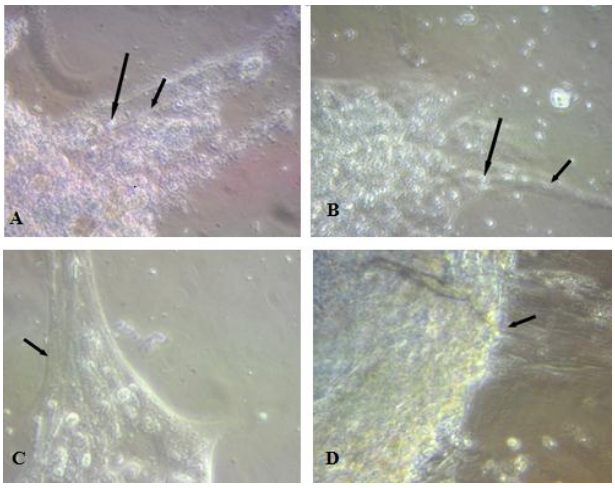

Fig. (4): *O. niloticus* primary brain culture. A., substantial growth with epithelial-like(long arrow) and fibroblast-like cells (short arrow) (15th day). B., epithelial-like(long arrow) and extended fibroblast-like cells(short arrow) (12th day). C., extended fibroblast-like cells (12th day). D., extended fibroblast-like cells (17th day).

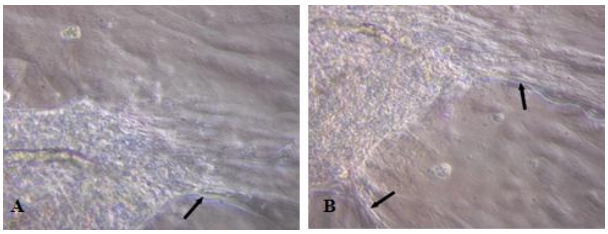

Fig. (5): *O. niloticus* primary brain culture. A., extended fibroblast-like cells (36th day). B., extended fibroblast-like cells (39th day).

Ovary primary culture:

Most of the ovarian tissues showed difficulties in the attachment to the surface of the flask. The clusters of cells left from detached tissues often showed well fixation to the surface of the flask on the 3rd day. Active growth was observed on the 7th day with fibroblast-like growth at the periphery (Fig. 6A). Substantial growth continued on the 10th day with epithelial-like cells (Fig. 6B and C). Obvious growth with fibroblast-like on the 17th day (Fig. 6 D).

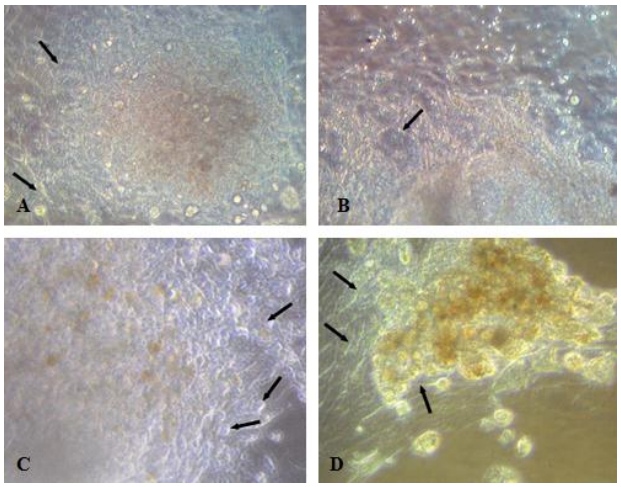

Fig. (6): *O.niloticus* primary ovarian culture. A., Active growths with fibroblast-like growth at the periphery (7th day). B., Substantial growth continued with epithelial-like (10th day). C., Substantial growth continued with epithelial-like cells (10th day), D., fibroblast-like growth (17th day).

Testis primary culture:

Testis primary culture from *Oreochromis niloticus* showed active growth (Fig. 7A) on 2nd day. Also, different types of cells of different sizes involved in spermatogenesis were noticed (Fig. 7B). Some fibroblast-like cells were observed when subcultured (Fig. 7 C and D) at different times.

