Application of Cell Culture From Hybrid Catfish (*Clarias gariepinus X Clarias macrocephalus)* in Screening Toxicity of Chemicals

Pornsawan Visoottiviseth[a,*] and Nantawan Chanwanna[b]

[a] Biology Department, Faculty of Science, Mahidol University, Rama VI Road, Bangkok 10400, Thailand.

[b] Chulabhorn Research Institute, Bangkhen, Bangkok 10200, Thailand.

* Corresponding author, E-mail: scpvi@mahidol.ac.th

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ABSTRACT Three types of primary cell cultures were established from organs of hybrid catfish (*Clarias gariepinus x Clarias macrocephalus*) namely, caudal trunk, gill and liver. Cell cultures from caudal trunk and gill were composed of mixed cells of fibroblasts and epitheloids, while cell cultures from the liver were composed of fibroblastic cells only. All cultures grew well in either Eagle's essential medium (MEM) supplemented with 15-20% fetal calf serum (FCS) or Leibovitz's L-15 medium supplemented with 5-20% FCS. The optimum growth temperature was 27°C in an atmosphere either with or without 5% carbon dioxide. The modal chromosome number as determined from the 19th passage of caudal trunk cell culture was 56. The cell cultures could be stored at 4°C for at least 2 months. Experiments were then performed to investigate the feasibility of the established catfish cell culture for screening toxicity of organotin pesticides (triphenyltin hydroxide, TPTH). The results showed that the culture was highly suitable for the purpose. The effect of TPTH on cell culture was recognized at the concentration of 3 mg l^{-1} when cell detachment was used as the criterion of the test. Established cell culture from hybrid catfish has great potential as a powerful tool in tropical aquatic toxicology as a method of screening chemicals prior to *in vivo* testing.

KEYWORDS: toxicity, organotin, triphenyltin, cell culture; catfish, *Clarias* spp.

INTRODUCTION

The initiation of fish cell cultures has been reviewed by Wolf and Quimby.[1] The first marine fish cell line "Gf-1" was established from grunt fish by Clen et al.[2] Wolf and Quimby[3] could develop the first freshwater fish cell line "RTG-2" from rainbow trout gonads. Subsequently a variety of other fish cell lines have been developed from economically important fish including *Tilapia* hybrid,[4] the rainbow trout,[5,6] eel,[4] Channel catfish, tench, Atlantic salmon,[7] okanee salmon,[8] and grass carp (*Ctenopharyngodon idella*).[9]

Fish tissue culture has been used as an important tool in research in the area of aquatic toxicology. Nowadays there is a large number of existing chemicals in commerce, between 700 and 3,000 new chemicals produced annually. Irrespective of the use of these compounds, they will eventually contaminate the aquatic ecosystem. Some of them may be highly toxic to aquatic living species especially those of commercial importance ie, fish, prawns and shellfish. Clean water, free from toxic chemicals, is needed for the aqua-industries. The aqua-industry of hybrid catfish (*Clarias gariepinus* × *Clarias macrocephalus*) is being promoted in Thailand as another source of protein food for the population. Thus cell cultures from hybrid catfish are established in this study for application in the determination of chemical toxicity to the hybrid catfish.

The application of cell and tissue culture systems to the study of toxic substances is not a new concept. Toxicologists have found that cells from different species respond differently to the same chemical.[10] This finding indicated that species differences are retained in *in vitro* systems, thereby making the study of basic toxicological responses possible in systems less complex than the intact animals. Toxicity tests with whole animals are frequently used to investigate the effect of environmental contaminants on aquatic species,[11] but such tests are limited by the number of animals which can be economically and conveniently studied, by problems in obtaining organisms of known background and parentage, as well as by the difficulties of extrapolating from one species to another. One way to overcome these difficulties is to use cultured cells, rather than whole animals, of different species for screening a large number of samples, and as a means of determining

