Epidermal Growth Factor Promotes E6 and CCL-81 Vero Cells Proliferation Under Serum-Free Condition

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Abstract

Vero cell culture as a platform for producing viral vaccines is an established and standardized process in vaccine manufacture. Generally, Vero cell culture requires media as a source of nutrition with serum supplementation to provide growth factors. However, the serum has several disadvantages including batch to batch variation and adventitious agent. Therefore, chemically defined serum-free media (SFM) are formulated by using standardized growth factors. Epidermal Growth Factor (EGF) is one of growth factors that showed adequate mitogenic support in serum-free medium system, especially in Vero cells. In this study, SFM-EGF media was compared with serum supplementation media, namely MEM 10% and MEM 5% FBS supplementation. The cell morphology was observed using an inverted microscope and their proliferation was evaluated using a MTT colorimetric-based assay. Vero E6 and Vero CCL-81 cells morphology did not show any morphological changes. Vero E6 and Vero CCL-81 proliferation in SFM-EGF media on day one to four did not show a significant difference compared to MEM 10% or MEM 5% serum supplementation media. However, the OD values of both Vero E6 and Vero CCL-81 cells given SFM-EGF media produced an average value below MEM 10% but higher than MEM 5% FBS supplementation. As such, this study proved that utilizing SFM-EGF could support the proliferation of Vero E6 and Vero CCL-81 cells.

Keywords: cell culture, cell-based vaccine production, conditioned media, vaccine

Received: 19 November 2023 **Revised:** 13 December 2023 **Accepted:** 16 January 2024

INTRODUCTION

Vero continuous cell line originates from African Green Monkey kidney epithelial cells which were developed into four subtypes, namely Vero JCRB0111, Vero CCL-81, Vero 76, and Vero E6 (Shen *et al*., 2019, Konishi *et al*., 2022). Vero cell culture has become common because its use has been approved and recommended by the World Health Organization (WHO) due to its safety in producing human viral vaccines (WHO, 1998). Generally, Vero cell culture requires media as a source of nutrition with serum supplementation to support cell growth (growth factors). Serum contains growth factors, albumin, transferrin, attachment factors, hormones, and minerals (Freshney *et al*., 2010). However, the use of serum was limited because it has many disadvantages, which were the risk of contamination from infectious agents such as mycoplasma and bovine spongiform encephalopathy (BSE), the incompatibility in the manufacturing process with animal welfare, the high price of producing the serum, and non-uniform variations between batches resulting in inconsistent results and the use of serum which could have implications for the haram or halal status of a product (Hasyim *et al*., 2012). Another disadvantage of using serum in cell culture media is that there have been reports of cases of allergic reactions to vaccines containing serum (Chruszcz *et al*., 2013). Along with scientific and

technological advances, there is also a lot of demand from various biopharmaceutical industries to produce animal component-free (ACF) raw materials and finished products to reduce serum usage (Hasyim *et al*., 2012).

Therefore, chemically defined Serum-Free Media (SFM) formulations have been carried out. However, until today, there is no serum-free media that can generally be used for all cells because each type of cell has different cell receptors, different types of cell growth, and differentiation (Posung *et al*., 2021). The SFM is an alternative to media that is free from serum SFM can be added with several other ingredients such as growth factors. Growth factors are proteins that bind to receptors on the surface of cells to activate the cells for proliferation and differentiation. The most commonly used growth factor ingredients are EGF, FGF, NGF, and PDGF. EGF consists of a single polypeptide of 53 amino acids (Boonstra *et al*., 1995) to promote mitogenesis, and cytoprotection in epithelial cells such as Vero cells through the binding to a specific tyrosine kinase receptor. (Acosta *et al*., 2008). By adding growth factors such as EGF to serum-free media (SFM-EGF), cells can continue to proliferate even in the absence of serum (Posung *et al*., 2021).

The study about formulating SFM-EGF for Vero cell culture is still limited. This study aimed to assess Serum Free Media–Epidermal Growth Factor (SFM-EGF) formulation which can support the proliferation of Vero E6 and Vero CCL-81 cells based on microscopic morphology and growth proliferation.

MATERIALS AND METHODS

Ethical Approval

This study did not require ethical approval because there was no treatment of animals.

Study Period and Location

The study was carried out from August to September 2023 at the Research Center for Vaccine Technology and Development Institute of Tropical Disease, Universitas Airlangga (RCVTD–ITD Unair), Surabaya.

Vero E6 and Vero CCL-81 Cell Culture

The cell line used in this study was Vero E6 (ATCC® CRL-1586™) Passage 25 (ATCC, USA) and Vero CCL-81 (ATCC® CCL-81™) Vero CCL-81 Passage 23 (ATCC, USA) from the cell bank of the Research Centre for Vaccine Technology and Development, Institute Tropical Disease, Airlangga University (RCVTD–ITD Unair). Vero E6 and Vero CCL-81 cells were cultivated at 2.1×10^6 density in T-flask 75 (Nest, China) maintained in Minimum Essential Media (MEM, Gibco, New York) supplemented with 10% Fetal Bovine Serum (FBS, Gibco, New York), 1% Amphotericin (Gibco, Israel) and 1% Penicillin Streptomycin (Gibco, New York) were then incubated in an incubator (Thermo, New York) at 37°C in a humidified environment with 5% CO_2.

