Study of Vero Cell Growth in A Modified Serum-Free Medium (SFM01-M)

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Abstract

The aim of this work was to study a modified serum-free medium (SFM01-M) in supporting Vero cell growth in both static and microcarrier cultures. SFM01-M was developed using a systematic design of experiments (DoE). In static cultures, a concentration of rhEGF in SFM01-M was first studied in order to find its concentration suitable for using in the long-term subcultures of Vero cells and in an attempt to assess the minimizing of a cost of SFM01-M as a result of the addition of rhEGF. The results shown here indicated that the addition of rhEGF at least 0.015 mg/L in SFM01-M had effect on cell yields, particularly during subcultures under a serum-free condition (P1-P3). Additionally, a comparison of cell yields obtained from P3 between the rhEGF-added SFM01-M and the rhEGF-free SFM01-M cultures was found to be significantly different (P-value < 0.05). Therefore, in the long-term subcultures of Vero cells, a concentration of rhEGF at 0.015 mg/L was chosen to add to SFM01-M. The results showed that the cell yields obtained during P1-P9 were in the range of 7.09±0.12–7.33±0.33x10^5 cells/well (6-well plate) and then decreased gradually from P10 to P12. With the gradual decrease of growth ability, the cell yields obtained during P12-P20 were rather lower (5.75±0.39–5.96±0.36x10^5 cells/well) than those obtained from previous passages. By increasing an initial cell density of 4x10^4 cells/cm^2, therefore, the cell yields could be increased in the range of 7.54±0.19–8.00±0.15x10^5 cells/well during P10-P20. In microcarrier cultures, the ability of Vero cells to attach and grow on Cytodex^{TM}1 solid microcarriers in SFM01-M in a spinner flask was found to be close to that of VP-SFM. From this study, Vero cells can be grown in SFM01-M in both static and microcarrier cultures. Moreover, a cost of SFM01-M is relatively cheap (approximately 70 US dollar per liter) and SFM01-M can also be an economical alternative for Vero cell cultures under a serum-free condition.

Key words: a modified serum-free medium, microcarrier, Vero cells

Introduction

Vero cell line, derived from African green monkey kidney, has been widely used for viral vaccine productions (WHO, 1998). To establish cell cultures *in vitro*, serum is of importance for promoting cell growth because it contains growth factors, albumin, transferrin, anti-proteases, attachment factors, minerals, hormones and inhibitors (Hewlett, 1991; Jayme, 1991; Castle and Robertson, 1998; Brunner et al., 2010). However, using serum in cell cultures has a number of problems, particularly the contamination with adventitious agents (mycoplasma, viruses, prions). Moreover, it can make a serious problem in the downstream processing of recombinant proteins (Castle and Robertson, 1998; Van der Valk et al., 2004). Therefore, a number of serum-free media (SFM) have been widely developed, although no a universal SFM available to fit all the cellular requirements (Van der Valk et al., 2004; Keenan et al., 2006). Since different cell types have their own receptors involving in their survival, growth and differentiation; in addition, they also have their own factors released to their environment (Van der Valk et al., 2010). At present, non-animal derived hydrolysates such as plant (soy, wheat gluten, rice) and yeast are often used as SFM additives in cell cultures (Jan et al., 1994; Keen and Rapson, 1995; Heidemann et al., 2000; Franěk et al., 2000; Sung et al., 2004; Chun et al., 2007; Kim and Lee, 2009; Rourou et al., 2009; Babcock et al., 2010; Lobo-Alfonso et al., 2010; Michiels et al., 2011). Additionally, the basic components (insulin, transferrin, sodium selenite, ethanolamine) have been essential for the development of serum-free medium (Taub et al., 1979; Murakami et al., 1982; Chuman et al., 1982; Jäger et al., 1988; Kovář and Franěk, 1989; Eto et al., 1991; Chen et al., 1993; Okamoto et al., 1996; Lee et al., 1999; Kim et al., 1998; Morris and Schmid, 2000; Liu and Chang, 2006; Parampalli et al., 2007; Cervera et al., 2011). Vitamin C is a micronutrient required for biological functions. It is an essential cofactor of α-ketoglutarate-dependent dioxygenases which play a role in the collagen and glycosaminoglycan biosynthesis (Kao et al., 1990; Arrigoni and Tullio, 2000; Traber and Stevens, 2011; Arigony et al., 2013; Lane and Richardson, 2014). Vitamin B12 is required for methionine synthase, folate and polyamine metabolism and *S*-adenosylmethionine metabolism, and is also in the methylation pathway of deoxyribonucleic acid (DNA), RNA, proteins, and lipids (Kenyon et al., 1996; Thomas and Thomas, 2001; Bjelakovic et al., 2006; Arigony et al., 2013). Epidermal growth factor (EGF) is a small mitogenic polypeptide. It plays an important role in the regulation of cell growth, proliferation, and differentiation (Gospodarowicz and Moran, 1976; Bettger et al., 1981; Zhaolie et al., 1996). In the present study, a modified serum-free medium (SFM01-M) was developed for Vero cell cultures under a serum-free condition. The growth of Vero cells in SFM01-M was studied by means of a direct adaptation. The effect of rEGF in SFM01-M on cell yields during the subcultures of Vero cells at passage P0,

