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Primary culture of hepatic cells from Metynnis roosevelti (Pisces, Teleostei, Characidae)

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Cultivo primário de células hepáticas de Metynnis roosevelti (Pisces, Teleostei, Characidae)

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SUMMARY

In order to achieve monolayers and adhesion in cell cultures of hepatocytes from the subtropical fish *Metynnis roosevelti* (Pisces, Teleostei, Characidae) three different methodologies were used: enzyme cell dissociation by means of a system containing trypsin and versene 0.25%; by means of bovine fetal serum and by the use of blood serum obtained from adult specimens of *M. roosevelti.* The cell medium used has been a modified F10-199 by the addition of 2 mM L-glutamine, 10 µg/mL insulin, 50 µg/mL fibronectin; and antibiotics. The best results in terms of cell dissociation as well as in terms of adhesion and confluence of the cells monolayer has been achieved by means of the enzymatic cell dissociation.

UNITERMS: Liver cells; Cell culture; Cells; Pisces; Metynnis roosevelti.

INTRODUCTION

The first account on trout embryos being maintained 24 hours in a medium of Ringer solution containing amphibian lymph goes back to 1914. A more systematic research work on this subject was carried out in 1935 emphasizing the importance of osmotic pressure for the integrity of marine teleosts tissues. Important advances in this methodology were achieved in 1949 by means of the use of antibiotics in cell culture medium and in 1958 by trypsinization of fish tissues, a technique that allowed, by the first time, to cultivate fish cells in monolayers[25,27].

In 1980, information on 61 lineages of fish cells from 17 families and 36 species of teleosts were brought together. Most of the fish lineages studied were either from continental waters or from anadromous fish, only 22% being from strict marine fishes. On the other hand, the EPC carp (*Cyprinus carpio*) lineages, the RTH and RTN rainbow trout (*Salmo gairdneri*) lineages, the WC-1 from *Stizostedium vitreum* and the PS-12 from *Esox lucius* lineages started from neoplasm tissues. Cell lineages from the following teleost families are nowadays known: Anabaenidae, Carangidae, Cyprinidae, Cichlidae, Clariidae, Esocidae, Ictaluridae, Percichthyidae, Percidae, Poeciliidae, Pomadasyidae, Salmonidae (18 species), Scianidae and Sparidae[17,26].

Fish cells and tissue cultures have been used for different research purposes as, for example, viral diagnostic and studies in different animals, invertebrates and vertebrates[23]; studies on the mechanism of termoregulation[6,20]; endocrinological research[5,9]; and more recently, as a suitable model for the study of oxidative stress of animal cells[2,12]. *In vitro* studies on cytotoxicity using fish cell cultures are being carried out for different scientific purposes[3]. One of the objectives of these studies is being the identification of the effects caused by chemical aggressors responsible for environmental impacts[1,4,6,16]. The main purpose of the present research work is to develop adequate methodology for the primary cultivation of hepatic cells from *Metynnis roosevelti*, a subtropical fish from the Characidae family.

METHODOLOGY

Specimens of *Metynnis roosevelti* with body length of about 5 cm were first acclimatized for a period of two weeks[14,24] in 140 liters aquarium under constant aeration. The photoperiod used was of 12 hours day/12 hours night and the temperature maintained at 29°C ± 1°C by means of an electronic thermostat. The fish were fed once a day with dry ration.

Primary Cell Cultivation

The fish were first maintained 30 minutes in 0.01% saline followed by immersion during 2 minutes in 70% ethanol solution. After this step to disinfect them, the fish were taken to a laminar flow, sacrificed by section of the spinal cord and the liver immediately excised and maintained for 30 minutes in Hank's solution containing penicillin, 100 IU; streptomycin, 20 mg/mL; enrofloxacine, 10 µg/mL; and anfotericine B, 25 µg/mL[18,27]. This solution has been renewed 5 times during this period.

