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Evaluation of substitution of fetal calf serum in VERO cell cultures by fish serum

El-Dakhly A.T[1], Azab El Sayed Azab[2*] Qutaiba K. Alrawi[3] and Nuri Mohamed Lashkham[4]

[1]Veterinary Serum and Vaccine Research Institute, Abassia, Cairo, Egypt
[2]Department of Zoology, Faculty of Science, Alejelat, Zawia University, Alejelat, Libya
[3]Department of Laboratory, Faculty of medicine technology, Surman, Zawia University, Surman, Libya
[4]Dean of Faculty of Medicine Technology, Surman, Zawia University, Surman, Libya
Corresponding author

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A B S T R A C T

To assess the effect of using fish serum as a growth medium supplement instead of fetal calf serum on the growth and proliferation of VERO cells culture, an investigation was performed. We added the same different concentrations of both serums separately to well established VERO cell cultures. It was found that the total lipid concentration in FS was more than threefold that in FCS. In addition, a decrease in FS concentration from 10 to 2% markedly increased cell density compared with FCS which gave the highest increase in cell density at 10% concentration. Consequently, the use of FS at low concentrations as a growth medium supplement may be useful for the growth VERO cells

Introduction

Animal cell culture is a basic technique in the fields of biology and medicine. The production of living cells in vitro, in the laboratory, permits numerous applications that would be difficult or impossible in vivo, in the living animal. The culture of animal cells requires a defined medium containing specific quantities of certain chemicals in addition to (for most cells), up to 15% of an undefined nutrient medium usually fetal bovine serum (FBS). Serum from newborn calves and other mammals is also used, but FBS is preferred because of its high level of growth factors and low cross-reactivity with other animal cells. FBS or other mammalian products are also used to coat the surface of culture-ware to promote cell attachment (Schinke *et al.*, 1996; Van der Valk *et al.*, 2004). The production of FBS costs a lot annually in many countries for example; in USA, it is estimated as 700,000 liters annually worth $300 to $400 million.

The industry obtains fetal calf serum by bleeding from slaughterhouses, or in some cases, rearing herds of cattle for this purpose. These herds are held in as isolated as possible in order to prevent diseases.

Whole blood is obtained aseptically (by syringe) from an animal, centrifuged to separate cells from serum, and the serum filtered to 0.22 microns to remove most bacteria. Often, serum is heated to 56°C to inactivate the complement system, and group of immune protein.

Contamination of cell cultures because of infectious organisms in serum can be a serious problem. Bacteria, fungi, viruses and *Mycoplasma* have been isolated from bovine serum. In the period from 1960 to 1980, *Mycoplasma* from bovine serum was the second major group of contaminants found in cell culture (Barile, 1977).

Now, FBS is usually screened for *Mycoplasma* and most viruses. However, a more serious cause for concern is an all-protein infectious agent called a prion for which no test is available (Kingman, 1993). This organism causes a fatal brain disease in mammals called Bovine Spongiform Encephalopathy (BSE), or "mad cow disease".

BSE occurs in sheep, cows, and other mammals, and is most likely the cause of similar neuro-degenerative diseases in humans. In Britain in 1986, BSE resulted in the destruction of cattle and fears for the safety of the meat supply and other bovine products. Since then, the disease has turned up in cattle in many other countries.

Consequently, serum from these countries cannot be imported for use in the U.S. and other countries. Basic texts in cell culture teach mammalian sera, especially FBS, for culture of mammalian cells (Freshney, 2005). So firmly established is this dogma of FBS, that some cell culture publications refer only to "serum" when FBS is intended (Pollard and Walker, 1990). Likewise, major serum suppliers such as Sigma Chemical Co. and Hy-Clone Laboratories include only bovine and some equine sera in their cell culture catalogs. In practice during the past thirty years, only mammalian sera have been used for the culture of mammalian cells.

Hence, trials have been made to find another source for nutrient medium instead of FBS to overcome the problem of microbial contaminants. Sights have been directed to fish as a source of nutrient medium. To date, no DNA or RNA viruses infecting fish have been reported to infect humans (Yoshimizu and Kasai, 2005). Thus, employing fish serum (FS) in mammalian cell culture for medically related use should be safer than employing FCS (Masashi *et al.*, 2009). However, the stimulating activities of FS for the adhesion and proliferation of most types of cell cultures are unknown.