the specific activity of a toxicant on the cells of an organism. Since *in vitro* cells do not possess multiple defense mechanisms, they are frequently more sensitive to the basic cytotoxic effects of a chemical and respond at concentrations at least as low as those that would affect the whole organism[12]. Since the earliest report of successful *in vitro* cultivation[3], fish cells have been used to study both infectious diseases and the toxicity of contaminants in the aquatic ecosystem.[13] It has many advantages, including low cost, rapidity, versatility, sensitivity and reproducibility. The cell line RTG-2, which originated from rainbow trout gonadal cells (*Salmo giardneri*) obtained by primary cultivation of pooled normal gonads of fingering fish, has been used for the evaluation of the toxicity of aquatic pollutants.[14,15] The RTG-2 cell line showed excellent sensitivity for detecting toxicity of aquatic pollutants as compared to *in vivo* classical bioassays. However, the RTG-2 cell line may not be appropriate for use in areas with a tropical climate, such as Thailand. Cell cultures originated from organs of local fish may be more suitable for screening tests of chemical toxicity in Thailand. Thus, cell cultures of hybrid catfish will be established and used for determination of toxicity of the triphenyltin hydroxide in this study.

Materials and Methods

Cell culture establishment

Hybrid catfish (*Clarias gariepinus* × *Clarias macrocephalus*) aged 1-2 weeks (1.5-2.5 cm body length) were used for caudal trunk cultivation. One month old fish (6.7-8.5 cm body length) was used for organ (gill and liver) cultivation.

The technique used in this study was modified from that of Wolf and Quimby.[3] Three techniques for tissue dispersion were tested, ie, mechanical dispersion, trypsinization and dispersion with collagenase enzyme. Mechanical dispersion was done by mechanically pressing tissue fragments with the piston of a disposable plastic syringe. Then, the cells were dispersed by pipetting the suspension through a sieve. Trypsinization of tissue fragments was done by immersing tissue fragments in 0.25% trypsin-versene pH 7.4 for 15 min and constantly pressing with the piston. For dispersion with collagenase enzyme, the technique was the same as trypsinization but 186 units ml^{-1} of collagenase were used instead of trypsin.

Testing for appropriate conditions for cell culture

Following tissue dispersion, the cells were then collected and cultured. Various parameters which had impacts on growth of cell culture establishment were determined namely, seeding volume and supplements; types of culture medium; incubation temperature; serum concentration; and carbon dioxide requirement.

Four types of commercial culture medium, ie, Leibovitz's medium (L-15), Eagle's minimum essential medium (MEM), Dulbecco's modified Eagle's medium (DMEM) and medium 199 (M199) were screened. With each culture medium, a different percentage of fetal calf serum (FCS) ie, 5%, 10%, 15% and 20%, was separately added into culture flasks. One hundred units each of penicillin and streptomycin, and 0.5 µg ml^{-1} of fungizone were added into each flask for protection from contamination by bacteria and fungi. Growth was evaluated at various incubation temperatures, ie, 25°C, 27°C, 30°C and 37°C by comparing the number of viable cells at alternate days for a total of one week. The CO_2 requirement for cell growth was also evaluated from the number of "viable cells".

Preservation of Cell Culture

Trypsinized cultured cells at a concentration of 10^6-10^7 cells ml^{-1} in medium containing 5% FCS were kept at 4°C in a refrigerator. The viability of cells was tested every month against those that were stored in growth medium containing 20% FCS and 10% dimethylsulfoxide (DMSO) at -70°C.

Karyology of Catfish Cultured Cell

Chromosome analysis was carried out from the 19^{th} passage caudal trunk cell culture by the method of Schroy and Todd[16] with some modifications, including pretreatment of the cells with 10^{-4} M sterile colchicine for 6 h at temperature 27°C.

Toxicity test of triphenyltin hydroxide

The effect of TPTH on chromosomes was also studied. The caudal trunk cell cultures of hybrid catfish were trypsinized. The cells were washed from the culture vessels and L-15 medium, supplemented with 20% fetal calf, serum was added. The cells were then distributed, grown on slides in sterile petridishes, and incubated at 27°C overnight. Then aliquots of 1 mg l^{-1} of TPTH stock solution were added to the culture medium. The final concentration of TPTH in each culture medium was 1 µg l^{-1}, 3 µg l^{-1}, 5 µg l^{-1} and 10 µg l^{-1}, respectively. The petridishes were reincubated at 27°C for 2 hours. After that the slides of the established cell culture were observed for the effect of TPTH on cell detachment. The cells

retained on the slides were then stained for chromosomal analysis by Schroy and Todd's methods[16] and chromosomal abnormalities were observed by comparing them with the untreated cells.