P1, P2 and P3 was first studied and then the maintaining of Vero cells in the long-term subcultures was also investigated. In order to develop the growth of Vero cells on Cytodex^{TM} 1 solid microcarriers, the spinner flask cultures were performed and were compared with a serum-containing medium and a commercial serum-free medium.

Research Objectives

The objective of this study was to study Vero cell growth in a modified serum-free medium (SFM01-M) in both static and microcarrier cultures

Research Methodology

Cell line

Vero cells (ATCC® CCL-81^{TM}) were purchased from the American Type Culture Collection (ATCC, Rockville, MD, USA). An initial cell density of 2x10^4 cells/cm^2 was grown in a 75 cm^2 tissue culture flask (T75 flask, Thermo Fisher Scientific, Jiangsu, P.R. China) containing 20-25 mL of a serum-containing medium (SCM) at 37 ^{o}C, 5% CO_2 in a CO_2 incubator (Thermo Electron Corporation, MA, USA) until confluence (about 3-4 days). SCM consisted of Eagle's minimal essential medium (MEM "*Nissui*" No.1, Nissui Pharmaceutical Co., Ltd, Japan), 10% (v/v) fetal bovine serum (FBS, EU Approved: South America, Thermo Fisher Scientific, Auckland, New Zealand), 1.0% (v/v) MEM non-essential amino acids (100X, Thermo Fisher Scientific, MA, USA), 1.0 mM sodium pyruvate (Sigma-Aldrich®, St. Louis, MO, USA), and 2.0 mM L-glutamine (Sigma-Aldrich®, St. Louis, MO, USA). Monolayer cells were washed once with 20-25 mL of phosphate buffered saline (Ca^{2+}, Mg^{2+}-free PBS, pH 7.4), and then detached with 1-2 mL of the trypsin-EDTA solution (0.25% w/v trypsin plus 0.02% w/v EDTA) for 2-5 min at 37 ^{o}C. Dislodged cells were then added with 5-10 mL of SCM in order to inactivate the trypsin activity and were resuspended several times until obtaining single cells. In the SCM culture, cells were subcultured for at least 3 times (passages). In the VP-SFM microcarrier culture, Vero cells were adapted to VP-SFM (Thermo Fisher Scientific, NY, USA) for at least 3 passages and TrypLE^{TM} Select (1X, Thermo Fisher Scientific, NY, USA) was used instead of the trypsin-EDTA solution. Briefly, an initial cell density of 4x10^4 cells/cm^2, which were obtained from SCM culture, were grown in a T75 flask containing 20-25 mL of SCM at 37 ^{o}C, 5% CO_2 for 3 h, and then a spent medium was discarded and replaced directly with an equal volume of VP-SFM. Cells were then grown at 37 ^{o}C, 5% CO_2 until confluence (3-4 days). The first culture of Vero cells in VP-SFM was established as P0 because cells were still contact with FBS. To continue the cell culture for the next

