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Comparative Growth Response of Fish Cell Lines in Different Media, Temperatures, and Sodium Chloride Concentrations

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Growth responses to three different media, temperatures, and sodium chloride concentrations in the media were determined for 13 salmonid and 14 non-salmonid fish cell lines. Most cell lines showed better growth in Eagle's MEM than in Medium 199 or in Leibovitz L-15 medium. Nine salmonid cell lines grew well in the normal sodium bicarbonate buffer in Eagle's MEM, while 11 non-salmonid cell lines grew better in Eagle's MEM buffered with either HEPES-bicarbonate or Tris-bicarbonate. Optimum temperature for growth ranged from 15 to 20°C for almost all salmonid cells and 20 to 30°C for non-salmonid cells. Most of the cell lines showed highest growth in commercial medium preparations with the lowest concentration of sodium chloride (0.116 M) examined. However, three of the six cell lines derived from rainbow trout, *Oncorhynchus mykiss*, and cell lines from eels, *Anguilla japonica*, showed optimum growth response at a higher sodium chloride concentration of 0.171 M in the medium.

The increase in incidences of fish viral diseases due to the intensification of the aquaculture industry had made fish cell culture an invaluable technique in fish disease diagnosis. Additionally, the interest in this aspect is widely advancing since fish cell lines are now popularly used not only in fish disease studies but as well as in other research fields (Hightower and Renfro, 1988; Nicholson, 1989; Bols, 1991).

Approaches in the use of growth media and maintenance of fish cell lines are very similar to those of mammalian and animal cell lines (Wolf and Quimby, 1969). Until lately, commercially available media are being applied without modification in the propagation of fish cell lines, although several attempts to modify and improve some of its additives are in progress (Barnes and Sato, 1980; Shea and Berry, 1983; Collodi and Barnes, 1990). The relatively convenient and quick way in the preparation of media, as they are commercially available, for fish cell propagation coupled with its need in fish disease studies had mounted to increasing number of cell lines to the present. Until in 1980, Wolf and Mann had reported at least 61 cell lines from teleost and probably this number had increased to 100 or more up to the present. Currently, there is just scattered information on appropriate media for fish cell pro-

pagation, incubation temperature, and sodium chloride requirements during which fish cells grow *in vitro*. In this report we attempted to define these aspects to provide more information to fish cell culturist and improve fish cell line propagation.

Materials and Methods

Cell Lines

Cell lines derived from salmonids: ASE, RTT, RTE (provided by Dr. B. J. Hill), CHH-1, CHSE-214, KO-6, RTH-149, STE-137 (provided by Dr. J. L. Fryer) SE, YNK (provided by Dr. T. Watanabe), RTG-2 (provided by Dr. K. Wolf), SEH and RTE-2 (by the authors); and from non-salmonid fish: BF-2, EPC, FHM (also from Dr. K. Wolf), CCO (also provided by Dr. J. A. Plumb), EO-2, EK-1 (provided by Dr. S.-N. Chen), EPG, SHH, WF-1, GSE, JSKG, KRE, KRE-2, and PAS (by the authors) (Table 1) were used in this study. Initially cell lines ASE, RTT, CHH-1, CHSE-214, KO-6, EPC, RTH-149, STE-137, RTG-2, RTE, RTE-2, BF-2, FHM were maintained in either Eagle's Minimum Essential Medium (Eagle's MEM, GIBCO) buffered with a combination of sodium bicarbonate (8.9 mM NaHCO₃) and tris-(hydroxymethyl)-aminomethane

Table 1. Fish species and type, tissue of origin and original reference of fish cell lines.