RESULTS

Cell culture establishment

Cells from hybrid catfish attached to culture flasks within 1-3 days after seeding. A monolayer was formed within two weeks. Among the three techniques for cell dissociation tested, cell dispersion with 186 units ml^{-1} collagenase was the most effective. This method yielded approximately 5.0 x 10^5 viable cells g^{-1} (Table 1). The effect of seeding volume and supplements on cell attachment is shown in Table 2. Pre-coating of culture flasks with fetal calf serum or 10 mg ml^{-1} concanavalin A did not enhance the cell attachment. But growth medium supplemented with 0.5 ng ml^{-1} epidermal growth factor (EGF) showed positive influence on the attachment and spreading of cultured cells.

Cultures from caudal trunks and gills of the hybrid catfish gave the mixed cultures of epitheloid and fibroblast cells, whilst culture from liver contained only fibroblast cells (Figs 1-3).

Table 1. Comparison of cell dissociation method on viability and plating efficiency of cells.

Method	Yield (Viable cells*g^{-1})	Plating efficiency (days before cell attachment)
Mechanical	2.83×10^5	5 -14
0.25% Trypsinin versene	0.86×10^5	2 - 3
186 units/ml collagenase	5.00×10^5	2 - 3

* The number is the average of 10 replicates

Table 2. Effect of seeding volume and supplements on cell attachment.

Pre-coating	Seeding volume (ml)	Supplement	Days for cell attachment
No	2.5-5.0	10 % FCS	2-5
No	0.5-1.0	10 % FCS	1-4
No	2.5-5.0	20% FCS	2-4
No	0.5-1.0	20 % FCS	1-3
FCS	2.5-5.0	10 % FCS	2-5
No	0.5-1.0	10 % FCS+ con.A	1-4
No	0.5-1.0	10 % FCS + EGF	1-3

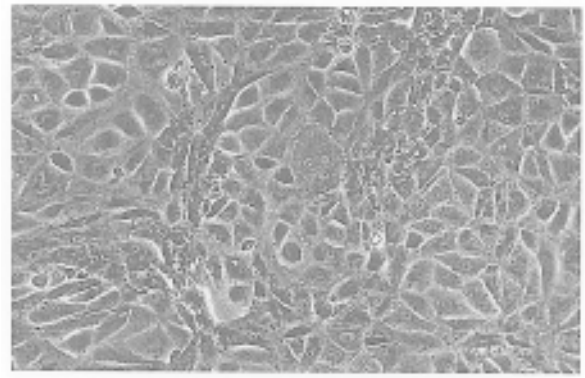

Fig 1. Cell culture originated from caudal trunks of hybrid catfish (passage 19th) commposes of the mixed cultures of epitheloid and fibroblast cells (Magnification 500X).

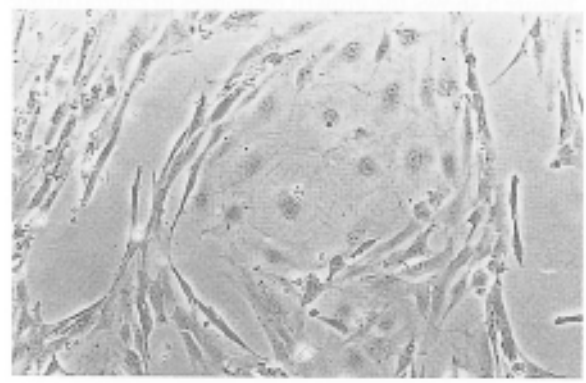

Fig 2. Cell culture originated from gills of hybrid catfish (passage 19th) commposes of the mixed cultures of epitheloid and fibroblast cells (Magnification 500X).

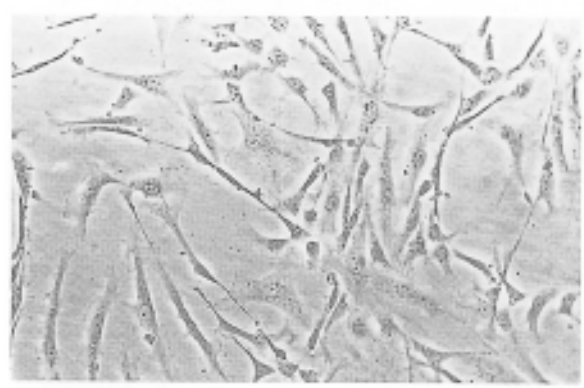

Fig 3. Cell culture originated from liver of hybrid catfish (passage 19th) commposes of the fibroblast cells (Magnification 500X).