passages, monolayer cells were washed once with PBS, and then detached with 1-2 mL of TrypLE™ Select (1X) at 37 ºC for 2-5 min. Dislodged cells were then added with 5-10 mL of VP-SFM containing 0.5 g/L of soybean trypsin inhibitor (Thermo Fisher Scientific, MA, USA). Cells were separated using centrifugation at 1,000 rpm for 5 min. Cell pellet was resuspended in 5-10 mL of VP-SFM and mixed several times until obtaining single cells. In each cell culture cycle, the initial cell density and the volume of VP-SFM were the same values as mentioned above for P0.

Cell cultures

In this study, the original SFM01-M was fundamentally comprised of Bacto™ yeast extract (0.1 g/L), Bacto™ soytone (0.1 g/L), vitamin C (9.719 mg/L), vitamin B12 (0.173 mg/L), SITE liquid media supplement (0.1% v/v), recombinant human epidermal growth factor (rhEGF)(0.058 mg/L), MEM non-essential amino acids (100X)(1.0% v/v), sodium pyruvate (1.0 mM), L-glutamine (4.0 mM), sodium hydrogen carbonate (2.2 g/L), and MEM medium (9.4 g/L). SFM01-M was prepared as a liquid form. Additionally, SFM01-M was not added with any adhesion factors such as fibronectin, laminin or collagen before used in the experiments.

Effect of rhEGF on Vero cell growth

To investigate the effect of rhEGF on Vero cell growth during the subcultures of Vero cells at passage P0, P1, P2, and P3, the concentrations of rhEGF in SFM01-M were varied as follows; 0, 0.015, 0.029, 0.049, 0.069 and 0.082 mg/L. Cell cultures were carried out in 6-well plates (Thermo Fisher Scientific, Jiangsu, P.R. China). Starter cells were obtained from SCM cultures. An initial cell density of 2.0 x 10^4 cells/cm^2 was inoculated to each well containing 3 mL of SCM, and then incubated at 37 ºC, 5 % CO_2 for 3 h. After that, a spent SCM was removed and replaced directly with each media formulations. Cell cultures were performed in triplicate for each medium formulation and were grown at 37 º C, 5 % CO_2 for 4 days. The first culture of Vero cells was established as P0 because cells were still contact with FBS. After 4 days of cultures, the spent media were discarded and monolayer cells were washed once with PBS. Monolayer cells were detached using TrypLE™ Select (1X) at 37 ºC for 2-5 min. Dislodged cells were added with each medium formulation containing 0.5 g/L soybean trypsin inhibitor in order to inactivate the trypsin activity and then mixed several times. Cells were separated using centrifugation at 1,000 rpm for 5 min. Cell pellet was resuspended with each medium formulation until obtaining single cells. In a series of cell cultures for next passages (P1, P2, and P3), the initial cell density and the volume of each medium formulation were fixed at the same values as mentioned above for P0.

The long-term subcultures of Vero cells in SFM01-M

In the long-term subcultures of cells in SFM01-M, the concentration of rhEGF at 0.015 mg/L was chosen and added to SFM01-M on account of the results derived from the previous experiment. Starter cells were obtained from SCM cultures. The cell culture method was carried out the same as mentioned as in the section “Effect of rhEGF on Vero cell growth”. However, the ability of cell growth in SFM01-M was evaluated for at least 20 passages.

