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A newly established cell culture from hybrid catfish (*Clarias gariepinus* × *Clarias macrocephalus*) for screening toxicity of triphenyltin hydroxide

Pornsawan Visoottiviseth[1]* and Nantawan Chanwanna[2]
[1]Biology Department, Faculty of Science, Mahidol University, Rama VI Road, Bangkok 10400, Thailand
[2]Chulabhorn Research Institute, Bangkhen, Bangkok 10200, Thailand

Three types of primary cell culture can be established from organs of hybrid catfish (*Clarias gariepinus* × *Clarias macrocephalus*) namely, caudal trunk, gill and liver. Cell cultures from caudal trunk and gill are composed of mixed cells of fibroblasts and epitheloids, whenever cell cultures from the liver are composed of fibroblastic cells only. All cultures grow well in either Eagle's minimum 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 is 27 °C in an atmosphere either with or without 5% carbon dioxide. The diploid chromosome number as determined from the 19th passage of the caudal trunk cell culture is 56. The cell cultures can be stored at 4 °C for at least 2 months. Experiments were then performed to investigate the possibility of using the established catfish cell culture for screening toxicity of organotin pesticides (triphenyltin hydroxide, TPTH). The results show that the culture is highly suitable for the purpose. The effect of TPTH on cell culture is recognized at a concentration of 3 μm l^{-1} when cell detachment is used as the test criterion. Established cell culture from hybrid catfish has a great potential as a powerful tool in tropical aquatic toxicology as a method of screening chemicals prior to *in vivo* testing. Copyright © 2001 John Wiley & Sons, Ltd.

* Correspondence to: Pornsawan Visoottiviseth, Biology Department, Faculty of Science, Mahidol University, Rama VI Road, Bangkok 10400, Thailand.
E-mail: scpvi@mahidol.ac.th
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INTRODUCTION

The initiation of fish cell cultures has been reviewed by Wolf and Quimby[1] The first marine fish cell line 'Gf-l' was established from grunt fish by Clen *et al*[2] Wolf and Quimby[3] developed 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] rainbow trout (*Salmo gairdneri*),[5,6] eel (*Anguilla anguilla*) channel catfish (*Ictalurus punctatus*), tench (*Tinca tinca*), Atlantic salmon (*Salmo sarla*), kokanee salmon (*Onchorhynchus nerka*) 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 exiting chemicals used in commerce, with between 700 and 3000 new chemicals being produced annually. Irrespective of how these compounds are used some may eventually contaminate the aquatic ecosystem. Some of them may be highly toxic to aquatic living species, especially those of commercial importance, i.e. fish, prawns and shellfish. Clean water, free from toxic chemicals is needed for the aqua-farms. 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 the hybrid catfish are studied here for application in the determination of chemical toxicity to hybrid catfish.

The application of cell and tissue culture systems to the study of toxic substances is not a new concept. Toxicologists found that cells from different species may respond differently to the same chemical.[10] The results indicate that species differences are retained in the *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 that can be economically and conveniently studied, by problems in obtaining organisms of known background and parentage and also 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, 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 defence 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 reports of successful *in vitro* cultivation,[3] fish cells have been used to study both infection diseases and the toxicity of contaminants in the aquatic ecosystem.[13] They have many advantages, including low cost, rapidity, versatility, sensitivity and reproducibility. The cell line RTG-2 originating from rainbow trout (*Salmo gairdneri*), gonadal cells obtained primary cultivation of pooled normal gonad of fingering fish, has been used for the evaluation of toxicity of aquatic pollutants.[14,15] The RTG-2 cell line showed excellent sensitivity for detecting toxicity of aquatic pollutants compared with *in vivo* classical bioassay. However, the RTG-2 cell line may not be appropriate for use in tropical climates, such as found in Thailand. Cell cultures originating from organs of local fish may be more suitable for screening tests on chemical toxicity in Thailand. Triphenyltin hydroxide (TPTH) is of special interest to us, since it is a pesticide for rice, a crop that is widely grown in Thailand. In order to protect both rice and the hybrid catfish farms, cell cultures of the hybrid catfish and used for determination of toxicity of the TPTH 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) are used for caudal trunk cultivation, and 1 month old fish (6.7–8.5/cm body length) are 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, i.e. mechanical dispersion, trypsinization and dispersion with collagenase enzyme. The mechanical dispersion is 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 continuously pressing with the piston. For dispersion with collagenase enzyme, the technique was 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 cell were then collected and cultured. Various parameter when have an impact on the growth of cell culture 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, i.e. 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), i.e., 5, 10, 15, and 20% was added separately into the culture flasks. Then 100 units of both penicillin and streptomycin, and 0.5 μm ml^{-1} of fungizone were added into each flask for protection from contamination by bacteria and fungi. Growth was evaluated at various incubation temperatures, i.e 25, 27, 30 and 37 °C, by comparing the number of viable cells at alternate days for a total of 1 week. The carbon dioxide requirement for cell growth was also evaluated from the number of cell viability.