Three methodologies were then used with the purpose of getting free cells from the liver samples prepared as described:

I. *Trypsin cell disruption.* The liver was cut in small fragments of about 1 mm, transferred to an erlenmeyer containing 2.5 mL of a 0.25% of trypsin-versene solution and then shaken 10 minutes at 50 g at room temperature by means of a magnetic stirrer. At the end of this period, trypsin is inactivated by adding to the system 5.0 mL of the F10-199 Nutrient Mixture (F-10 HAM and Medium 199, Sigma) containing 10% of bovine fetal serum (FBS). Trypsin is inactivated by the action of a_2-microglobulin from the FBS. After spun down the whole suspension 2 minutes at 50 g, the supernatant is discarded and the pellet resuspended in 5 mL of F10-199 medium containing penicillin, 100 IU; streptomycin, 200 µg/mL; and Fungizon, 250 µg/mL. The final cell suspension is then filtered through sterile gauze and transferred to 75 cc polystyrene Corning flasks and to sixwelled Falcon microplates for monolayer cell cultivation. Flasks and microplates were previously treated with a 10 percent bovine serum albumin (BSA) in sterile distilled water and then kept at 30°C in a 5% CO_2 oven.

II. *Bovine foetal serum.* The liver was cut in small fragments of about 1 cm, which were then placed in the sterile flasks and microplates for cultivation. This was followed by the addition of 0.1 mL of FBS over each fragment from tissue and then, flasks and microplates were incubated for about 4 hours at 30°C in a 5% CO_2 oven. This step was followed by the addition of 6.0 mL of medium F10-199 to each flask and 2.0 mL to each microplate, which were then kept again at 30°C in a 5% CO_2 oven.

III. *Homologous serum.* Blood samples were obtained in Vacuntainer tubings by means of a vein puncture of the caudal vein of adult specimens of *M. roosevelti.* The whole blood was then spun down 10 min at room temperature and the serum kept in sterile flasks at 4°C up to its use. For cell cultivation, the fish liver was reduced to small fragments as described above. To each fragment of tissue placed in sterile flasks and microplates, 0.1 mL of fish serum was added and the whole system maintained 4 hours at 30°C in a 5% CO_2 oven. After this step, 6.0 mL of F10-199 medium were added to each flask and 2.0 mL to each microplate the whole system being then kept again at 30°C in a 5% CO_2 oven.

Besides the addition of antibiotics, F10-190 medium has been modified by the addition of 2 mM L-glutamine[7,8,15], 10 µg/mL insulin[11,12,18,24] 50 µg/mL fibronectin (Gibco BRL, Bovine Plasma Fibronectin)[7,13].

The Corning polystyrene flasks and the Falkon microplates were previously treated with 10% solution of bovine serum albumin (BSA) in sterile distilled water and kept at 30°C in a 5% CO_2 oven.

For cell counting and determination of cell viability the classical Fuchs-Rosenthal chamber as well as Trypan Blue as cell staining, were used.

Subculture of Cells

With the purpose to remove the proteins from the BFS, the monolayer cells from each flask were washed out with 3 mL of phosphate buffer solution (PBS) and each well from the microplates with 1 mL as well. For their enzymatic dissociation, the monolayer cells from the flasks were treated for about 15 minutes at room temperature with 1.0 mL of a 0,05% trypsin-versene solution. For the microplates this same operation is carried out with 0.5 mL of the trypsin-versene solution. After this step of trypsinization, trypsin has been inactivated by transferring to each system F10-199 medium in a volume equivalent to half of that of the trypsin-versene solution. The preparation is then spun down 1 minute at 50 g at room temperature. The supernatant is discarded and the pellet suspended in 5 mL of the F10-199 medium. Cell suspensions containing 3.10^{-4} cells/mL were stored in Corning polystyrene flasks and in Falcon microplates previously treated with a 10% bovine serum albumin solution in steril distilled water and kept at 30°C in a 5% CO_2 oven.

RESULTS

Upon the trypsin effect the monolayer *M. roosevelti* hepatic cells underwent dissociation, they began differentiation towards fibroblasts from the 4th day on, reaching confluence of about 80% of monolayer at the 15th day (Fig. 1) from the beginning of the experiment. At the first passage, 2.5 mL of a 0.25% trypsin solution was added to the cells. The mixture was carried out 15 minutes, at 50 g, and at room temperature, by means of a magnetic stirrer. It has been found, under this experimental condition, adhesion of most of the cells after the first passage with 90% of confluence after the 5th day of the experiment.