Microcarrier cultures

The ability of Vero cells grown on Cytodex^{TM} 1 solid microcarriers in SFM01-M was evaluated. Cytodex^{TM} 1 solid microcarrier (GE Healthcare, Uppsala, Sweden) was prepared according to the manufacturer’s instructions. Briefly, a concentration of 2.0 g/L (dry weight) was added in a 500 mL siliconized spinner flask (Techne, UK) containing 50-100 mL of PBS and then left for 3 h at room temperature with a gentle agitation (20-30 rpm). After that, a spent PBS was removed and then replaced with fresh PBS. Microcarriers were autoclaved at 121 ^{o}C, 15 psi for 30 min. Prior to using, microcarriers were washed once with a serum-free MEM medium for 5-10 min with a gentle agitation (20-30 rpm). Starter cells were obtained from SFM01-M cultures at passage P5. Cell inoculums were prepared in 100 mL of SFM01-M with a 5 cells/microcarrier ratio and then transferred to a spinner flask. A mixture of both cells and microcarriers in suspension was cultured at 37 ^{o}C and 5% CO_2 with a gentle agitation (10-20 rpm). For SCM and VP-SFM cultures, a concentration of microcarriers, a ratio of cells/microcarrier, the preparation of microcarriers and the microcarrier culture method were carried out the same as mentioned as in the SFM01-M culture unless starter cells were obtained from its own culture.

Cell counting

The growth of Vero cells was determined daily. Spent media in well plates were removed and washed once with PBS. Cultured cells were detached with the trypsin-EDTA solution for 2-5 min at 37 ^{o}C. Dislodged cells were resuspended in SCM in order to inactivate the trypsin activity. A small volume of cell suspension was sampled to a 1.5 mL microcentrifuge tube and viable cells were counted after staining with the 0.4% (w/v) trypan blue solution using an improved Neubauer hemocytometer (Boeco, Hamburg, Germany). In SFM01-M cultures, the trypsin-EDTA solution was replaced by TrypLE^{TM} Select (1X). Briefly, a spent medium in a well was removed and washed once with PBS and then 0.1 mL of TrypLE^{TM} Select (1X) was added to the well. Cultured cells were detached at 37 ^{o}C for 2-5 min until cells were dislodged. Dislodged cells in the well were added with 0.9 mL of SFM01-M containing 0.5 g/L soybean trypsin inhibitor.

Cells were mixed several times and then transferred to a 1.5 mL microcentrifuge tube. Cells were separated using centrifugation at 1,000 rpm for 5-10 min. The spent medium was removed and replaced with 1.0 mL of SFM01-M. Cell pellets were mixed several times before cell counting. For microcarrier cell cultures, a cell counting was carried out by using a mixture of 0.1% (w/v) crystal violet and 0.1 M citric acid. Briefly, 1 mL of sample was taken from the spinner flasks and placed in a 1.5 mL microcentrifuge tube and then left for about 5 min until microcarriers were settled down to the bottom of microcentrifuge tube. The supernatant was removed and replaced with 1.0 mL of the mixture of 0.1% (w/v) crystal violet and 0.1 M citric acid. Sample was left for 1 h at 37 ^{o}C. Viable cells were counted as released cell nuclei by an improved Neubauer hemocytometer.

Statistical analysis

Data were representative of three independent experiments unless otherwise stated and were expressed as mean ± standard deviation (SD). Two-tailed Student's t test was used to compare mean and P-value < 0.05 was regarded as statistically significant.

Results

Effect of rhEFG on Vero cell growth

The results showed that the addition of rhEGF in SFM01-M had effect on cell yields (Fig. 1) during P1, P2, and P3 where cells were subcultured continuously under a serum-free condition whereas in the rhEGF-free SFM01-M cultures, cell yields gradually decreased during those passages. The cell yields obtained from P0 from the rhEGF-added SFM01-M cultures and the original SFM01-M cultures were relatively higher than that obtained from the rhEGF-free SFM01-M culture. However, the cell yields of the rhEGF-added SFM01-M cultures and the original SFM01-M cultures obtained from P1, P2, and P3 were still rather lower than those obtained from P0, particularly in the rhEGF-free SFM01-M cultures. In a group of rhEGF-added SFM01-M cultures and the original SFM01-M culture, the cell yields obtained from P3 were not significantly different and were in the range of $6.5\pm0.1x10^{5}$-$7.0\pm0.4x10^{5}$ cells/well, and were significantly higher than that of the rhEGF-free SFM01-M culture ($4.2\pm0.4x10^{5}$ cells/well).

