ACTA SCIENTIFIC MICROBIOLOGY (ISSN: 2581-3226)

Volume 3 Issue 1 January 2020

Research Article

Efficient Establishment of Cell Culture from *Xenopus laevis* as a Model for *In Vitro* Studies

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Received: November 25, 2019; **Published:** December 09, 2019

DOI: 10.31080/ASMI.2020.03.0451

Abstract

Cell lines are useful tools to facilitate in vitro studies in virology, epizootology and many other biological field of research. We describe an efficient method of primary cell culture obtaining from pool of internal parenchymal organs of tadpoles *Xenopus laevis*. The enzymatically digested tissue attached well to previously treated cell culture surface and start of exponential cell growth ranged from 10 to 14 days. Cells had fibroblastic morphology and normal diploid karyotype up to ten passage. African clawed frog cell culture was propagated continuously, cryopreserved and recovered successfully. Acceptable cell growth occurred at 29°C in standard medium DMEM after osmotic pressure correction and FBS adding. This cell culture would be used in the study of viral diseases of amphibian, as well as fundamental virological and epizootological investigation.

Keywords: African Clawed Frog (*Xenopus laevis*); Cell Culture; Amphibian; Fibroblast

Introduction

We are living in a time of rapid change. This applies to all sectors of the economy, knowledge, including science, health and the environment. New factors are emerging that directly affect biological objects, leading to unknown consequences of their action. There are nanoproducts, new chemicals that are part of medicines and various products, as well as new pathogens of infectious diseases, including those that cause emergent, re-emergent diseases. Viral infectious diseases (both well-known and researched, as well as new, emergent ones) are widespread worldwide and cause significant economic damage not only to livestock, but also to the threat to wild, rare and endangered animal populations [1]. Given the rapid globalization of the world, for some pathogens the boundaries of their circulation are disappearing, they are spreading to new continents, territories, hosts.

Therefore, the problems of biological security in the current environment is an important component of the overall security of the state. And one of the biggest mode of the pathogen spread is animal trade. Since the mid 20th century, a wide range of amphibian species has been represented in the international pet trade. It may be surprising to hear that 220 of the world's amphibian species are used for food [2] and that at least 278 species of amphibian were in the pet trade alone [3]. The majority of animals in trade (all species) coming from wild sources and commercial collection of wild amphibians has often been resulted in significant reductions in amphibian populations and the international trade in frog legs still largely depends on the collection of wild animals. The main centers of export for the amphibian pet trade are the wet tropics, with species largely coming from the rainforests of Africa (including Madagascar), Central America, South America and Southeast Asia, India and Bangladesh [4]. And the largest consumption market is Western Europe, particularly France, Belgium, the Netherlands, Luxembourg, and Switzerland [5,6,7]. In order to meet market demands for frogs, commercial frog aquaculture has developed in some countries. parts of the world. Fishery statistics available from the FAO indicate that farms in China alone annually produce more than 70 tons of frogs (all species) [4]. Unfortunately, the one of the biggest problem of frog aquaculture is the escaping of large, often non-native amphibians (such as the American Bullfrog) and their introducing new pathogens into surrounding environments [8-12]. Apart of this the illegal animal trade the vast bulk of the international pet trade in amphibians comprises wild-caught specimens. Although most amphibian species kept as pets have been now bred in captivity on occasion, the great bulk of all animals in trade are wild-caught. The emergence of a large number of different animal species that have not previously been found in this territory has a significant impact on the epizootic, epidemic and environmental situation and biosecurity of the state as a whole, since the role of these animals in the epizootic/epidemic chains of many infectious diseases remains. In the world, infectious diseases of amphibians have received more attention since 2009. Despite the fact that ranavirosis and chitridiomycosis of amphibians have been recommended by the OIE for diagnosis "to be monitored, given their importance for wildlife, animal and people welfare'' [13] - the number of studies in this area remains very limited. One of the least studied biological objects in terms of epizootology is amphibians. This is despite the fact that they are numerous and are quite ancient ani-

mals, considering evolutionary studies. But, unfortunately, these animals are poorly researched as possible reservoirs and vector of different pathogens, as well as their potential for risks and their role in the epizootic or epidemiological chain [14]. Cell cultures are still indispensable methods of in vitro studies of many biological and molecular processes, and remain the "gold standard" for the study of various infectious agents. And it is known that one of the most widely used laboratory objects in the world of science is frogs. It was frog cells that were the first cell culture to be obtained, thanks to the work of Harrison, who in 1907 cultivated the nerve cells of the frog embryo [15]. This achievement led to a revolution in world science.