Table 1 Comparison of cell dissociation method on viability and plating efficiency of cells (each number is the average of ten replicates)

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

Preservation of cell culture

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

Karyology of catfish cultured cell

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

Toxicity test of TPTH

The criteria for toxicity testing of the chemical by the established caudal trunk cell culture in this study are cell detachment and genotoxicity (chromosome abnormality). Only the caudal trunk cell culture was used in this experiment because of the ease of maintaining the culture are long person in the laboratory.

The caudal trunk cell cultures of hybrid catfish were trypsinized. The cells were washed off from the culture vessels and L-15 medium, supplemented with 20% FCS, was added. The cells were then distributed, grown on the slides in sterile Petridishes, and incubated at 27 °C overnight. Then aliquots of 1 mg $^{-1}$ of TPTH stock solution were added to the culture medium. The final concentrations of TPTH in each culture medium were 1, 3, 5 or 10 μg l^{-1}. The Petri dishes was reincubated at 27 °C for 2 h. After that the slides of the established cell culture was observed for the effects of TPTH on cell detachment. The cells retained on the slides were then stained for chromosomal analysis by the Schroy and Todd method[16] and chromosomal abnormalities were observed by comparing them with the untreated cells.

RESULTS

Cell culture establishment

Cells from the hybrid catfish attach to culture flasks within 1–3 days after seeding. A monolayer was formed within 2 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 5.0×10^5 viable cells per gram (Table 1). The effect of seeding volume and supplements on cell attachment is shown in Table 2. Pre-coating of culture flasks with FCS or 10 μg ml^{-1} concanavalin

Table 2 Effect of seeding volume and supplements on cell attachment (each number is the average of ten replicates)