Nine permanent cell lines have been established from five species of salmonids native to America's Pacific Northwest. With the exception of a hepatoma from an adult trout, the lines were derived from normal tissues of embryonic or juvenile fish. Cells were routinely grown in Eagle's minimum essential medium with 10% fetal bovine serum. Six of these lines were demonstrably free of any microbial contamination but Mycoplasmas were found in three (Lannan *et al.*, 1984).

Previous research has shown interesting results on the utilization of fish serum (FS) prepared from wash water of surimi processing line. FS was used as a substitute for FBS in two types of hybridoma cell cultures (Zakaria-Runkat *et al.*, 2006). It was shown to strongly stimulate cell proliferation (Rosa *et al.*, 2010).

We can use fish sera to avoid the cross reactivity and contamination especially by Mycoplasmas so that employing fish serum (FS) in VERO cell culture for medically related use should be safer than employing

FCS. However, the stimulating activities of FS for the adhesion and proliferation of VERO cells are unknown. Some searcher used fish sera to fish cells and mammalian tissue culture.

The trials for use of fish sera had mixed results, some searchers found the growth response excellent (Kunst, 1961; Tomasec *et al.*, 1964; Stephenson and Potter, 1967; Rathore *et al.*, 2001). But other searchers found that fish sera had been inhibitory or toxic.

The present study aimed to investigate the influences of using fish serum on VERO cell line' growth compared with those of FCS.

Materials and Methods

Fish serum

Fish serum (FS) was supplied by Biotech, Inc. It was obtained from aseptically drawn whole blood from salmonids. This serum has not been heat-treated because it will get gel if it is heated alone.

To prevent gelling, we added twice the volume of defined media (Minimum essential medium) (MEM), before heating to 55^{o}C for 30 minutes.

Fetal calf serum

Fetal calf serum (FCS), (virus and *Mycoplasma* screened) was used as supplement for cell culture media. It was supplied by Gibco–USA.

Tissue culture

African Green Monkey Kidney (VERO) cells (Yasumura and Kawatika, 1963) were used in this study. The cells were seeded at density of 2×10^4 per well into 24-well micro titer plate.

Tissue culture media

Minimum essential medium (MEM), produced by Gibco with Hank's salts, L-glutamine and without sodium bicarbonate was used for propagation and maintenance of VERO cells. It was prepared according to the manufacturer's instructions. The medium was supplemented with Fish serum (FS) or Fetal calf serum (FCS), as growth medium and 0.1 mg/mL streptomycin (Sigma, St. Louis, MO, USA), and 100 U/mL penicillin (Sigma) at PH 7.2. The medium containing FS or FCS was prepared by adding the serum at various concentrations (0%, 2%, 4%, 6%, 8%, and 10%). The evaluation of tissue cultures growth was performed on micro titer plate (24-well plates) each plate representing one level of growth medium supplementation 0%, 2%, 4%, 6%, 8% and 10% of FS or FCS.

Tissue culture

VERO cells microtiter plates were established and cells were seeded at density of 2×10^4 cells/cm^2 into 24-well microtiter plates. Then the inoculated plates were incubated at temperature of 30^{o}C.

Evaluation of tissue culture growth

A - Density of VERO cells in the culture was determined by counting cells nuclei using haemocytometer after nuclei staining, at which the adhering cells were incubated in a solution of 21 g/L citrate and 1 g/L crystal violet, and the stained nuclei were counted under a microscope (Sanford *et al* 1951).

B - The resazurin reduction test is the ideal test in vitro to evaluate the rate proliferation of VERO cell. It is the most simple and rapid test (Fields and Lancaster, 1993).

Resazurin (blue and nonfluorescent) is reduced to resorufin (pink and highly fluorescent) whereby 10% of commercially available solution is added to the cell medium and measured by fluorimetry. It exhibits an absorption peak at 600 nm (Brien *et al.,* 2000).

C - Lipid content in FS and FCS was determined using an automatic biochemical method (Masashi *et al.,* 2009).

Statistical procedures

The data was analyzed by ANOVA to determine the significance of differences among means (Dean *et al.,* 1994).