In the presence of bovine fetal serum and in the absence of proteolytic enzymes, the differentiation of *M. roosevelti* hepatic cells began after 10 days. After 21st days

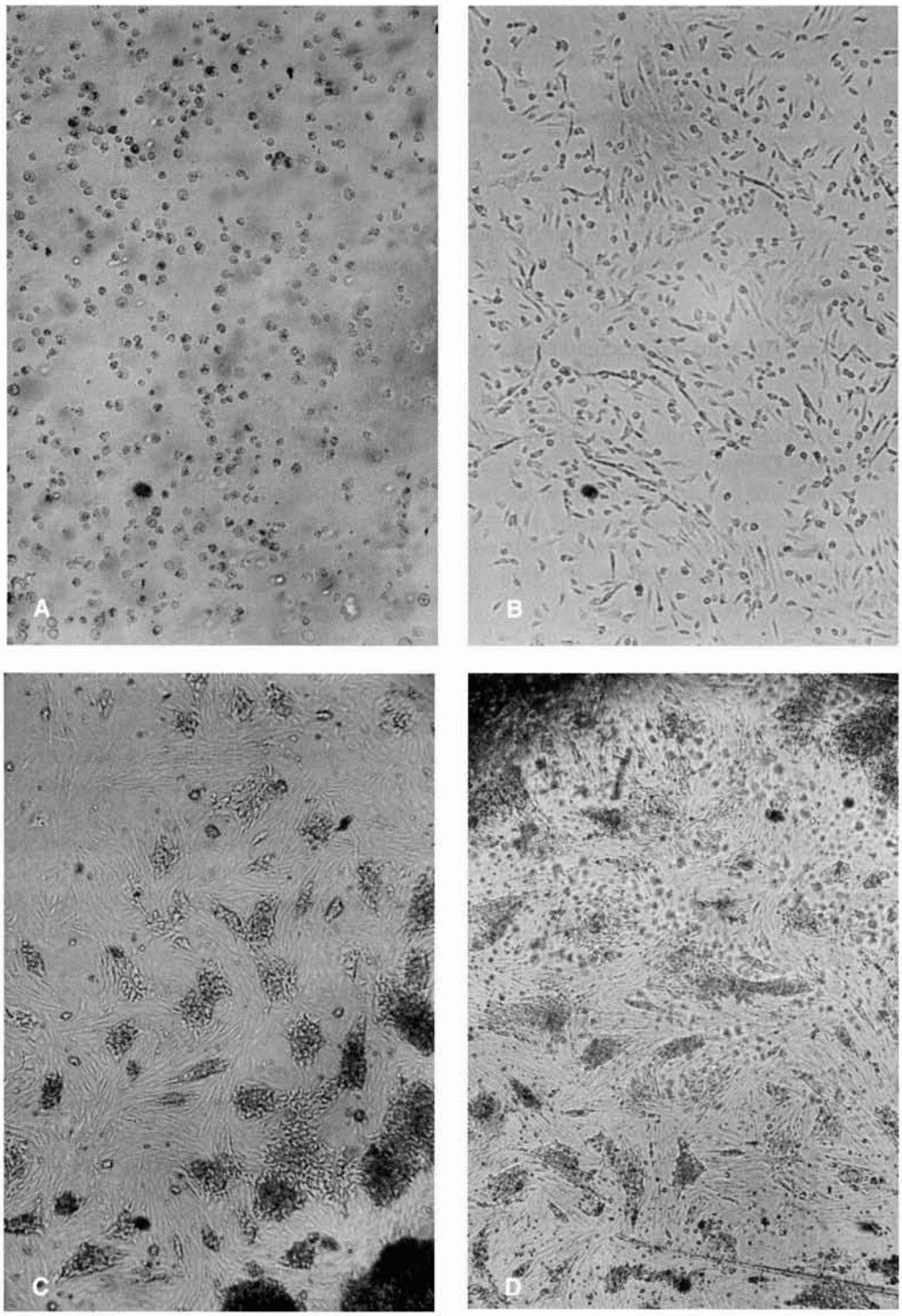

Figure 1

(A) -Trypsin versene enzyme dissociation of hepatic cells from Metynnis roosevelti; (B) - Cells differentiated in fibroblasts after seven days in F10-199 culture medium; (C) - Cells after 15 days of enzyme dissociation displaying 80 per cent confluence in the monolayer; (D) - Cells showing 100 per cent confluence in the monolayer after 21 days in culture medium.