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

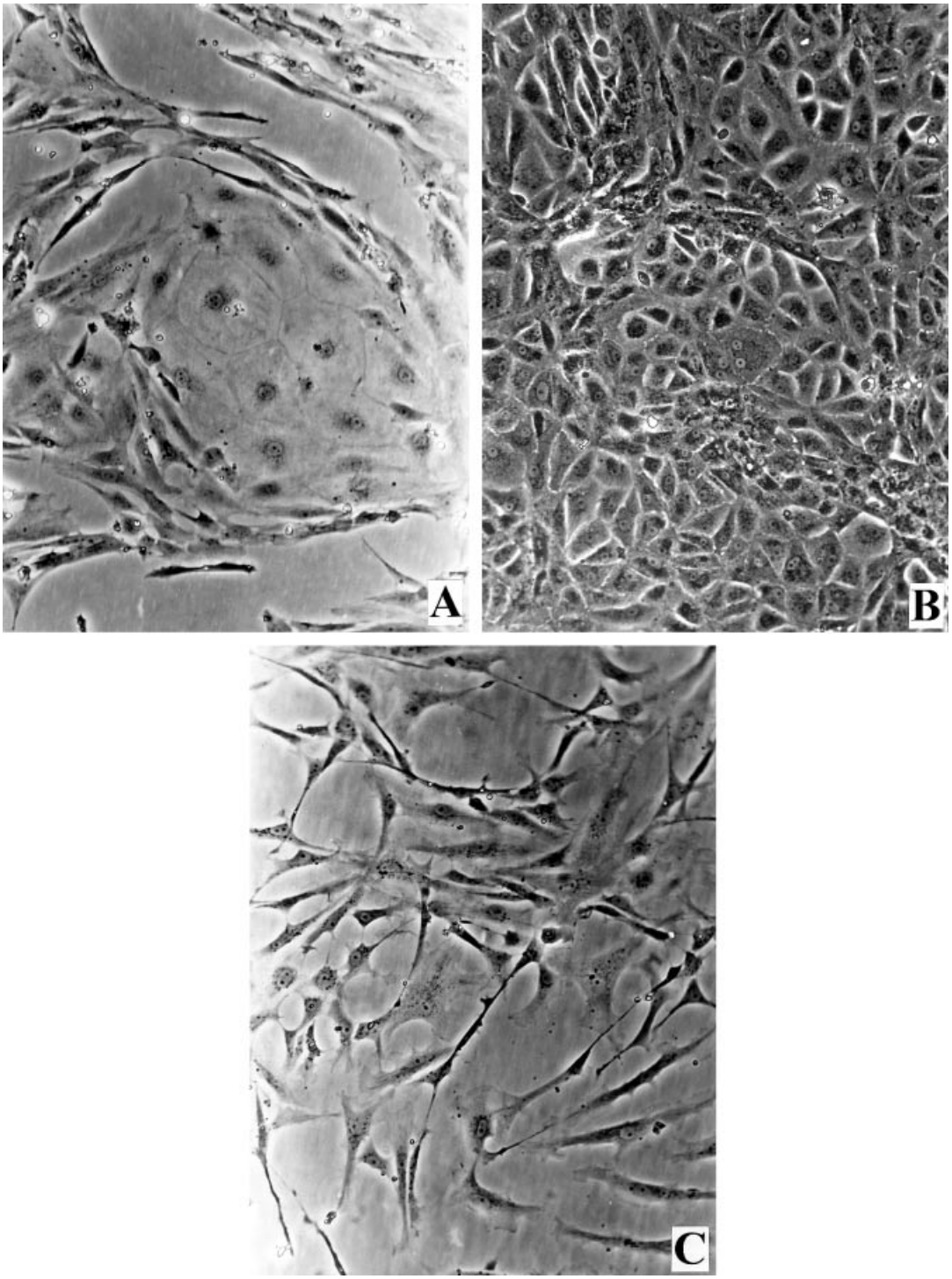

Figure 1 Cell culture originating from various organs of hybrid catfish 19th passage: (A) from caudal trunk; culture is composed of epitheloid and fibroblast cells; (B) from gills; culture is composed of epitheloid and fibroblast cells; (C) from liver; culture is composed of fibroblast cells magnification 225×.

Table 3 Effect of temperature on growth of caudal trunk cells of hybrid catfish (each number is the average of four replicates)

Temperature (° C)	Cell Growth (Number of cells × 10^5)				
	Day 0	Day 1	Day 3	Day 5	Day 7
25	1.00	0.75 ± 0.09	1.39 ± 0.17	1.88 ± 0.18	2.33 ± 0.25
27	1.00	0.75 ± 0.09	1.55 ± 0.11	2.08 ± 0.17	2.71 ± 0.18
30	1.00	0.63 ± 0.08	0.40 ± 0.06	0.15 ± 0.04	0
37	1.00	0.44 ± 0.09	0.13 ± 0.04	0	0

A does not enhance the cell attachment. Growth medium supplemented with 0.5 ng ml^{-1} epidermal growth factor (EGF) showed a positive influence on the attachment and spreading of cultured cells.