The potential value of amphibians to modern medicine is coming under increasing scientific study, and the most part of it was made with cell culture method using. Chemical secretions identified so far fall into the four categories of biogenic amines, bufodienolides (bufogenins), alkaloids and steroids, and peptides and proteins and many of these considered to be potentially very useful chemicals [16-18]. Even three amphibian antimicrobial peptides that inhibit Human Immunodeficiency Virus (HIV) had obtained from a study of amphibians and these peptides have also been found to be effective at preventing transmission of HIV from dendritic cells to T cells [19].

Materials and Methods

All The tadpole African clawed frog (*Xenopus laevis, Pipidae, Anura*) were bought from local animal trade company. All experimental procedures using animals conformed to the guidelines established by the Animal Care Committee, Nagoya University [20]. The euthanized tadpoles were washed extensively during 3 min with Decasanum and were washed twice with sterile 1x PBS after this. Animals were dissected in the BSL-2 laminar hood with aseptic technic maintenance. The pool of internal parenchymal organs were collected separately from each animal (n=9). Four methods of primary cell culture obtaining were used: the primary explants method and three different trypsization methods. All obtained tissue specimens were disaggregated in sterile Petri dish with sterile surgical instruments. 1xPBS with Penicillin 100IU/cm^3, Streptomycin 100µg/cm^3 Gentamicin 50µg/cm^3 were used for periodically disaggregated tissue washing. For the primary cell culture a 12-well cell culture plate were applied. The half wells in plate were pretreated for better cell growth by coating with FBS in 14 days before cell seeding by application of 200 µl of FBS per well and storing at 4°C prior using.

For primary explants method implementation the 1 g of tissue after primary mechanical disaggregation were immersed in the cell culture medium and distributed evenly between 6 wells of 12-well plate. The rest of the minced tissue were digested with 0,25% trypsin with magnetic stirrer using at different temperature condition and exposure time. The first method consisted of digestion of tissue at temperature condition 4°C over night. The second one is using of 0,25% trypsin at 18-22° C and the third one is using of preheated to 37°C 0,25% trypsin. After that the cell suspension were aspirated by sterile Paster pipette, equal volume of complete culture media were added and centrifuged at 300g 10 min. Cell pellet were resuspended in complete medium, viability was measured by Trypan blue staining and seeded at concentration $10x10^3/cm^2$. In case of tissue explant method using the 0,1 g of tissue per well were seeded. For the best primary cell culture method determination, the three critical factors contributing to cell growth were evaluated: viability and adhesion to substrate [21,22].

The cell culture was maintained at 29°C under 5% CO_2 in a humidified chamber of incubator in 75% DMEM medium (Sigma) with 15% FBS (HyClone). After the first week, the medium was changed twice per week until cells reached 90% confluence. For passaging cells were gently washed with versene 0,02%, treated with 0,25% trypsin and passaged in three-fold dilutions.

The cryopreservation of frog cells was made in a manner similar to the method used for mammalian cells. The cryoprotective medium consisted of 10% dimethyl sulfoxide (DMSO) (Arterium), 30% FBS and 60% grows media with cells.

The karyotype of African clawed frog (*Xenopus laevis*) was determined using the cells obtained from fifth passage. Chromosome preparations were made following standard protocol [23].

Results and Discussion

The mechanically chopped non-digested tissue fragments and cells obtained as enzymatic digestion result, have demonstrated different quantity and viability levels as well as levels of attachment to the treated and non-treated substrate at 24 and 48 h after seeding.

The method of primary explants provided a high percentage (90-96%) of living cells obtained from amphibian tissues, but low yield of the cells per 1 g of tissue 1,2 x 10^6 cells/g in compare with enzymatic disaggregation methods. The low adhesion efficiency (10-25% of the total number of inoculated cells after 48 hours) also was noticed. It was less effective than the enzymatic disaggregation method. The cold trypsinization method (at 4-8°C) during 12 hours ensured the cell yield per gram of tissue on average higher by 54,1% than the primary explant method. The number of viable cells was 55-68%, and adhesion was the lowest among all the methods used (it was 4-5.5% after 48 hours). The method of fractional trypsinization (without preheating) with the use of 0,25% trypsin solution at a temperature of 22°C provided the highest cell

yield — $11.6x10^6$ cells/g of tissue. The viable cells quantity were in the range of 90 - 91%; the adhesion efficiency was 41% for 48 hours. The enzymatic disaggregation at 37°C had more damaging effect on cells, than trypsinization at temperature 22°C insofar as adhesion efficiency and viability of cells were approximately on 14% and 20% lower.

The optimal substrate for adhesion and proliferation of primary amphibian cell cultures was the serum-coated surface, which provided adhesion of largest cell counts at 24-48 h and was cost/result effective enough.