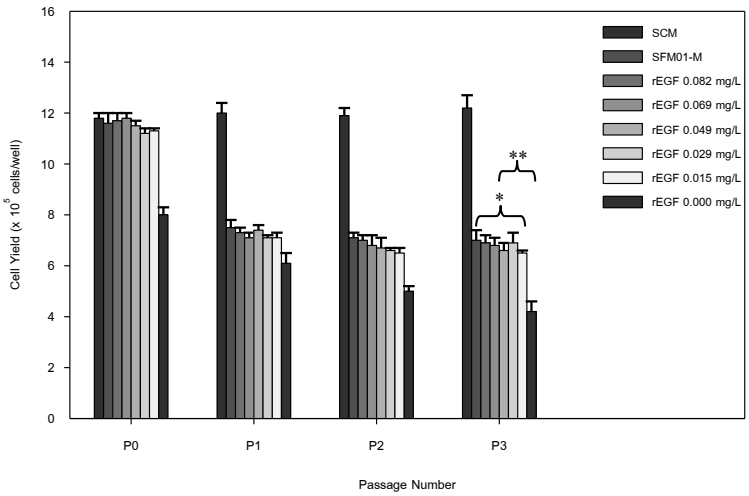

Fig. 1 Cell yields obtained from SFM01-M supplemented with different concentrations of rhEGF (rhEGF-added SFM01-M), compared with SCM and the original SFM01-M. A comparison of cell yields at P3 between the rhEGF-added SFM01-M and the original SFM01-M cultures was not significantly different (* P-value > 0.05). A comparison of cell yields at P3 between the rhEGF-added SFM01-M and the rhEGF-free SFM01-M cultures or between the original SFM01-M and the rhEGF-free SFM01-M cultures was significantly different (** P-value < 0.05). Error bars represent standard deviations with n = 3.

The long-term subcultures of Vero cells in SFM01-M

In this study, SFM01-M was supplemented with 0.015 mg/L rhEGF. The growth of Vero cells in SFM01-M was compared with SCM during the long-term subcultures for 20 passages (Fig. 2). The results showed that cell yields obtained from P1–P9 of SFM01-M cultures were in the range of 7.09±0.12–7.33±0.33x10^5 cells/well and then gradually decreased from P10 to P12. After P12 of cultures, cell yields were relatively constant until reaching P20. The cell yields obtained from P12-P20 were in the range of 5.75±0.39–5.96±0.36x10^5 cells/well with a mean cell yield of 5.86±0.05x10^5 cells/well. To evaluate the ability of Vero cell growth with another cell density, an initial cell density of 4x10^4 cells/cm^2 was chosen (starter cells were obtained directly from P9 of the same culture) for using in a parallel culture during P10–P20. The results showed that the increased cell yields in the range of 7.54±0.19–8.00±0.15x10^5 cells/well with a mean cell yield of 7.72±0.04x10^5 cells/well could be observed. For SCM subcultures, in contrast to SFM01-M cultures, throughout 20 passages a mean cell yield of higher than 11x10^5 cells/well could be

obtained. Therefore, the addition of rhEGF at least 0.015 mg/L to SFM01-M can maintain efficiently the growth and proliferation of Vero cells, particularly in the long-term subcultures. Moreover, a cost of SFM01-M supplemented with 0.015 mg/L rhEGF can be reduced up to approximately 70 US dollars per liter.