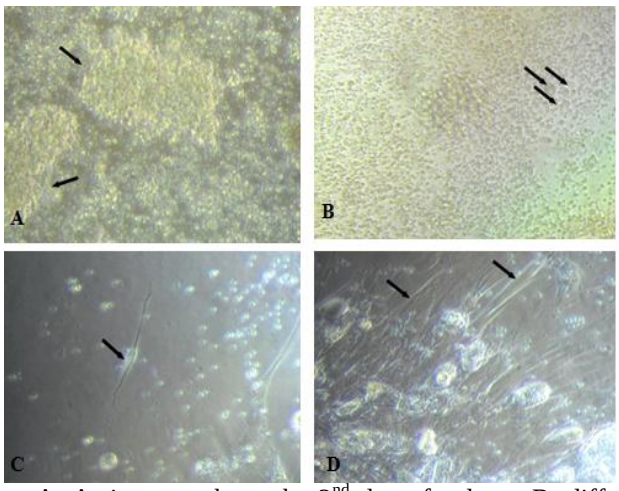

Fig. (7): Testis tissue culture .A, Active growth on the 2nd day of culture. B, different types of cells of different sizes. C, Fibroblast-like cell on the 9th day of subculture. D, Extended fibroblast-like cell on the 11th day of subculture.

Spermatozoa were regularly removed by changing media. Motile spermatozoa were continuously produced *in vitro*. Motile sperm decreased in number and speed gradually. Spermatogenesis was maintained through motile sperms production *in vitro* between 2 and 10 days of primary culture and during 11 days of first subculture.

DISCUSSION

Increased knowledge about fish cells has shown that there are many fundamental similarities between fish and mammalian cells for cellular mechanisms and toxic response (**Eriksson *et al*., 2010**). Fish cells also reflect many fish-specific traits that cannot be assessed with mammalian cells (**Castaño *et al*., 2003**). As a consequence, from a practical point of view, fish cell lines seem a more promising alternative than mammalian cells (**Braunbeck and Lammer, 2006**).

In the present study, L-15 medium was used for the establishment of primary culture from different organs of *Oreochromis niloticus*. L-15 was designed to be used in CO_2-free systems (**Qin *et al*., 2006**). It was reported to be the most suitable medium in terms of its efficacy to support rapid cell attachment and cell proliferation (**Servilli *et al*., 2008; Khamiss and Hashem, 2012 and Wei *et al*., 2014**).

L-15 was previously used for brain cultures (**Servili *et al*., 2008; Wen *et al*., 2008 and Hasoon *et al*., 2011**), ovarian cultures (**Khamiss and Hashem, 2012 and Wei *et al*., 2014**), testis cultures (**Tokalov and Gutzeit, 2005 and Leal *et al*., 2009**), and hepatocyte cultures (**Zhou *et al*., 2006 and Yanhong *et al*., 2008**) of fishes.

The medium was supplemented with 15% FBS in agreement with **Ganassin *et al*. (1999), Wen *et al*. (2008), Hasoon *et al*. (2011), Zhang *et al*. (2011) and Wei *et al*. (2014)**. **Hasoon *et al*. (2011)** reported that cells from the brain tissue of Asian sea bass exhibited maximum growth with FBS concentrations of 15 and 20%. **Kumar *et al*. (2001)** used 10% FBS with ovarian tissue cell culture of African catfish *Clarias gariepinus*. **Khamiss and Hashem (2012)** found that L-15, when supplemented with 20% FBS, supported a higher percentage of attachment of cells leading to cell proliferation during primary culture. **Wei *et al*. (2014)** found that ovarian cell line from Southern catfish multiplied preferentially in L-15 medium with 15 % FBS, but they used 10% of FBS during primary culture. However medium used for testis primary culture was supplemented with 15% FBS without further supplementation in the present work; heterogeneous testis primary culture was adequate to maintain organ function. Spermatogenesis was maintained through motile sperms production *in vitro* between 2 and 10 days from primary culture and during 11 days of first subculture. Meanwhile, previous studies involved different supplements in testis cultures. Reduced glutathione, Zn sulfate, Na selenite, Mn sulfate, tocopherol acetate, ascorbic acid, Ultroser SF and bovine serum albumin (BSA) were supplemented to the culture medium for establishment of testis primary culture from trout **(Loir, 1999)**. Ketotestosterone (KT), BSA, and retinoic acid were added to the system supported complete spermatogenesis in zebrafish testis culture **(Leal *et al*., 2009).** Primary cultures of tilapia testis cells were maintained for up to 16 days in the presence of 11-ketotestosterone (KT), insulin-like growth factor I (IGF) and/or human chorionic gonadotropin (hCG) (**Tokalov and Gutzeit, 2005**). The primary culture medium was supplemented with 20% of FBS and bFGF for testis primary cultured of tongue sole while FBS reduced to 15% after 45 passages (**Zhang *et al*., 2011**).