Appropriate conditions for cell growth

Optimum growth temperature: Caudal trunk cell culture in L-15 medium supplemented with 10% FCS, 100 units ml^{-1} penicillin, 100 mg ml^{-1} streptomycin and 0.5 mg ml^{-1} fungizone was used for determining the optimal temperature for cell growth. The incubation temperature at 27°C gave a significantly higher growth rate of cells than at other incubation temperatures tested (Fig 4). At a temperature of 30°C and above, the cultured cells detached from the surfaces of culture flasks and finally degenerated.

Type of culture medium and percentage of fetal calf serum: The growth responses of caudal trunk cells in 4 types of media and 4 concentrations of fetal calf serum in each medium were evaluated. Figure 5 shows the growth responses of caudal trunk cells in MEM, DMEM, M 199, and L-15, repectively.

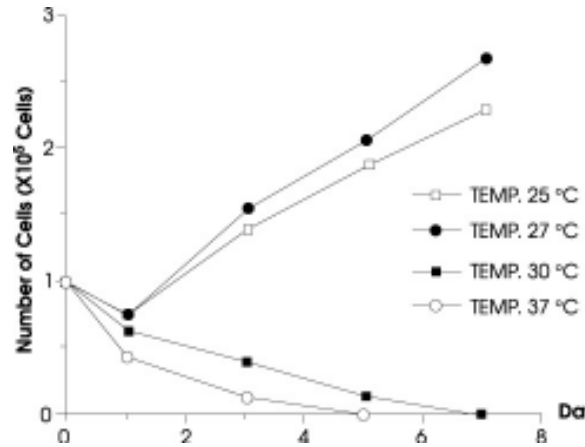

Fig 4. The effect of incubation temperature on growth of hybrid catfish caudal trunk cell culture. The plotted values are mean of 4 replicates.