Cell line	Species	Passage number	Tissue of origin/morphology	Original Reference
A. Salmonids				
ASE	Atlantic salmon (<i>Salmo salar</i>)	83	embryo/E*	Yoshimizu <i>et al.</i> , 1988
CHH-1	chum salmon (<i>Oncorhynchus keta</i>)	100	heart/E	Lannan <i>et al.</i> , 1984
CHSE-214	chinook salmon (<i>Oncorhynchus tshawytscha</i>)	255	embryo/E	Fryer <i>et al.</i> , 1965
KO-6	kokanee salmon (<i>Oncorhynchus nerka</i>)	145	ovary/E	Lannan <i>et al.</i> , 1984
SE	chum salmon	201	embryo/E	Watanabe <i>et al.</i> 1980
SEH	chum salmon	58	embryo/E	Yoshimizu <i>et al.</i> , 1988
YNK	yamame (<i>Oncorhynchus masou</i>)	233	kidney/F**	Watanabe <i>et al.</i> 1978
RTE	rainbow trout (<i>Oncorhynchus mykiss</i>)	98	embryo/E	Yoshimizu <i>et al.</i> , 1988
RTE-2	rainbow trout	67	embryo/E	Yoshimizu <i>et al.</i> , 1988
RTG-2	rainbow trout	304	gonads/E	Wolf and Quimby, 1962
RTH-149	rainbow trout	216	hepatoma/E	Fryer <i>et al.</i> , 1981
RTT	rainbow trout	83	tail/F	Yoshimizu <i>et al.</i> , 1988
STE-137	rainbow trout	206	embryo/E	Fryer <i>et al.</i> , 1965
B. Non-salmonids				
BF-2	bluegill (<i>Lepomis macrochirus</i>)	114	caudal trunk/F	Wolf <i>et al.</i> , 1966
CCO	channel catfish (<i>Ictalurus punctatus</i>)	275	ovary/E	Bowser and Plumb, 1980
EO-2	Japanese eel (<i>Anguilla japonica</i>)	99	ovary/F	Chen and Kou, 1981
EK-1	Japanese eel	88	kidney/F	Chen <i>et al.</i> , 1982
EPC	carp (<i>Cyprinus carpio</i>)	156	epithelioma/E	Fijan <i>et al.</i> , 1983
EPG	goldfish (<i>Carassius auratus</i>)	57	epithelioma/E	Yoshimizu <i>et al.</i> , 1988
FHM	fathead minnow (<i>Pimephales promelas</i>)	237	caudal trunk/E	Gravell and Malsberger, 1965
SHH	snakehead (<i>Channa striatus</i>)	77	heart/E	Yoshimizu <i>et al.</i> , 1988
WF-1	pond smelt (<i>Hypomesus olidus</i>)	52	fin/E	Yoshimizu <i>et al.</i> , 1988
GSE***	gizzardshad (<i>Clupanodon punctatus</i>)	78	embryo/E	Yoshimizu, 1991
JSKG***	Japanese stripeknifefish (<i>Oplegnathus fasciatus</i>)	64	gonad/E	Fernandez <i>et al.</i> , 1993, in press
KRE***	hybrid of kelp and red spotted grouper (<i>Epinephelus moara</i> × <i>E. akaara</i>)	92	embryo/E	Fernandez <i>et al.</i> , 1993, in press
KRE-2***	hybrid of kelp and red spotted grouper	93	embryo/E	Yoshimizu, 1991
PAS***	purplish amberjack (<i>Seriola dumerili</i>)	124	skin/E	Fernandez <i>et al.</i> , 1993, in press

* epithelioid

** fibroblastic

*** marine fish cell lines.

(16 mM Tris). Cell lines EO-2, EK-1, EPG, SE, SEH, YNK, WF-1, SHH, CCO, GSE, JSKG, KRE, KRE-2 and PAS were maintained in Leibovitz L-15 Medium. Both media were supplemented with 10% FBS and with added antibiotics, 100 IU penicillin (Sigma) and 100 μ g streptomycin (Sigma) per ml. Salmonid cell lines were maintained at 15°C including EO-2, EK-1, WF-1, EPC and EPG, while non-salmonid cell lines at 20°C.

Growth Response to Various Culture Media

Growth response of the cell lines in three kinds of semi-synthetic media were observed: Eagle's MEM, Leibovitz L-15 Medium and Medium 199. In Eagle's MEM, aside from the usual sodium bicarbonate buffer (26 mM), two other organic buffers were used; HEPES (N-[2-hydroxyethyl]piperazine-N'-2-ethanesulfonic acid) (14 mM) and Tris (16 mM), in combination with sodium bicarbonate (8.9 mM). Cells were seeded in 48 wells of a 96-well microplates (Corning) and incubated at either 15° or 20°C. Cells in Eagle's MEM with sodium bicarbonate alone was incubated in a CO₂ incubator (5% CO₂).