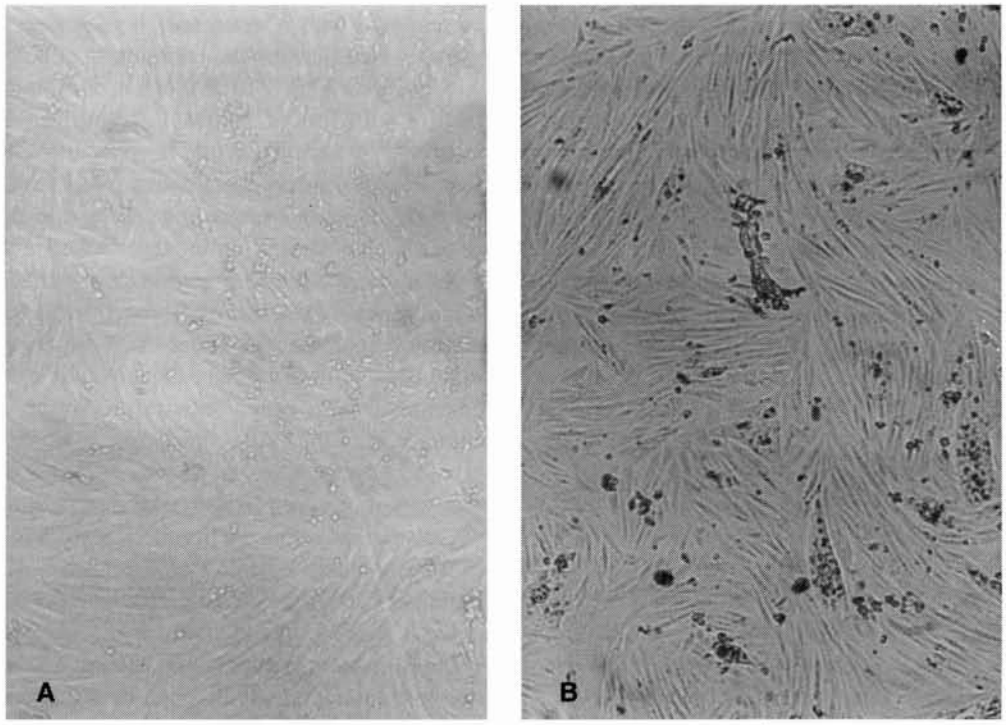

Figure 2
Metynnis roosevelti hepatic cell culture (A) from liver fragments, in bovine fetal serum displaying (B) 70 per cent confluence in the monolayer after 21 days cultivation.