Cultures from caudal trunks and gills of the hybrid catfish give mixed cultures of epitheloid and fibroblast cells, whilst culture from liver contains only fibroblast cells (Fig. 1A–C)

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 μg ml^{-1} streptomycin and 0.5 μg ml^{-1} fungizone was used for determining the optimal temperature for cell growth. An incubation temperature of 27 °C gave a significantly higher growth rate of cells than at other incubation temperatures tested (Table 3 Fig. 2). At a temperature of 30 °C and above, the cultured cells detach from the surfaces of culture flasks and finally degenerate.

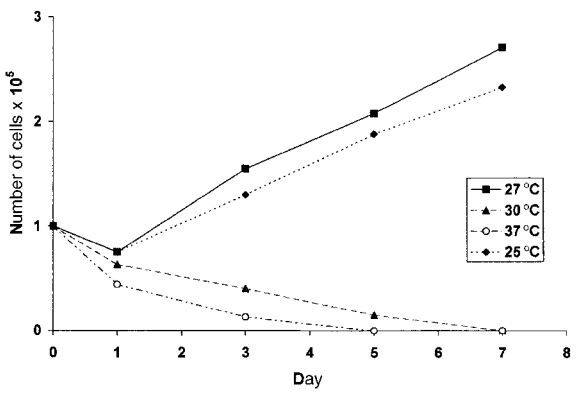

Figure 2 The effect of incubation temperature on growth of hybrid catfish caudal trunk cell culture.

Type of culture medium and percentage of FCS

The growth responses of caudal trunk cells in four types of medium and four concentration of FCS in each medium were evaluated. Figure 3A–D shows the effects of 5%, 10%, 15% , and 20% FCS on growth of caudal trunk cells of the hybrid catfish in MEM, DMEM, M199 and L-15. From the results of this experiment, L-15 medium supplemented with 5–10% FCS or MEM medium supplemented with 15–20% FCS were selected for subsequent experiments.

Carbon dioxide requirement

Caudal trunk cells in L-15 medium supplemented with 10% FCS, 100 units ml^{-1} penicillin, 100 μg ml^{-1} streptomycin and 0.5 μg ml^{-1} fungizone were used for determining the effect of carbon dioxide on cell growth. Carbon dioxide gas (5%) was found to have no effect on cell growth and can be omitted from the incubating chamber (Table 4).

Karyotype of cultured cells

The diploid chromosome number of the cell from the catfish caudal trunk is 56 (Fig. 4). The karyotype shows that most of the chromosomes are in the metacentric and submetacentric group (Fig 5).

TPTH toxicity test by the newly established hybrid catfish caudal trunk cell cultures

Cell detachment from substrate

The effect of TPTH on cell detachment at four different concentrations of TPTH i.e. 1, 3, 5, and 10 μl^{-1} was observed by comparison with untreated cells. The results are shown in Table 5. It was found that cell detachment is first observed at 3 μg l^{-1} of TPTH exposure. The effect of cell detachment is more pronounced at higher concentrations of TPTH, i.e. 5, and 10 μg l^{-1}.