Growth Response to Various Temperature

The effect of various temperatures on the growth of the cell lines was likewise determined. Cell lines derived from salmonids were incubated from 10°C to 30°C at 5° difference while non-salmonid cells were further incubated up to 35° and 40°C. The highest growth response obtained in the test for various media was used as the growth medium for this test.

Growth Response of the Cell Lines to Various Concentrations of Sodium Chloride in the Media

Eagle's MEM buffered with Tris and sodium bicarbonate was adjusted to 0.171 M, 0.256 M, 0.341 M, and 0.512 M of sodium chloride. A stock solution of Eagle's MEM containing 3.40 M sodium chloride was prepared and was used to adjust the above test concentrations. Unadjusted medium was used as control. All cell lines were exposed to these concentrations, except for KRE-2. Cells were incubated at its optimum temperature based on the above results.

Cells were seeded at initial concentrations of 1–2 $\times 10^5$ cells per ml at 100 μ l per well in all of the above experiments.

Determination of Cell Growth Response

Cell growth responses were observed based on the methods described by Fernandez *et al.* (1993). After 7 days incubation period, cells in microplates were fixed with 10% formalin for 30 min to 1 h and carefully rinsed with tap water. Cells were then stained with 0.1% crystal violet for 1 h and were rinsed again with several washings. Rinsed microplates were then thoroughly air-dried. Absorbance of stained microplates was read in a Microplate Spectrophotometer (Corona MTP-22) at 600 nm.

Results

Growth Response to Various Culture Media

Relative growth response of the cell lines in various media was calculated as the ratio of mean absorbance reading at 600 nm on the final day (day 7) to that on the initial (day 1) of incubation (Table 2). Interestingly, high response was observed in Eagle's MEM with the usual sodium bicarbonate buffer in 9 (69%) of the 13 salmonid cell lines. Eagle's MEM with Tris-bicarbonate was shown also as the second media of choice for salmonid cell lines. On the other hand, growth responses of cell lines from non-salmonid fish were high when Eagle's MEM was buffered with either HEPES-bicarbonate or Tris-bicarbonate. Growth responses of the two groups of fish cell lines were poor in both Medium 199 and Leibovitz L-15.

Growth Response to Various Temperatures

Optimum growth response of both salmonid and non-salmonid lines were observed at varying temperature exposures (Figs. 1–3). Cell lines from salmonid fish, both from *Oncorhynchus* spp. and *Oncorhynchus mykiss* had an optimum growth temperature from 15° to 20°C and with very little or no response at 25° or 30°C (Fig. 1). Among the non-salmonid cell lines, only EPG, a cell line from an epithelioma of goldfish, behaved similarly to the salmonid cell lines. For the non-salmonid cell lines, optimum growth response varied from 20° to 25°C and 30°C. EO-2 and EK-1, cell lines derived from eels, and BF-2 had its optimum growth temperature at 25°C; FHM, CCO, and EPC at 30°C; SHH at 20°C; and a broad optimum temperature ranged from 20° to 30°C for WF-1 (Fig. 2). Marine fish cell lines JSKG and PAS had their optimum growth temperature at 25°C; KRE at 30°C; and GSE and KRE-2 at 20°

Table 2. Relative growth response of fish cell lines in three commercial media after 1 and 7 days incubation

Cell Lines	Relative growth*				
	Medium			Leivobitz L-15	Medium 199
	Eagle's MEM		NaHCO ₃		
	HEPES	Tris			
A. Salmonid					
ASE	0.63	1.21	1.02	1.37*	0.67
CHH-1	4.29	5.24	8.45*	3.75	6.68
CHSE-214	5.57	6.00	8.94*	4.57	5.19
KO-6	4.42	4.78	6.20*	4.10	4.86
RTE	2.53	2.70	2.84*	2.22	2.64
RTE-2	2.34	2.69	2.98*	1.94	2.44
RTG-2	4.22	3.99	4.73*	3.74	4.05
RTH-149	6.32*	4.75	6.30	3.89	4.79
RTT	2.37	2.64	3.11*	2.02	2.35
SE	1.55*	1.42	1.35	1.41	1.22
SEH	1.36	1.41	1.35	1.38	1.43*
STE-137	2.54	2.67	3.34*	2.32	2.70
YNK	2.03	2.31	3.02*	1.58	2.55
B. Non-salmonid					
BF-2	0.5	1.95*	0.97	1.89	0.43
CCO	12.81*	6.54	7.00	4.96	9.86
EO-2	2.09*	1.70	1.50	2.05	2.00
EK-1	2.38	2.06	2.43	2.26	2.80*
EPC	2.41	3.51	3.64*	2.61	3.39
EPG	3.91	3.20	4.05*	2.00	3.94
FHM	1.11	3.29*	0.94	2.26	0.86
SHH	2.90	2.94	3.83*	2.23	2.55
WF-1	3.33*	2.55	3.18	1.88	2.52
GSE**	1.66	1.95*	1.70	1.71	1.64
JSKG**	4.56*	3.99	3.86	2.94	4.53
PAS**	3.75	4.24*	3.49	3.71	2.28
KRE**	3.55*	3.17	2.87	3.31	2.95
KRE-2**	1.71*	1.62	1.32	1.68	1.49