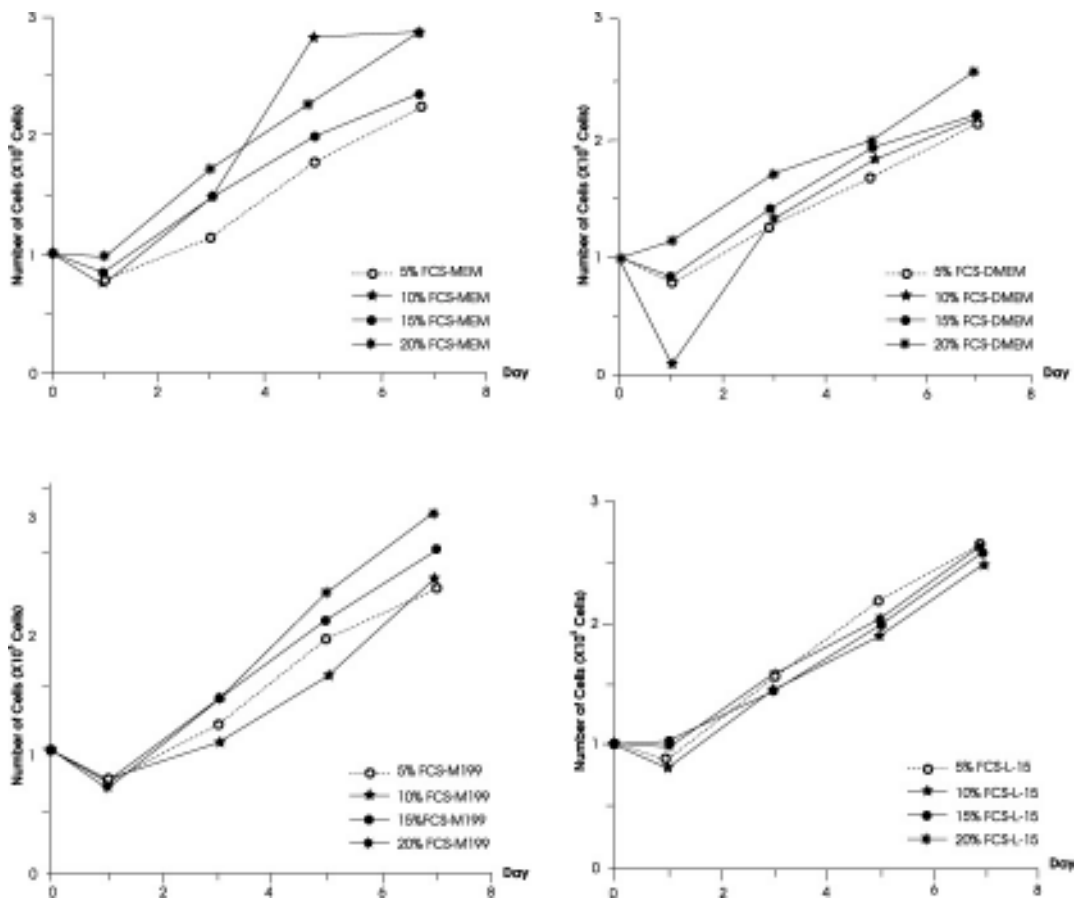

Fig 5. Growth curves of caudal trunk cells in commercially available media tested:
(A) Eagle's minimal essential medium (MEM).
(B) Dulbecco's modified Eagle'smedium (DMEM)
(C) M 199 medium
(D) Leibovitz's L-15 medium
The plotted values are averages of 5 replicates.

The effects of percentage of FCS in growth medium, ie, 5%, 10%, 15% , and 20% on growth of the caudal trunk cell culture were also tested. From the results of both experiments, L-15 medium supplemented with 5-10% FCS or MEM medium supplemented with 15-20% FCS were selected for use in later experiments.

Carbon dioxide requirement: Caudal trunk cells in L-15 medium supplemented with 10% FCS, 100 units ml^{-1} penicillin, 100 mg ml^{-1} streptomycin and 0.5 µg ml^{-1} fungizone were used for determining the effect of carbon dioxide on cell growth. It was found that carbon dioxide gas (5%) had no effect on cell growth, no matter which type of commercial medium was used. Thus carbon dioxide was omitted from the incubating chamber in later experiments.

Karyotype of cultured cells

The diploid chromosome number of cultured cells as determined from the catfish's caudal trunk cells varied from 49-60 with a modal chromosome number of 56. The karyotype showed that most of the chromosomes were in the metacentric and submetacentric groups (Fig 6).

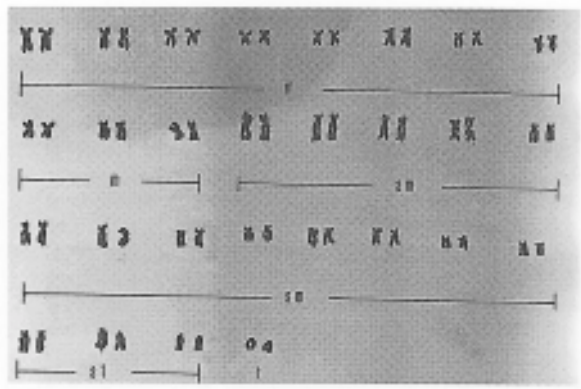

Fig 6. Karyotype of cells derived from the caudal trunk's cell culture.
m = metacentric sm = submetacentric
st = subtelocentric t = telocentric

Toxicity test of triphenyltin hydroxide (TPTH) using newly established hybrid catfish caudal trunk cell cultures

The criteria used for establishing the toxicity of the chemical in the established caudal trunk cell culture in this study were cell detachment and genotoxicity (chromosome abnormality).

1. Cell detachment from substrate

The effect of TPTH on cell detachment at four different concentrations of TPTH, ie, 1 µg l^{-1}, 3 µg l^{-1}, 5 µg l^{-1} and 10 µg l^{-1}, was observed by comparison with untreated cells (Fig 7). It was found that cell detachment was first observed at 3 µg l^{-1} treated cells. The effect of cell detachment was more pronounced at higher concentrations of TPTH, ie, 5 µg l^{-1} and 10 µg l^{-1}, in which the cells hardly attached to the surfaces of culture slides.