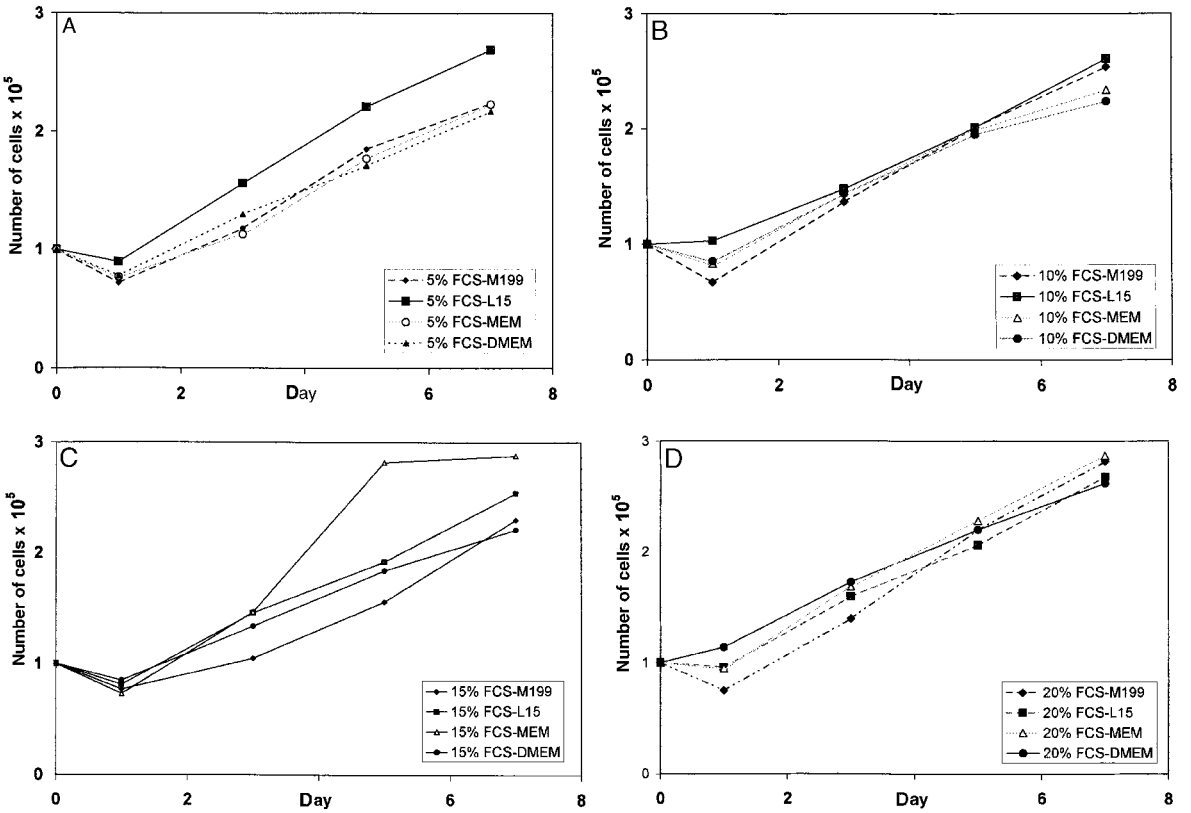

Figure 3 Growth of the caudal trunk cells in the four kinds of commercial culture medium tested (MEM DMEM, M199, L-15): (A) with 5% FCS (B) with 10% FCS; (C) with 15% FCS; (D) with 20% FCS; The values plotted are averages of five replicates.

Genotoxicity of TPTH

The effect of TPTH on chromosome abnormality was studied. The chromosome number of the 1, 3, 5, and 10 $\mu g\,l^{-1}$ TPTH-treated cells is compared with the untreated cells. The result (Fig. 6) shows that the chemical at the four TPTH concentrations tested exerts no effect on chromosome number. However, it was observed that chromosomes from the TPTH treated cells showed structural changes. Chromosome arms of the TPTH treated cells tend to twist round each other (Fig. 7). Chromosomal aberration, i.e. deletion or duplication of chromosomes[3] could occur from these abnormal chromosomes.

Preservation of established cell culture

The techniques for cell preservation including preservation at temperatures 4 and $-75\,^{\circ}C$ were compared. Caudal trunk cells were kept in growth media supplemented with 10% FCS at 4 °C in a refrigerator without changing media for 2 months; the percentage of viability on reculturing was *ca*

Table 4 Effect of carbon dioxide on growth of the caudal trunk cells of hybrid catfish (each number is the average of ten replicates)

	Cell Growth (Number of cells $\times 10^5$)				
CO_2 incubation	Day 0	Day 1	Day 3	Day 5	Day 7
5% CO^2	1.00	0.66 ± 0.06	1.34 ± 0.16	1.83 ± 0.10	2.37 ± 0.29
CO_2	1.00	0.65 ± 0.06	1.31 ± 0.15	1.76 ± 0.08	2.38 ± 0.18