Result and Discussion

To evaluate the effect of culturing VERO cells in a medium containing fish serum, VERO cells were cultivated for six days in media containing different concentrations 0%, 2%, 4%, 6%, 8% and 10% of fish serum. At the same time another VERO cells were cultivated in media containing the same different concentrations of fetal calf serum. Where the cell cultivated in media containing 0% and 10% fetal calf and fish serum considered as negative and positive control respectively.

The total lipid concentration in the used two types of serum

Phospholipids, triglyceride, cholesterol and total lipid concentrations were determined in both tested types of serum. It was found that the total lipid concentration was 2120×10^4 mg/L and 99×10^4 mg/L in 10% FS and FCS respectively as shown in table 1 and 2. While, in 2% concentration of FS, the total lipid concentration was 460×10^4 compared to the concentration of 10% FCS was 99×10^4 mg/L as shown in table 1 and 2.

The effect of different levels of both tested serum types on VERO cell density

The analysis of the results showed positive effect ($P < 0.05$) of the increasing levels of FCS on the proliferation of VERO cells.

On the other hand, it was found that VERO cell density in 2% fish serum containing media was the highest between the tested fish serum concentrations (2.9×10^5 cell) ($P < 0.05$) and relative fluorescent units (RFU) was 228 (p □ 0.01). While the cell density in 10% fish serum media was the lowest between the tested concentrations (0.42×10^5 cell) and RFU were 56 as shown in table 3 and panel 1, respectively.

The highest density of VERO cells noticed in the medium containing fetal calf serum was (3.9×10^5 cell) at 10% concentration and RFU was 394. On contrary, the cell density in 2% fetal calf serum containing media was the lowest between the tested concentrations (2.7×10^5 cell) and RFU was 394 as shown in table 3 and panel 2. Meanwhile the cell density in 2% fish serum containing media was slightly lower than those in 10% FCS media. While, the cell density in 10% fish serum containing media were sharply decline than those in 10% FCS media. Generally, it was observed that, VERO cell density was markedly increased as FS concentration decreased up to 2% compared to increased cell density with 10% FCS.

The results were conventional to a certain degree, since FCS (10%) is the choice growth media concentration supplement for many cultured VERO cells and mammals' cells as shown in our results and previous works of many authors (Freshney, 1987, 1989; Allen, 1987; Konigsberg, 1979; Hay *et al.,* 1992 and Dayton and Allen, 1987). In the same time, the results cleared that decreasing FS concentration to 2% markedly

increased the cell density approximately to a level that did not differ significantly from using 10% concentration of FCS. That comes in accordance with results of Sawyer Evelyn and Sawyer Philip (1995), Zakaria-Runkat *et al.* (2006) and Masashi *et al.* (2009).

Fanous (2010) mentioned that the use of salmon blood as a component of growth media. It contains easily digested proteins and a high concentration of poly-unsaturated omega-3 acids. He added that the use of salmon blood thus could be extended to mammalian cell culture.

The supplementation of growth medium with increasing levels of FS (4% up to10 %) showed a negative decreased stepwise effect on the proliferation of VERO cells which may be due to increasing lipid concentrations as exposed in FS (10%) media where total lipids reached 2120×10^{-4} mg/L as shown in table 1 and 3.

While, supplementation of growth medium with decreasing levels of FS (2%) showed a positive effect on the proliferation of VERO cells wherever the total lipids at these concentration was 460×10^{-4} mg/L as shown table 1 & 3 and panel 1.

Therefore, the difference in response to serum concentration between FS and FCS mentioned above may be due to the markedly higher concentration of lipids in FS than in FCS, an attribution agreed with that stated by Masashi *et al.* (2009)

Conclusion

It can be concluded that, the use of fish serum at low concentrations as a growth medium supplement may be useful for the growth VERO cells. Decreasing concentrations of fish serum might be successful in obtaining suitable growth of VERO cells in adhesion culture without using fetal calf serum.