In the present investigations, the primary cultures from different organs of *Oreochromis niloticus* were incubated at 25^{o}C. This is in agreement with **Wen *et al*. (2014)** who incubated tilapia brain culture at 25^{o}C. As well as, **Khamiss and Hashem (2012)** reported maximal growth at 25° C for *in vitro* propagation of selected Nile tilapia ovarian culture.

The present results showed that brain primary cultures grew slowly. This comes in agreement with **Servili *et al*. (2008)** when they passaged cellular preparations of brains of adult sea bass within 2 or 3 weeks of growth. Also, **Wen *et al*. (2008)** passaged adult tilapia brain tissue 6 weeks after the culture was initiated. Besides, **Hasoon *et al*. (2011)** obtained monolayer from the brain tissue of Asian sea bass during the first 3 weeks. As well as the results indicated that brain primary culture from *Oreochromis niloticus* exhibited both epithelial-like and fibroblast-like cells. Fibroblast-like cells were the most common cells in the primary culture. This comes in agreement with **Servili *et al.* (2008)** and **Wen *et al*. (2008)**. Epitheloid cells also were observed in the primary brain cultures as found by **Wen *et al*. (2008).** As well as, **Hasoon *et al*. (2011)** reported that the initial subcultures of cell line from brain tissue of Asian sea bass showed both epithelial-like and fibroblast-like cells.

Meanwhile, ovary primary culture showed epithelial-like cells during the early days of culture, while fibroblast-like cells observed later in agreement with **Khamiss and Hashem (2012)** and **Wei *et al*. (2014).**

In the present work, heterogenous testis primary culture was capable of maintaining organ function. Testis primary culture from *Oreochromis niloticus* showed different types of cells involved in spermatogenesis. Spermatozoa were regularly removed by media changing. Motile spermatozoa were continuously produced *in vitro*. Spermatogenesis exhibited during primary culture and subculture. Certain steps of spermatogenesis have been reported under organ culture conditions in Salmonid fish (**Amer *et al*., 2001**). Spermatogenesis is a developmental process that is amenable to *in vitro* studies in mammals (**Jeyaraj *et al*., 2002**) and fish (**Sakai, 2002; Song and Gutzeit, 2003; Hong *et al*., 2004 and Zhang *et al*., 2011**). Although *in vitro* spermatogenesis in mammals has so far ended up with the spermatid stage (**Feng *et al*., 2002**), spermatogenesis in lower vertebrates can proceed fully *in vitro*. The cystic mode of spermatogenesis was characterized by a less complex Sertoli-germ cell interaction may contribute to allow *ex vivo* spermatogenesis in testis organ culture. Specifically, sperm production *in vitro* has been reported in primary testicular cultures from several distantly related fish species. In zebrafish, dissociated testicular cells during 15 days of co-culture on a feeder layer of Sertoli-like cells gave rise to fertile sperm (**Sakai, 2002 and Kawasaki *et al*., 2012**). Remarkably, **Hong *et al*. (2004)** have found that spermatogonia from the medaka testis can develop into a stable stem cell line, which is capable of full recapitulation of spermatogenesis *in vitro*, including differentiation through meiosis till generating test-tube sperm. Monolayer of primary cultured testis of tongue sole was obtained after 2 weeks (**Zhang *et al*., 2011**).

Finally, the establishment of a cell culture system from Egyptian *Oreochromis niloticus* is a must to make use as a powerful tool in various applications concerning fish diseases diagnosis and treatment, pharmacology, toxicology, carcinogenesis, functional genomics studies, in addition to lacking *in vivo* complexities.

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