2. Genotoxicity of TPTH

The effect of TPTH on chromosome abnormality was also studied. The chromosome number of the TPTH- treated cells (1 µg l^{-1}, 3 µg l^{-1}, 5 µg l^{-1} and 10 µg l^{-1}) was compared with the untreated cells. The results (Fig 8) show that the chemical at the four TPTH concentrations tested exerted no effect on chromosome number. In addition, abnormal chromosomes were not observed in the TPTH-treated cells.

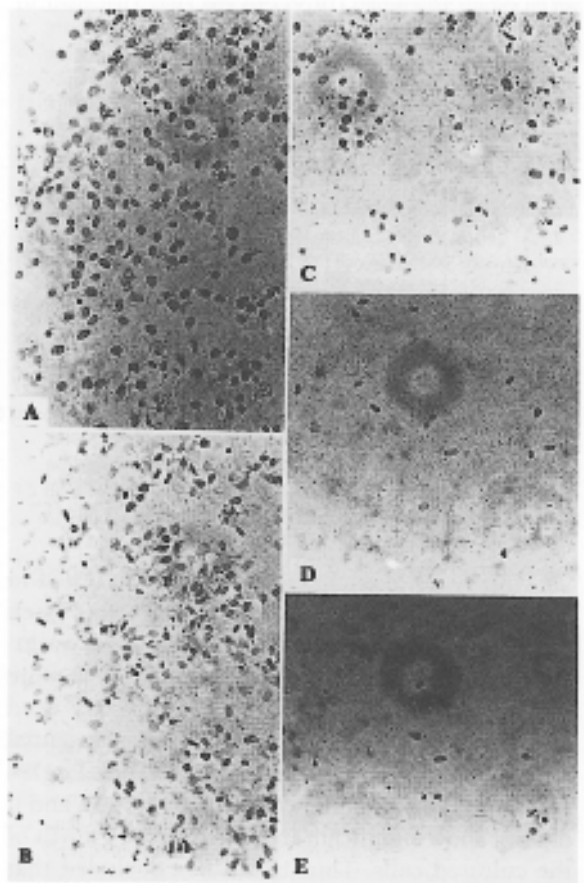

Fig 7. Caudal trunk cells retained on glass slides : (Magnification 500 X)
(A) untreated cells
(B) 1 ppb TPTH treated cells
(C) 3 ppb TPTH treated cells
(D) 5 ppb TPTH treated cells
(E) 10 ppb TPTH treated cells