Table.1 Lipid concentration in the used fish serum at different concentrations

Type of lipid Serum Concentration	Phospholipids mg/L	Triglycerides mg/L	Cholestrol mg/L	Total lipids mg/L
Pure serum	6.45	2.95	2.1	11.5
2 % diluted serum	258×10^{-4}	118×10^{-4}	84×10^{-4}	460×10^{-4}
10 % diluted serum	1290×10^{-4}	590×10^{-4}	240×10^{-4}	2120×10^{-4}

Table.2 Lipid concentration of the used fetal calf serum at different concentrations

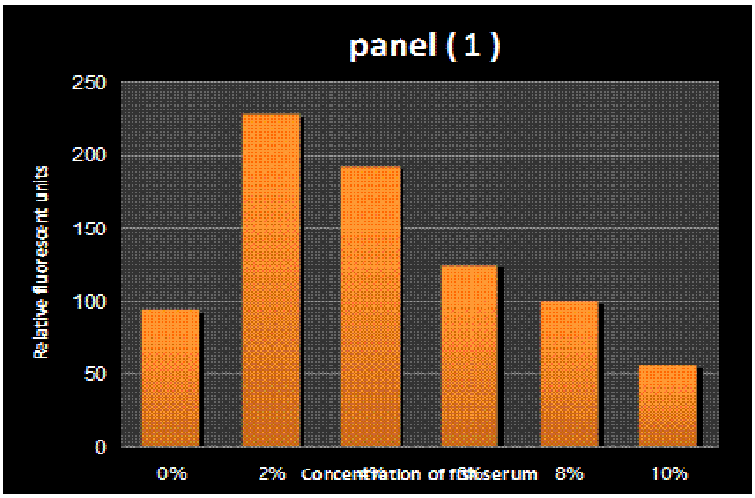
Type of lipid Serum Concentration	Phospholipids mg/L	Triglycerides mg/L	Cholestrol mg/L	Total lipids mg/L
Pure serum	2.35	1.4	1.2	4.95
2 % diluted serum	94×10^{-4}	56×10^{-4}	48×10^{-4}	198×10^{-4}
10% diluted serum	470×10^{-4}	280×10^{-4}	240×10^{-4}	99×10^{-4}

Table.3 The effect of the used different concentrations of fish and fetal calf serums on growth of VERO cells after being cultivated for 6 days

Type of serum concentrations	0 %	2%	4%	6%	8%	10%
F. C. S	0.63	2.7	3.15	3.5	3.8	3.9
F. S	0.63	2.9	2.2	1.4	0.6	0.42

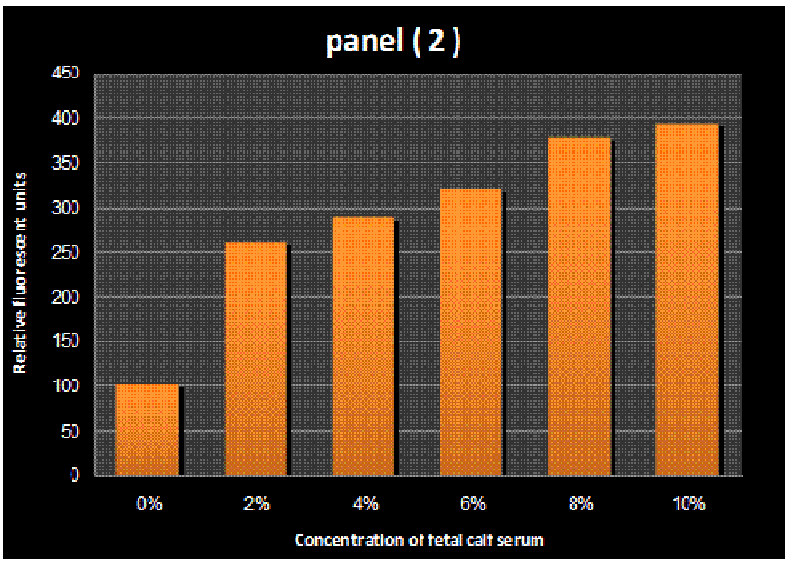
*This values are multiplied by 10^5. Treatment means did not differ significantly (P > 0.05).

Fig.1 The effect of the used different concentrations of fish serum on growth of VERO cells after being cultivated for 6 days



Treatment means did not differ significantly (P □ 0.01).

Fig.2 The effect of the used different concentrations of fetal calf serum on growth of VERO cells after being cultivated for 6 days



Treatment means did not differ significantly (P □ 0.01).

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