The frog cells have been storing successfully in liquid nitrogen for 6 month without losing of viability. The recovery was made in 29-30°C water bath until the ice was completely thawed, after that cell suspension were immediately transferred to flask with complete growth medium and cultured at 29°C. After thawing, more than 70% of the cells were viable and grew well.

The results of the karyotyping showed that obtained cells were diploid, exhibited the expected chromosome number (2n=36) and had normal diploid karyotype which is the same as that of African clawed frog (*Xenopus laevis*) (data not shown).

In our study, a primary cell culture from the pool of internal parenchymal organ of tadpoles' African clawed frog (*Xenopus laevis*) have been obtained successfully.

The cells obtained in result of enzymatically digestion of tissues at temperature conditions 18 - 22 °C, which close to preferred optimal temperature zone (POTZ) of this frog, have demonstrated the most quantity viable and attached cells. By contrast, enzymatic digestion of the tissues at 37°C led to decrease of attached cells approximately at two times, which may indicate the temperature damage of cells. Tissue pieces that had not been enzymatically digested were not easy to attach to the substrate. However, proper attachment of cells to the growth surface is essential for normal cell physiology and one of the critical factor in initiating cell proliferation [24]. To increase of tissue attachment to the flask bottom, we investigated modification of the growth surface. The most common methods for increasing attachment of cells is coating of surface with attachment factors, such as collagen IV, gelatin, fibronectin, but they are not always at hand and can be expensive. We used cheap and effective enough method of treating of cell culture flasks with FBS, which contain the most of this attachment factors. We observed that the surface treated with FBS surpassed the untreated growth surfaces, both in terms of adhesion and proliferation.

Primary cells proliferation from the pool of internal parenchymal organ of tadpoles' African clawed frog (*Xenopus laevis*) were observed 10-14 days after the start of cell culture. The cells displayed fibroblasts like, spindle shaped morphology, unlike the majority of other amphibian cell lines what have been previously described in African clawed frog (*Xenopus laevis*) [25-28] (Figure 1).

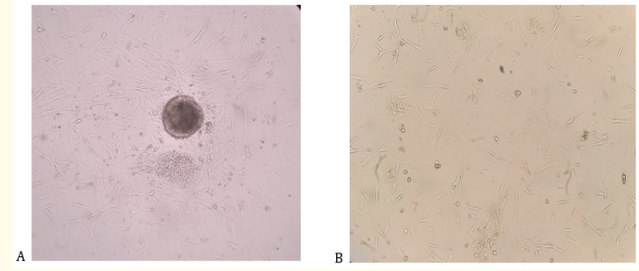

Figure 1: *X. laevis* primary fibroblasts. (A) Fibroblasts proliferating from an explant (100×). (B) Fibroblasts obtained in result of enzymatically digestion (100×).

Formation of monolayer depended on the type of surface. Non-treated surface to a lesser extent contributed to the attachment and proliferation of explants and cells, respectively, time until monolayer became confluent was markedly longer than in the wells with FBS coated surface. In our case, it was 18-25 days from establishment of primary cell culture until first passage. The proliferation speed of *X. laevis* cell line was relatively slow in compare with mammalian cell lines, such as Vero, BHK-21, MDBK etc and tend to reach confluence in 9–12 days.

Another one big difference between warm-blooded and cold-blooded animal cell culture was the optimal temperature of proliferation. Previous literature suggests, that optimal temperatures for proliferation of poikilothermic animal cell have to be near the POTZ or slightly above. In our case the optimum growth of subcultures was observed at 29°C, with the highest proliferation index at 72-hour and the fastest confluent formation occurred within 10 ± 2 days.

Appropriate osmotic pressure, as well as, temperature conditions are important for cellular survival and proliferation [29,30]. The most body fluids of amphibian have the osmotic pressure lower than that of mammals, 180-250 mOsm/L [31], and cell culture medium used for mammalian cell cultures may not be appropriate for amphibian cell culturing. The osmotic pressure of the standard medium was reduced to necessary for amphibian body fluids by adding of sterile water for injection.

All of the above, emphasizes the differences in biology and physiology between mammalian and poikilothermic animals' cells, highlighting the importance of using appropriate cell lines as a platform for virological and epizootological studies.

Our karyotype results showed that our cells possess a stable chromosome number. This data confirmed that the cells were indeed derived from the species *Xenopus laevis.*

Conclusion

In conclusion, we successfully developed easy and chip method of African clawed frog (*Xenopus laevis*) cell culture obtaining. Amphibian cell lines like this one will be useful for virological and epizootological studies.

Conflict of Interest

The authors declare no conflict of interest.

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