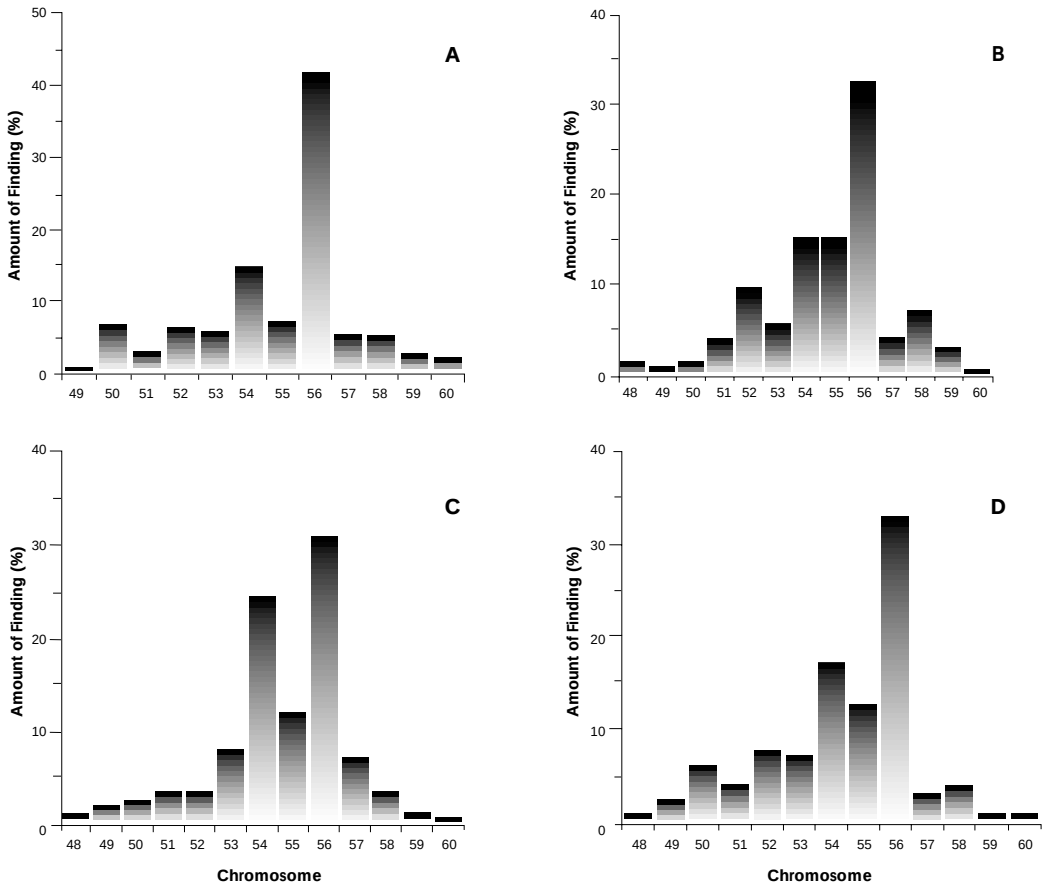

Fig 8. Chromosome counts from (A) 1 ppb TPTH treated cells, (B) 3 ppb TPTH treated cells, (C) 5 ppb TPTH treated cells, (D) 10 ppb TPTH treated cells.

Discussion

Four types of commercial media were tested in this study. Leibovitz's L-15 medium has been recommended for growing cell cultures from warm-water fish species.[4] However, this medium is more expensive than the other three commonly used media for cell culturing, ie, Earle's minimal essential medium (MEM), Dulbecco's modified Eagle's medium (DMEM) and medium 199 (M199), and it did not show significant improvement of growth of the cultured cells. Thus it can be concluded that cultured cells from hybrid catfish can be successfully grown in any of the 4 commercial cell culture media tested. This result corresponds with those of other investigators.[17,18] The concentration of fetal calf serum at 5% can support good growth of cell culture as at 20%. These cell cultures grow well at a temperature of 27°C without adding 5% CO_2. This finding leads to a big saving on the cost of maintaining these cell cultures in the laboratory. Moreover, as the passage number increased the growth rate did not decrease. The present study also indicated that catfish cultured cells can be stored at a refrigerator temperature (4°C) without a change of medium for at least 2 months. But if the culture is kept at -70°C in a freezer, it can be kept longer than that. This is in agreement with the report of Nicholson.[18]

The modal chromosome number or the chromosome number which most of the cells contained, as determined from the 19th passage caudal trunk cultured cells, was 56. This is similar to the report of Supamart and Donskul.[19] However, the modal chromosome number obtained in this study was contrary to the result of Sungpetch[20] who determined the diploid chromosome number from *in vivo* study of the hybrid catfish.

Hybrid catfish cell culture was used for screening TPTH toxicity by using two criteria, ie, cell detachment and genotoxicity. In this study, cell detachment was determined to be an important

parameter for the TPTH toxicity test. The results correspond with those of other investigators[15, 21] who found that changes in the ability of cells to attach to a substratum can be used as an indicator for cytotoxicity assays. Although the chromosomal abnormality can be observed in TPTH treated cells, the frequency of this phenomenon is quite low (1%) and is not dose-dependent. Thus, it should not be used as a criterion for TPTH toxicity test. Babich et al [22] stated that genotoxicity of fish was difficult to discern or interpret because of the small size and large number of chromosomes in the cells. TPTH has no effect on chromosome number. The modal chromosome number of cells from hybrid catfish cell cultures was 56 whether it was treated or untreated with TPTH.

Other cell lines which have been used in chemical toxicity tests, including rainbow trout gonad (RTG-2, fibroblast cells), fathead minnow (FHM), bluegreen sunfish fibroblast (BF-2) and brown bullhead catfish fibroblast (BB), are developed from cold-water fish species. According to Babich and Borenfreund,[21] temperature exerts its effect on cytotoxic responses of the fish cells to the tested xenobiotic. Therefore, cells that originate from cold-water species may give different responses to those that originate from warm-water fish species. For this reason, hybrid catfish cell culture may be more suitable for toxicity tests of xenobiotics in tropical areas than cell lines that originate from cold-water fish species.