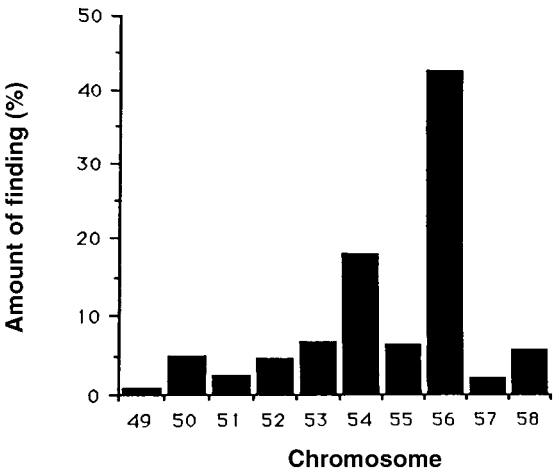

Figure 4 Frequency distribution of the diploid chromosome numbers of cells derived from the caudal trunk cell culture. Total number of cells counted is 399.

65%. When cell cultures were kept for a longer time the cell vaibility was decreased to 48%, but they were weak. The other technique of cell culture storage was done by keeping the culture in growth medium with 20% FCS and 10% DMSO in a cryopreservative tube and stored at −75 °C freezer for 3 months. After that the cells were thawed and recultured in fresh growth medium supplemented with 10% FCS. The percentage of cell viability was 58%.

Table 5 Effect of TPTH on cell detachment (each number is the average of ten replicates)

Treatment	cells retained on each slide (%) of
Untreated cells (control)	100
1 μg^{-1} TPTH	90–100
3 μg^{-1} TPTH	50
5 μg^{-1} TPTH	25
10 μg^{-1}TPTH	0–10

DISCUSSION

Four types of commercial medium were tested in this study. Leibovitz's L-15 medium has been recommended for growing cell culture from warm-water fish species However, this medium is more expensive than the other three commonly used media for cell culturing, i.e. MEM, DMEM and M199 and it does not show a significant effect on supporting growth of the cell cultures. Thus it can be concluded that cultured cells from hybrid catfish can be successfully grown in any one of the four commerical cell culture media tested. This result corresponds with those of other investigator.[17,18] A concentration of FCS at 5% can support as good a growth of cell culture as that at 20%. These cell cultures grow well at a temperature of 27 °C without adding 5%. This finding leads to a big

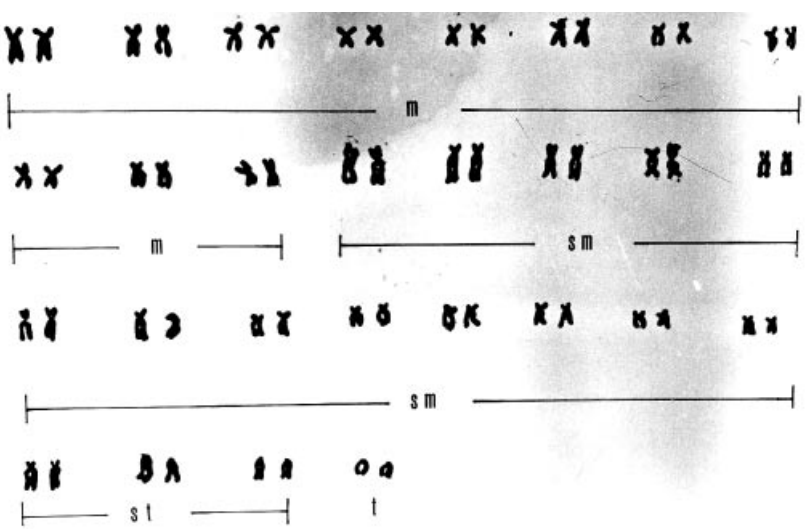

Figure 5 Karyotype of cells derived from the caudal trunk cell culture; m: metacentric; sm: submetacentric; St: subtelocentric; t: telocentric.