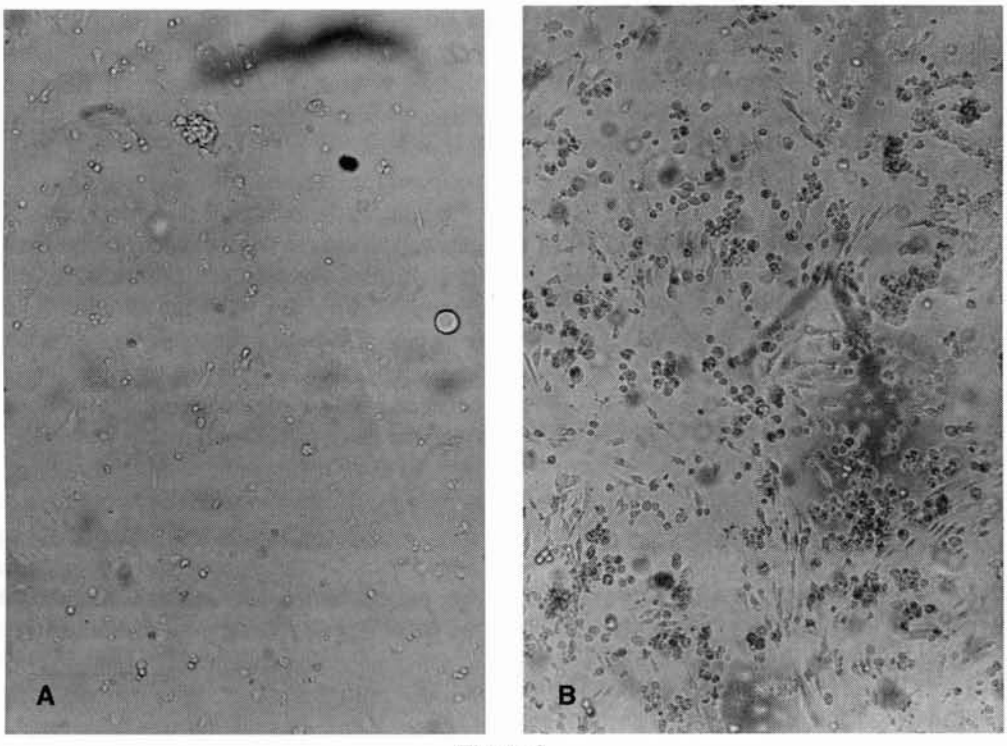

Figure 3
Metynnis roosevelti hepatic cell culture (A) from liver fragments, in homologous serum displaying (B) beginning of differentiation after 21 days cultivation.

of the beginning of the experiment, the monolayer confluence reached 70% (Fig. 2). At the first passage, 2.5 mL of a 1:5 dilution of trypsin in phosphate buffer solution (PBS) was transferred the cells. It has been found that under these conditions, a small number of cells underwent adhesion resulting in a low rate of confluence of the monolayers.

In the presence of homologous serum, the cells underwent their process of adhesion and differentiation very slowly around the 21st day of the beginning of the experiment. However they did not reach adequate development to allow confluence to monolayer enough for a cell subculture (Fig. 3).

DISCUSSION

Due to the fact that fish possesses a quite diversified bacterial population, in the attempt to obtain a primary culture of *M. roosevelti* hepatic cells, the liver samples were maintained in Hank's solution containing 100 IU/mL of penicillin, 200 µg/mL of streptomycin, and 250 mg/mL of Fungizone. In studies with another tropical fish, *M. roosevelti*, it has been found several bacteria resistant to the antibiotics usually employed in primary cell cultivation. The use of 10 µg/mL of enrofloxacine (Baytril) reduced the index of contamination in the culture medium in about 80%.

In the present experiments, fish hepatic cells began their process of differentiation towards fibroblasts after four days, upon being treated with trypsin, displaying 80% confluence of the monolayer in about 15 days. These results contradict other experiments[8,22] concluding that enzyme dissociation destroys cell interactions affecting this way cell functions and differentiation.

Fetal bovine serum showed not to be very efficient for the purpose of cell cultivation and confluence to monolayers. In the presence of FBS, cell differentiation began after 10 days with 70% of confluence towards monolayer, a result reached after 21 days.

The use of homologous serum showed a negative effect in both adhesion and formation of monolayer. Studies carried out with elasmobranchs[11], showed that shark and ray sera displayed a toxic effect in monolayer primary cells cultivation, not allowing adequate adhesion and further cell growing. On the other hand, experiments[15] using 1.25% solutions of trout serum showed a neat increase in the rate of adhesion and propagation of the fish hepatocytes in the culture media. However, these experiments with trout serum raised contradictory results[10,15,19], in one case[27] showing that the fish homologous serum was rate limiting in regard to the efficiency of cell adhesion when compared with FBS; in another set of experiments[20] trout serum as well as in the present ones with *M. roosevelti*, homologous fish serum did not alter significantly cell adhesion in culture medium.

The original F10-199 culture medium was modified by the addition of 2 mM L -glutamin[7,15], 10 µg/mL insulin[11,12,18], and 50 µg/mL fibronectin[7,13]. In fish hepatic cells insulin displays an effective role in mitosis and DNA synthesis[24], besides promoting increase in the intracellular fluid of glucose and aminoacids[6]. Cell culture medium deficient in insulin concentration clearly affects hepatic cells morphology[1]. Addition of fibronectin to the fish culture medium[6] increased in about 80% the confluence of the monolayer in trout primary hepatic cells cultivation, when compared with other substrates. Being an important component of the extracellular matrix, fibronectin seems to provide a better adhesion as well as cell differentiation. In this way, fibronectin regulates the protein organization of the cytoskeleton. Plasma fibronectin, a filamentous resistant protein is classified into two categories, according to its functional activity. One category includes proteins, which possess specific ligand sites for macromolecules such as collagen, proteoglycans, fibrin, heparin and actin. The other category is of proteins that can be recognized by specific cells or bacteria. The fibronectin region that links to the cells is in an area of the molecule that possesses 250-310 pairs of bases of terminal amino acids of the sequence Arg-Gly-Asp-Ser. In malignant or transformed cells, fibronectin is generally highly reduced or even absent. However, in normal fibroblastic cells, fibronectin amount in the cell glycoproteic surface is high. The extracellular fibronectin might be indirectly attached to the intracellular cytoskeleton through a transmembrane fibronectin receptor and several other peripheral membrane proteins[21].