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References

1. Wolf K and Quimby MC (1969) Fish cell and tissue culture. In: *Fish Physiology.* (Edited by Hoar WS and Randall DJ), Academic Press. New York
2. Clen LW, Moewus L and Sigel MM (1961) Studies with cells from marine fish in tissue culture. *Proc Soc Expy Biol Med* 108, 762-6.
3. Wolf K and Quimby MC (1962) Established eurythermic line of fish cells *in vitro*. *Science* 135, 1065-6.
4. Chen SN and Kou GH (1987) Establishment, characterization and application of 14 cell lines from warm water fish. Invertebrate and fish tissue culture. *Proceeding of the 7th International Conference on Invertebrate and Fish Tissue Culture. Japan.* pp 218-27.
5. Li MF and Stewart JE (1965) A quantitative study of the effects of naturally occurring supplements on the growth of rainbow trout (*Salmo gairdneri*) gonadal cells. *Can J Microbiol* 11, 9-14.
6. Li MF and Jordan C (1969) Factors affecting rainbow trout ovary cells cultivated *in vitro*. *J Fish Res Bd Canada* 26, 461-3.
7. Wolf K and Mann JA (1980) Poikelotherm vertebrate cell lines and viruses: a current listing for fishes. *In Vitro* 16, 168-79.
8. Lennan CN, Winton JR and Fryer JL(1984) Fish cell lines: Establishment and characterization of nine cell lines from salmonids. *In Vitro* 20, 671-6.
9. Lu Y, Lannan CH, Rohovec JS and Fryer JR (1990) Fish cell lines: establishment and characterization of three new cell lines from grass carp (*Ctenopharyngodon idella*). *In Vitro Cell Dev Biol* 26, 69-81.
10. Diamond L and Clark HF (1970) Comparative studies on the interaction of benzo (a) pyrene with cells derived from poikelothermic and homeothermic vertebrates I. Metabolism of benzo (a) pyrene. *J Natl Cancer Inst* 45, 1005-10.
11. Buikema AL and Cairns JC (1980) Aquatic invertebrate bioassays (Edited by American Society of Testing and Materials). *ASTM Special Technical Publication no* 715. 209 pp.
12. Ekwell B (1983) Screening of toxic compound in mammalion cell cultures. *In* : *Cellular systems for toxicity testing* (Edited by Williams GM, Dunkel VC, and Ray VA), New York Academy of Science. pp 14-7.
13. Kocan RM, Sabo ML and Landoft ML (1985) Cytotoxicity/ genotoxicity: the application of cell culture technique to the measurement of marine sediment pollution. *Aqua Tox* 6,165-77.
14. Marion M and Denizeau F (1983) Rainbow trout and human cells in culture for the evaluation of toxicity of aquatic pollutants: A study with cadmium. *Aqua Tox* 3, 329-43.
15. Bols N, Boliska SA, Dixon DC, Hodson PV and Kaiser KLE (1985) The use of fish cell culture as an indication of contaminant toxicity test. *Aqua Tox* 6, 147-55.
16. Schroy CB and Todd P (1976) Methods for preparing chromosome spread of glass attached cultured animal cells. In: *TCA Manual* 2 (1), pp 287-9.
17. Wolf K and Ahne W (1982) Fish cell culture. In : *Advances in Cell Culture* (Edited by Maramorosch K), Vol. 2, Academic Press, New York, pp. 305-28.
18. Nicholson BL (1988) Fish cell culture: an update. *Adv Cell Cult* 7, 1-18
19. Supamart K and Donskul R (1991) *Thai J Sci* 35, 388-93.
20. Sungpetch A (1993) Effects of triphenyltin hydroxide on the anatomy and chromosomes of catfish. *M Sc Thesis,* Faculty of Graduate Studies, Mahidol University
21. Babich H and Borenfreund E (1987) Fathead minnow FHM cells for use in *in vitro* cytotoxicity assay of aquatic pollutants. *Ecotox Envi Safety* 14, 78-87.
22. Babich H, Rosenberg DW and Borenfreud E (1991) *In vitro* cytotoxicity studies with the fish hepatonema cell line, PLHC (*Poeciliopsis lucida*). *Ecotox Envi Safety* 21, 327-36.