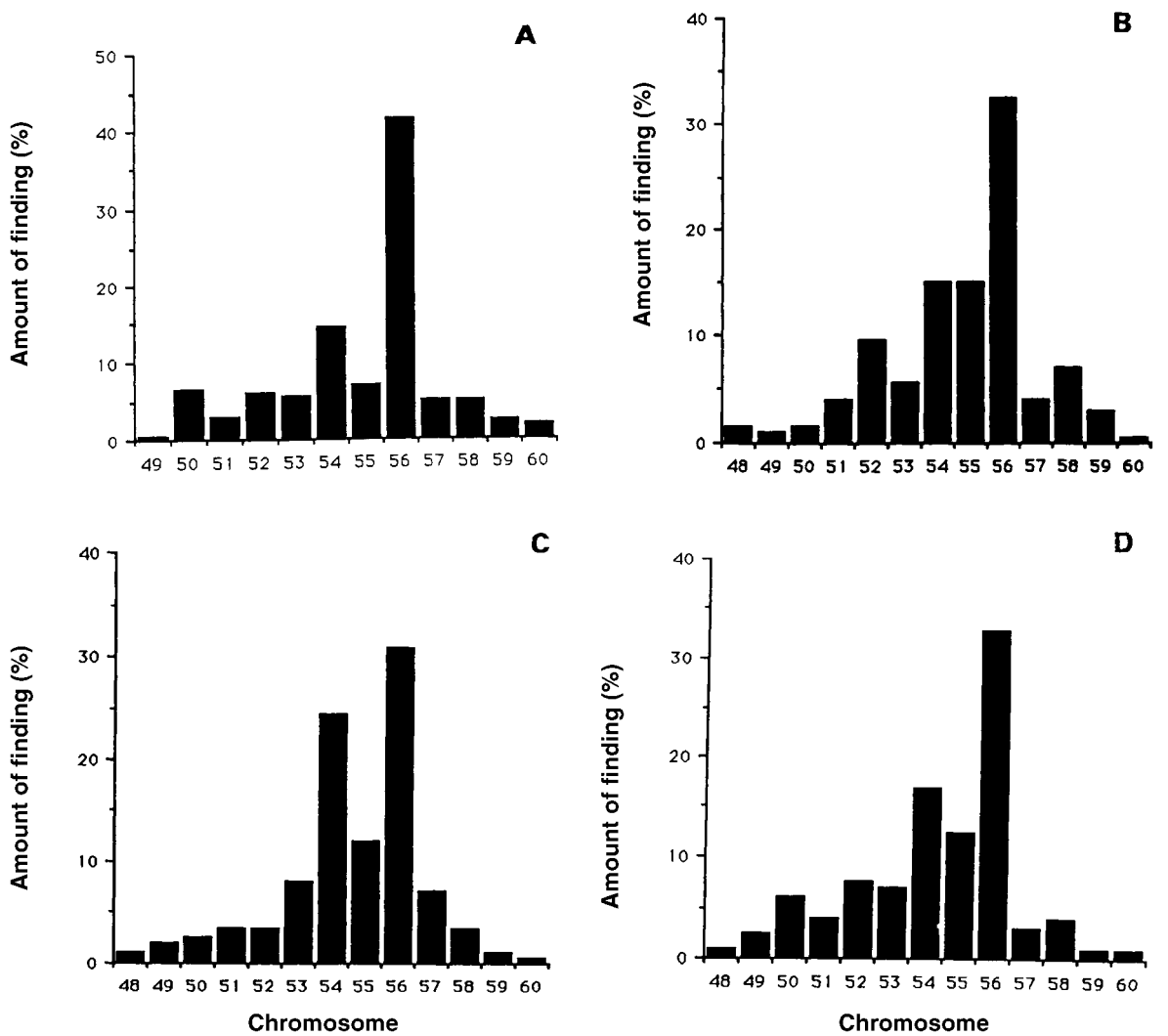

Figure 6 Chromosome counts from (A) 1 μg^{-1} TPTH-treated cells, (B) 3 $\mu g\, l^{-1}$ TPTH-treated cells, (C) 5 μg^{-1} TPTH-treated cells; (D) 10 $\mu g\, l^{-1}$ TPTH-treated cells.