* Final (day 7) mean absorbance reading at 600 nm

Initial (day 1) mean absorbance reading at 600 nm

** cell lines from marine fish.

^a highest response.

and 25°C (Fig. 3). ASE, a new cell line from Atlantic salmon also produced high growth response at 25° and 30°C. Except for WF-1, all non-salmonid lines produced little or no response at 35°C.

Growth Response of the Cell Lines to Various Sodium Chloride Concentrations in the Media

The effect of various sodium chloride concentrations in the media on the growth of several fish cell lines was evaluated (Figs. 4 and 5). Among the sodium chloride concentrations used, the unadjusted medium, which contained approximately 0.116 M

sodium chloride, showed the highest growth response to all salmonid lines of *Oncorhynchus* spp. and all non-salmonid lines including marine fish cell lines, except KRE. However, it is interesting to note that 3 of the 6 cell lines from rainbow trout had their highest growth response at a higher sodium chloride concentration of 0.171 M. Little or no response was observed from higher concentrations of 0.256 M, 0.341 M and 0.512 M sodium chloride.

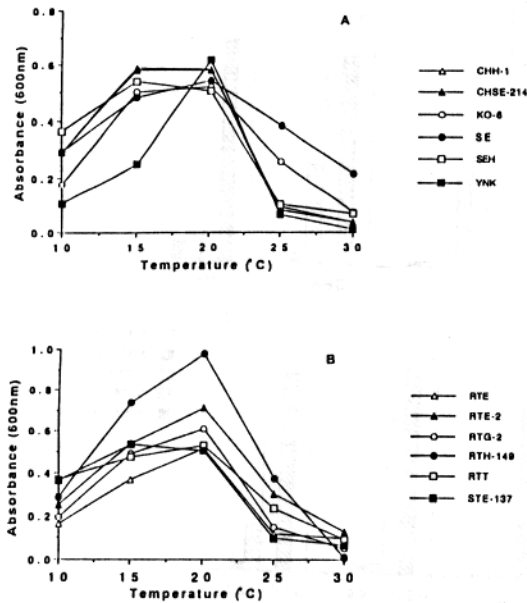


Fig. 1. Growth response of cell lines from salmonids to various temperatures.

A. derived from *Oncorhynchus* spp.; B, derived from *Oncorhynchus mykiss*

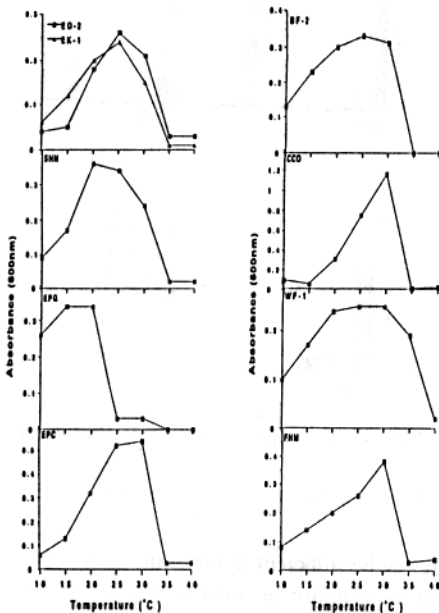


Fig. 2. Growth response of cell lines from non-salmonids, derived from warm freshwater fish, to various temperatures.