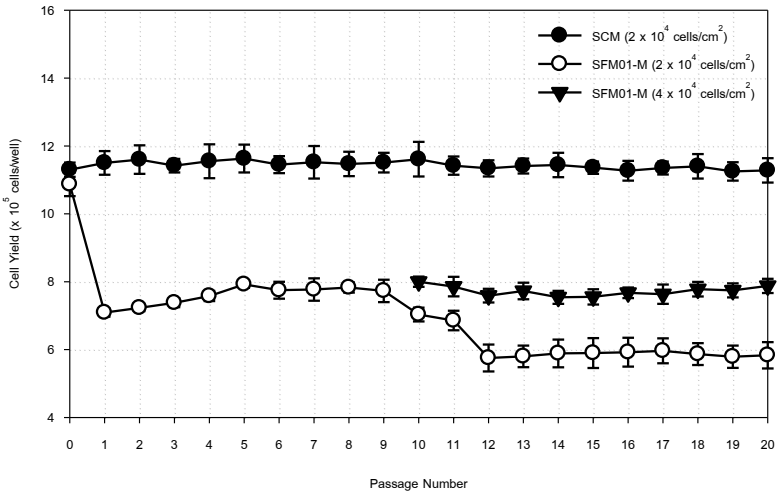

Fig. 2 Cell yields obtained from the long-term subcultures of Vero cells in SFM01-M containing 0.015 mg/L rhEGF compared with SCM. An initial cell density of 2 x 10^4 cells/cm^2 was used in both SCM and SFM01-M subcultures. An initial cell density of 4 x 10^4 cells/cm^2 was used in SFM01-M subcultures during P10–P20. Error bars represent standard deviations with n = 3.

Microcarrier cultures

In microcarrier cultures, the results showed that unattached cells of all cultures rapidly decreased during 60 min of cultures and then were relatively constant until 180 min of cultures (data not shown). The cell attachment rate of SFM01-M was 0.012 min^{-1}, equal and close to that of SCM culture (0.012 min^{-1}) and VP-SFM culture (0.013 min^{-1}) during 30 min of cultures. The percentage of cell attachment of SFM01-M culture was 98.37 % , relatively higher than that of SCM culture (94.65%) and almost close to that of VP-SFM culture (98.60 %) after 180 min of cultures. Cell growth curves (Fig. 3A) showed that the cell concentration of SCM culture was increased rapidly during 96 h of culture with the maximum cell concentration of 38.26±0.75 x 10^4 cells/mL. For two other serum-free media cultures, the cell concentrations were also increased, but gave a much lower concentration of cells at that time. The maximum cell concentrations of VP-SFM and SFM01-M cultures were 17.60±0.36x10^4 cells/mL and 15.27±0.73x10^4 cells/mL, respectively, on the 144th h of cultures. The specific growth rate of microcarrier grown cells

obtained from SCM, VP-SFM, and SFM01-M cultures were 0.029, 0.014 and 0.012 h^{-1}, respectively.