saving on the cost of maintaining these cell cultures in the laboratory. In agreement with Nicholson,[18] the present study indicates that catfish cultured cells can be stored at a refrigerated temperature (4 °C) without change of medium for at least 2 months. But, if the culture is kept at −75 °C in a freezer, it can be kept longer than that.

The diploid chromosome number, as determined from the 19th passage caudal trunk cultured cells, is 56. This is similar to the report of Supamart and Donskul[19]. However, it is not in agreement with the result of Sungpetch,[20] who determined the diploid chromosome number from *in vivo* study of the hybrid catfish. This may be due to a difference in the origin of the hybrid catfish used in the study.

In the case the TPTH is used in agriculture to control pests, the chemical would contaminate water. The Thai government wants to promote both agriculture and fish farming. Thus the newly established hybrid catfish cell culture is tested on the application for screening of TPTH toxicity by using two criteria, i.e.) cell detachment and genotoxicity. In this study, cell detachment is an important parameter in the TPTH toxicity test. The results correspond with those of other investigators.[15,21] Changes in the ability of cells to attach to a

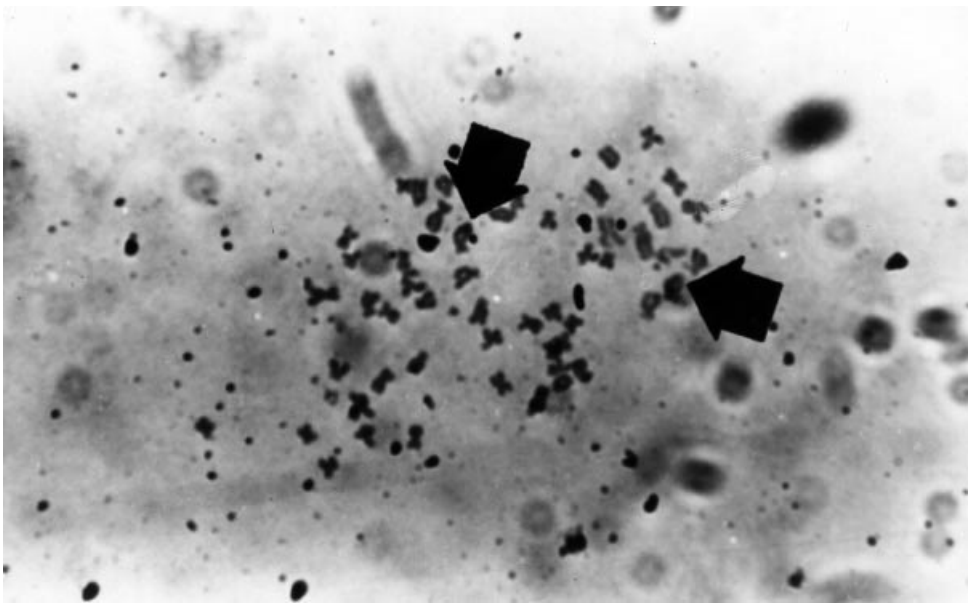

Figure 7 Chromosome twisted arms from TPTH-treated hybrid catfish cells.

substratum can be used as an indicator for cytotoxicity assay. 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 the 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.

Other cell lines that have been used in chemical toxicity tests including rainbow trout gonad (RTG-2, fibroblast cells), fathead minnow epithelial (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, the cells originating from cold-water fish species may give different responses from those originating from warm-water fish species. For that reason, hybrid catfish cell culture may be more suitable for toxicity test of xenobiotics in tropical areas than cell lines originating from cold-water fish species.

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