It has been found[6] that fibronectin might promote connection between fetal hepatic cells and prolong its growing and ability to produce a-fetalprotein albumin (AFP). Immunofluorescent tests for albumin in preparation of hepatocytes showed a high percentage of albumin-positive, amounting this way more than 90% of the cells from adult livers and 80% of the cells from fetal or neonatal livers. On the other hand, it has been found[7,8] that RNAm actin levels in cultures of fish liver cells are a little higher than *in vivo* while RNAm albumin showed lower rates than *in vivo*[7,8]. In the present research work, polystyrene flasks and microplates were previously treated with bovine serum albumin in a 10% solution, easing this way, cell adhesion and the quickness of cell growing and formation of the monolayer. On the other hand, *M. roosevelti* hepatic cells showed to be very adequate for the monolayers *in vitro* primary cell cultivation. Enzymatic cell dissociation was considered to be the best methodology for cell adhesion and posterior formation of monolayers.

RESUMO

A fim de obter culturas celulares e monocamada de células hepáticas do peixe subtropical *Metynnis roosevelti*, foram comparadas três metodologias: dissociação celular enzimática com tripsina versene 0,25%; utilização do soro fetal bovino; e soro homólogo, obtido por meio da punção da veia caudal, de exemplares adultos da mesma espécie de peixe. O meio de cultura utilizado foi F10-199 acrescido de L-glutamina 2 mM, insulina 10 µg/mL, fibronectina 50 µg/mL; e antibióticos. A dissociação celular enzimática apresentou maior eficiência na adesão e confluência da monocamada celular em comparação a outras metodologias utilizadas.

UNITERMOS: Hepatócitos; Cultura de célula; Células; Peixes; Metynnis roosevelti.

REFERENCES

1- BABICH, H.; BORENFREUND, E. Aquatic pollutants tested: *in vitro* with early passage fish cells. **ATLA**, v.15, p.116-22, 1987.

2- BABICH, H.; PALACE, M.R.; STERN, A. Oxidative stress in fish cells: *in vitro* studies. **Archives Environmental Contamination and Toxicology**, v.24, p.173-8, 1993.

3-BOLS, N.C.; BOLISKA, S.A.; DIXON, D.G.; HUDSON, P.V.; KAISER, K.L.E. The use of cell cultures as indicator of contaminant toxicity to fish. **Aquatic Toxicology**, v.6, p.147-55, 1985.

4- CASTAÑO, A.; VEGA, M.M.; TARAZONA, J.V. Acute toxicity of selected metals and phenols on RTG - 2 and CHSE - 214 fish cell lines. **Bulletin Environmental Contamination Toxicology**, v.55, p.222-9, 1995.

5- CHANG, J.P.; YU, K.L.; WONG, A.O.; PETER, R.E. Differential actions of dopamine receptor subtypes on gonadotropin and growth hormone release in vitro in goldfish. **Neuroendocrinology**, v.51, n.6, p.664-74, 1990.

6- CHEN, J.D.; YEW, F.H.; LI, C.G. Thermal adaptation and heat shoock response of Tilapia ovary cells. **Journal Cell Physiology**, v.134, n.2, p.99-189, 1988.

7- CHENG, L.L.; BOWSER, P.R.; SPITSBERGEN, M. Development of cell cultures derived from lake trout liver and kidney in a hormone-supplemented, serum-reduced medium. **Journal of Aquatic Animal Health**, v.5, p.119-26, 1993.

8- FLOURIOT, G.; VAILLANT, C.; SALBERT, G.; PELISSERO, C.; GUIRAUD, J.M.; VALOTAIRE, Y. Monolayer and aggregate cultures of rainbow trout hepatocytes: long-term and stable liver-specific expression in aggregates. **Journal of Cell Science**, v.105, p.407-16, 1993.

9- GREEN, J.; BAKER, B.I. Melanin concentrating hormone: influence of nerves and hormones on the control of trout melanophores. **Life Science**, v.45, n.13, p.1127-32, 1989.