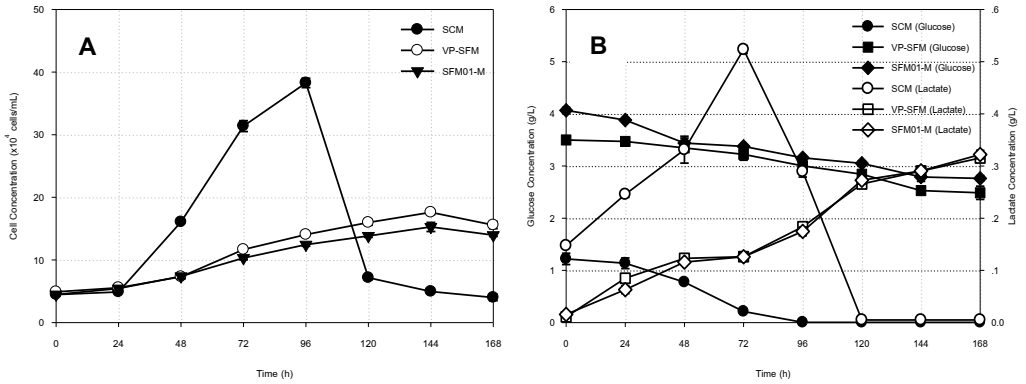

Fig. 3 A) Cell growth curves of SFM01-M culture compared with SCM and VP-SFM cultures and B) Glucose and lactate concentrations of SFM01-M culture compared with SCM and VP-SFM cultures.

Concerning the glucose metabolism during the SCM culture (Fig. 3B), it was found that glucose was consumed rapidly during 96 h of culture as lactate was increased rapidly as well during 72 h of culture. The cell concentration of SCM culture still increased continuously until the maximum point on the 96th h of culture (Fig. 3A) and then decreased sharply until the end of culture. After 72 h of culture, however, the growth and proliferation of cells in the SCM culture was still continued, although a limitation of glucose could be observed as shown in Fig. 3B. The reduction of lactate concentration after 72 h of culture was correlated to the increase of the cell concentration during 72-96 h of culture. For VP-SFM and SFM01-M cultures, a concentration of glucose was decreased slowly whereas a concentration of lactate was increased gradually during 168 h of their cultures. Glucose consumption and lactate production in both serum-free cultures were found to be similar as shown in Fig. 3B.

Discussion

Effect of rhEFG on Vero cell growth

In the original SFM01-M, a concentration of rhEGF at 0.058 mg/L was found to be optimal for Vero cell cultures after the first optimization of the original SFM01-M by using the central composite design (data not shown). However, the ability of Vero cell growth and proliferation has been carried out only at P0 where Vero cells were still contact with serum.

Therefore, the increased cell yields at P0 might occur by a small number of serum components that attach on the cell surface; in addition, the cells are internalized by themselves during a short time of contact with FBS (Hewlett, 1991). Consequently, the ability of Vero cell growth and proliferation after P0 in SFM01-M supplemented with other concentrations of rhEGF should be further investigated in order to find its optimal concentration for using in the long-term subcultures of Vero cells and also in an attempt to assess the minimizing of a cost of SFM01-M preparation due to the addition of rhEGF. From this study, it showed clearly that the cell yields obtained from P0 for all cultures were much higher than those obtained from the next passages (P1, P2 and P3). After P0, the cell yields of rhEGF-added SFM01-M cultures as well as the original SFM01-M cultures at P1, P2 and P3 were rather lower than those obtained from P0. However, at the last passage (P3), the cell yields obtained from the rhEGF-added SFM01-M cultures compared to the original SFM01-M culture were found to be not significantly different. Whereas the comparison of either the rhEGF-added SFM01-M cultures or the original SFM01-M culture with the rhEGF-free SFM01-M were found to be significant different, this indicated obviously that the addition of rhEGF in SFM01-M can improve significantly cell yields. Generally, epidermal growth factor (EGF) is a small mitogenic polypeptide. It plays an important role in the regulation of cell growth, proliferation and differentiation (Gospodarowicz and Moran, 1976; Bettger et al., 1981; Zhaolie et al., 1996). In previous studies, the addition of EGF at 0.020 mg/L in IPT-AFM medium (Rourou et al., 2009), 0.100 mg/L (Clark et al., 1982), and 5 mg/L (Zhaolie et al., 1996) in serum-free medium was found to be essential for Vero cell growth in both static and microcarrier cultures. Additionally, Pettiot et al. (2010b) also reported that EGF was a crucial component for Vero cell culture during the screening of serum-free medium components. From this study, the optimum concentration of rhEGF was relatively wider in the range of 0.015–0.082 mg/L (Fig. 1) corresponding to that used in previous studies. Moreover, the co-culture of embryos with Vero cells was demonstrated obviously that EGF could not be produced by Vero cells (Desai and Goldfarb, 1998). Therefore, the addition of rhEGF to SFM01-M is of much importance for Vero cell growth.