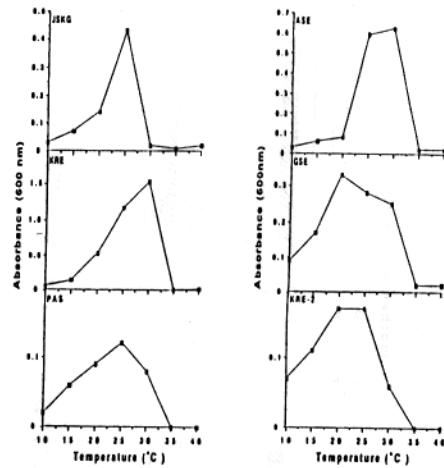


Fig. 3. Growth response of cell lines from non-salmonids, (derived from marine fish) and ASE cell line (derived from Atlantic salmon) to various temperatures.

Discussion

The amenability of fish cell lines to the use of media intended for mammalian cell lines allowed the continued use of semi-synthetic media in fish cell line propagation without further modifications. Three types of media formulations are so far popular both in the initial and in the continued propagation of fish cells: Eagle's MEM, Medium 199, and Leibovitz L-15 medium. Of these three, it is noteworthy that Eagle's MEM had been preferred as the choice of media by most fish tissue culturists, needless to say that almost all of the present cell lines had been initially propagated using this medium. This report further confirms and agrees with the report of Fryer *et al.* (1965) on the superiority of Eagle's MEM over that of other commercial preparations.

The stability of pH in the media is of important consideration in the growth and metabolism of cells and depends in part on the buffering capacity of the media. Although it appears not to be so critical in fish cell culture (Wolf and Quimby, 1969), sub-optimal limits may often result in deleterious changes in the cells such as sloughing, vacuolation, and contraction of cellular processes (Wolf and Ahne, 1982). Added bicarbonates in the medium are essential metabolites for many cells (Geyer and Chang, 1958; Swim and Parker, 1958; Eagle, 1971) and that their use in conjunction with organic buffers such as

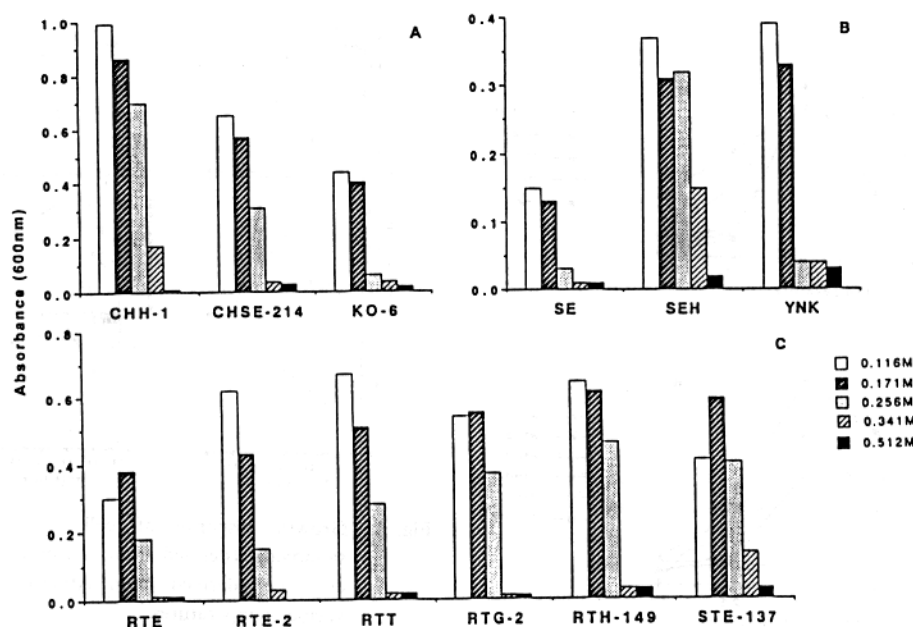


Fig. 4. Growth response of cell lines from salmonids to various molarities (M) of NaCl in the media. A and B, derived from *Oncorhynchus* spp.; C, derived from *Oncorhynchus mykiss*