10- HARTMANN, J.X. Routine establishment of primary elasmobranch cell cultures. **In Vitro Cellular & Development Biology**, v.28A, p.77-9, 1992.

11- HAYASHI, S.; OOSHIRO, Z. Primary culture of the cell hepathocytes in the serum-free medium. **Bulletin of the Japanese Society of Scientific Fisheries**, v.52, n.9, p.1641-51, 1986.

12- HIGHTOWER, L.E.; RENFRO, J.L. Recent applications of fish cell culture to biomedical research. **The Journal of Experimental Zoology**, v.248, p.290-302, 1988.

13- HIRATA, K.; YOSHIDA, Y.; SHIRAMATSU, K.; FREEMAN, A.E.; HAYASAKA, H. Effects of laminin, fibronectin and type IV collagen on liver cell cultures. **Experimental Cell Biology**, v.51, p.121-9, 1983.

14- KLAUNIG, J.E.; RUCH, R.J.; GOLDBLATT, P.J. Trout hepatocyte culture: isolation and primary culture. **In Vitro Cellular & Developmental Biology**, v.21, n.4, p.221-8, 1984.

15- KOCAL, T.; QUINN, B.A.; SMITH, J.R.; FERGUSON, H.W.; HAYES, M.A. Use of trout serum to prepare primary attached monolayer cultures of hepatocytes from rainbow trout (*Salmo gairdneri*). **In Vitro Cellular & Developmental Biology**, v.24, n.4, p.304-8, 1987.

16- KOCAN, R.M.; SABO, K.M.; LANDOLT, M.L. Cytotoxicity/ genotoxicity: the application of cell culture techniques to the measurement of marine sediment pollution. **Aquatic Toxicology**, v.6, p.165-77, 1985.

17- LANNAN, C.N.; WINTON, J.R.; FRYER, J.L. Fish cell lines: establishment and characterization of nine cell lines from salmonids. **Toxicology in Vitro**, v.20, n.9, p.671-6, Sept., 1984.

18- LIPSKY, M.M.; SHERIDAN, T.R.; BENNETT, R.O.; MAY, E.B. Comparison of trout hepatocyte culture on different substrates. **In Vitro Cellular & Developmental Biology**, v.22, n.6, p.360-2, Jun., 1986.

19- MAITRE, J.L.; VALOTAIRE, Y.; GUGUEN, C.G. Estradiol 17b stimulation of vitellogenin synthesis in primary culture of male trout hepatocytes. **Toxicology in Vitro**, v.22, p.337-43, 1986

20- PAULSON, H.; CLAUDIO, T. Temperature-sensitive expression of all-Torpedo and-rat hybrid in mammalian muscle cells. **Journal Cell Biology**, v.110, n.5, p.1705-17, 1990.

21- PIERSHBACHER, M.D.; RUOSLAHTI, E. Cell attachment activity of fibronectin can be duplicated by small synthetic fragments of the molecule. **Nature**, v.309, p.30-3, 1983.

22- SEGLEN, P. Preparation of rat liver cells, 3: enzymatic requirements for tissue dispersion. **Experimental Cell Research**, v.82, p.391-8, 1973.

23- SHORS, S.T.; WINSTON, V. Detection of infectious hematopoietic necrosis virus in invertebrate. **American Journal Veterinary Research**, v.50, n.8, p.1307-9, 1989.

24- WATANABE, T.; NAKANO, M.; ASAKAWA, H.; MORITOMO, T. Cell culture of rainbow trout liver. **Nippon Suisan Gakkaishi**, v.53, n.4, p.537-42, 1986.

25- WOLF, K.; DUNBAR, C.E. Cultivation of adult teleost tissues *in vitro*. **Procedure Society Experimental Biology Medicine**, v.9, p.455-8, 1977.

26- WOLF, K.; MANN, J. Poikilothermic vertebrate cell lines and viruses: a current listing for fishes. **Toxicology in Vitro**, v.16, p.168-79, 1980.

27- WOLF, K.; QUIMBY, M.C. Fish cell and tissue culture. **Fish Physiology**, v.3, p.253-305, 1969.

28- WOLF, K.; QUIMBY, M.C. Procedures for subculturing fish cells and propagating fish cell lines. **Tissue Culture Association Manual**, v.2, n.4, p.471-4, 1976.

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