Microcarrier cultures

As reported in previous studies, it was possible that lactate was probably consumed by Vero cells (Mendonça and Pereira, 1998; Quesney et al., 2003; Trabelsi et al., 2006). Generally, about 80% of glucose is converted to lactate and only 4–5% enters the tricarboxylic acid (TCA) cycle for energy production. The accumulated lactate is then converted into pyruvate by lacate dehydrogenase, and pyruvate is utilized by the cells via the TCA cycle to produce energy. For VP-SFM and SFM01-M cultures, glucose decreased slowly, whereas lactate increased gradually.

Glucose consumption and lactate production in Vero cell serum-free cultures were found to be quite different as reported in the previous studies depending on culture conditions studied (Souza et al., 2009; Petiot et al., 2010a; Thomassen et al., 2014). The reduction of glucose was correlated with the gradual increase of cell concentration obtained from both cultures until 144 h of culture. However, the increase of lactate concentration after 144 h of both cultures did not reach the inhibitory level as reported by Hassell et al. (1991) at about 20 mM. The reduction of cell growth after 144 h of both cultures might involve in the limitation of growth surface areas for some high cell densities on microcarriers (contact inhibition), and thus could lead to stop the growth of cells. From this study, the cell growth performance in SFM01-M was relatively equal to VP-SFM. The ability of cell growth and proliferation in SFM01-M was relatively similar to that obtained from MDSS2 and MDSS2N (animal protein-free medium) as reported by Merten et al. (1999). Consequently, SFM01-M can be an alternative for Vero cell cultures in both static and microcarrier cultures.

Conclusion

The aim of this study was to study Vero cell growth in a modified serum-free medium (SFM01-M) in both static and microcarrier cultures. The effect of rhEGF concentration in SFM01-M on the cell yields at P0, P1, P2, and P3 was first studied to find its optimal concentration for using in the long-term subcultures. The results showed that the cell yields obtained from P3 between the rhEGF-added SFM01-M cultures and the original SFM01-M culture were not significantly different whereas a comparison of both of cultures with the rhEGF-free SFM01-M culture were found to be significantly different. For the long-term subcultures of cells, therefore, the concentration of rhEGF was adjusted to 0.015 mg/L in SFM01-M. Cells were subcultured continuously for at least 20 passages in SFM01-M. During P1-P9, the cell yields of 7.09±0.12–7.33±0.33x10^5 cells/well were obtained and then gradually decreased from P10 to P12. A mean cell yield obtained during P12–P20 was 5.86±0.05 x 10^5 cells/well. However, in another culture with a higher cell density of 4x10^4 cells/cm^2, the improved growth of cells could be observed during P10–P20 with a mean cell yield of 7.72±0.04 x 10^5 cells/well. Consequently, the addition of rhEGF at least 0.015 mg/L in SFM01-M is of much importance and can also maintain effectively cell growth throughout 20 passages. In microcarrier cultures, the ability of Vero cells to attach and grow on Cytodex^{TM}1 solid microcarriers in SFM01-M in a spinner flask was found to be close to that of VP-SFM. From this study, the growth of Vero cells in SFM01-M depends mainly on the addition of rhEGF. Vero cells adapted to SFM01-M can attach and grow well on Cytodex^{TM}1 solid microcarriers. Since a cost of SFM01-M is relatively cheap (approximately 70 US dollars per liter),

compared to a commercial serum-free medium (VP-SFM) of approximately 125 US dollars per liter, SFM01-M is probably an economical alternative for serum-free Vero cell cultures. In the future work, SFM01-M will be further studied by culturing Vero cells on Cytodex^{TM}1 solid microcarriers in a larger bioreactor in order to find the optimal condition.

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