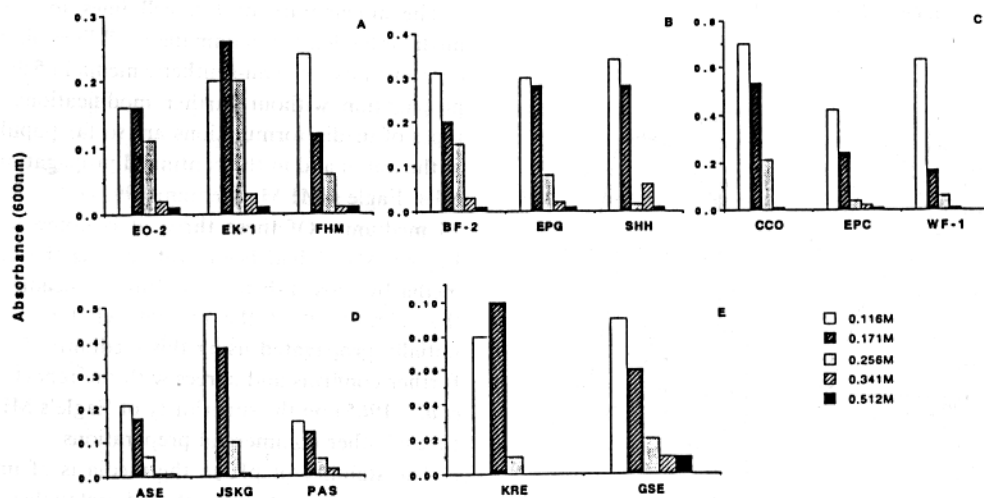


Fig. 5. Growth response of cell lines from non-salmonid fish to various molarities (M) of NaCl in the media.

A, B, and C, derived from warm freshwater fish; D and E, derived from marine fish.

HEPES was required for maximum virus production and for long term cell cultivation (Fisk and Pathak, 1969; Wolf and Quimby, 1973; Massie *et al.*, 1974). It appears from our results that the normal bicarbonate buffering is adequate for most salmonid cells but

may not be sufficient in non-salmonid cells. The high growth response of most non-salmonid cell lines in Eagle's MEM with the addition of organic buffers such as HEPES and Tris indicate that normal bicarbonate system is not sufficient to promote growth of

these cells. On the other hand, there are reports on the toxic effect of Tris in mammalian cell cultures (Swim and Parker, 1955; Good *et al.*, 1966; Eagle, 1971). However, since the scope of our experimental set up is very limited rendering no further cell observation, we recommend that further careful observation should be made on the cells if Tris should be incorporated in the medium on the basis of the above reports. Our laboratory had been employing Tris with sodium bicarbonate as its buffering system in the media for fish cell lines. We have observed that cells from two or three fish cell lines tended to form clumps a few weeks after propagation, but this was not exhibited when we shifted to other media. This clumping of cells is similar to the observation of Swim and Parker (1955) on the toxicity of Tris on HeLa cell lines. On the other hand, HEPES had been unquestionably used as an efficient medium and produced no toxic effect when used in cell cultures (Good *et al.*, 1966; Shipman, 1969; Medzon and Gendies, 1971; Eagle, 1971; Chagnon and Corbeil, 1973) but normally costs higher than Tris. Although Medium 199 was reported as the first medium in fish cell line propagation, it has not been popularly used lately. Aside from its high cost (Wolf and Quimby, 1969), it had not convincingly shown effectiveness in promoting cell growth in this experiment. The same was also observed for Leibovitz L-15 medium. However, Leibovitz L-15 medium is also commonly used by some fish cell culturists since it eliminates the use of CO₂ incubators and preparation of buffers.

In general, fish cell lines can be grown at lower temperatures and others at higher temperatures. Thus, depending upon the choice of cells, they could be very useful in a variety of purposes. This report further justifies (although in a limited manner) the temperature ranges of cell lines from fish. Also our results support what has been previously reported on the ranges of temperature for optimum cell growth (Wolf and Ahne, 1982; Nicholson, 1988, 1989; Bols and Lee, 1991). Nevertheless, according to Wolf and Ahne (1982) "fish cell lines could still be adapted, or selected, for growth at higher-than-normal temperatures within reasonable limits."

This paper also confirms that fish cell lines could well grow in the same amount of salt as that of the mammalian cell lines. The high tolerance limit of the three salmonid cell lines RTE, RTG-2 and STE-137, two cell lines derived from eels, EO-2 and EK-1, and a cell line from grouper, KRE in abrupt situations

seems to indicate that fish cell lines could still be adapted to higher amounts of sodium chloride in the medium, however, within reasonable limits. This additional observation presented here may reinforce the versatility and functionality of teleost cell lines to various applications in biological research.

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