Epidermal Growth Factor Treatment

After the cells reached approximately 80% confluence, the cells were cultivated at 0.05×10^6 density per well in multiwell 24 (M24) (Nest, China) with MEM 10% and incubated in an incubator at 37°C and 5% CO_2 for 24 hours. After 24 hours, the media were replaced with various treatment media such as SFM with 2 ng/mL EGF supplementation (SFM-EGF) (human recombinant EGF; Gibco, USA), MEM 5% FBS supplementation, and MEM 10% FBS supplementation as a control. Cell cultures were maintained for four days in an incubator with a temperature of 37°C and a CO_2 level of 5%, which media was changed every 48 hours. Cell morphology was observed every day for four days using an inverted microscope (Invitrogen™ EVOS™ XL Core Configured Cell Imager; Invitrogen, USA) at 100× magnification.

Tetrazolium-based Colorimetric Assay for Cell Proliferation

Cell proliferation assay was carried out on days 1, 2, 3, and 4 after media changes with treated media using a 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide (MTT) colorimetric-based method. Working solution MTT 5 mg/mL (Sigma-Aldrich, Netherlands) was used in this assay. First, culture medium was

discarded, followed by washing using 100 µL of Dulbecco's Phosphate Buffered Saline (dPBS; Gibco, New York), and discarded, then 100 µL of MTT working solution reagent was added and incubated for 30 minutes at a temperature of 37°C and 5% CO_2 humidity. The MTT reagent was then discarded and 100 µL of DMSO solvent (Merck, USA) was added, then put into an orbital shaker at a speed of 150 rpm and read on plate reader (Biobase, China) with a wavelength of 570 nm to obtain results in the form of Optical Density (OD).

Data Analysis

The data of the study was presented in the form of graphs and images. Parametric statistics were used if the study data met the Kolmogorov-Smirnov normality test with three technical replications (n=3) used in this study. Statistical analysis was carried out using SPSS Statistics Version 26. The F test or ANOVA was used in this study to differentiate between three or more treatment groups, if there were differences between groups, it was continued with Duncan's post-hoc test. Significance is achieved if the $p < 0.05$.

RESULTS AND DISCUSSION

Vero E6 and Vero CCL-81 cells grown for four days in SFM-EGF were compared with commonly used serum supplementation media, namely MEM 10% FBS supplementation and MEM 5% FBS supplementation. In microscopic observations that can be seen in Figure 1, Vero E6 and CCL-81 cells that were given SFM-EGF media from the first to the fourth day showed no change in morphology compared to MEM 10% and MEM 5% FBS supplementation. Both cells' morphology retained typical adherent epithelial shape and formed a well-defined monolayer. The results of the MTT assay on the SFM-EGF did not show a significant difference to the MEM 10% or MEM 5% FBS supplementation which can be seen in Figure 2. Vero E6 and Vero CCL-81cells given SFM-EGF media produced mean values below MEM 10% FBS supplementation but higher than MEM 5% FBS supplementation. As such, it can be concluded that SFM-EGF media was able to support the proliferation of Vero E6 and Vero CCL-81 cells. Average OD results are presented in Table 1. The first day on Vero E6 cells given SFM-EGF media showed significantly different results compared to MEM 10% and MEM 5%. On the second day, SFM-EGF media showed significantly different results ($p < 0.05$) compared to MEM 10% and MEM 5% FBS supplementation. On the third day, the SFM-EGF treatment media did not show significantly different results ($p < 0.05$) compared to MEM 10% and MEM 5% FBS supplementation. On the fourth day, SFM-EGF media showed significantly different results ($p < 0.05$) compared to MEM 10% and MEM 5% FBS supplementation.

Media is a source of nutrition for cell culture. The media commonly used was media with the addition of serum. One of the important nutrients contained in serum is growth factors such as platelet-derived growth factor (PDGF), transforming factor beta (TGF-β), and epidermal growth factor (EGF) which stimulate cell growth (Verma *et al*., 2022). However, the addition of serum to culture media has many disadvantages, such as variations between batches that are not uniform, resulting in inconsistent product results, the risk of contamination by infectious agents, expensive costs, and implications for the halal status of the product (Hashim *et al*., 2012). Therefore, efforts are being made to develop SFM, which is currently continuing (Posung *et al*., 2021). To formulate SFM, in this study the growth factor EGF was added to replace serum. According to the study, EGF consists of a 53 amino acid polypeptide isolated from rat salivary glands (Acosta *et al*., 2009) that can stimulate the proliferation of various types of cells, especially epithelial cells such as Vero cells (Jung *et al*., 2008). Another study mentioned that EGF derived from human and rat sources are highly similar in terms of their amino acid sequence and overall function. Both human and rat EGF share a high degree of sequence homology, typically around 80–90%. This means that the majority of the amino acid residues in human and rat EGF are identical or very similar (Petch *et al*., 1990).

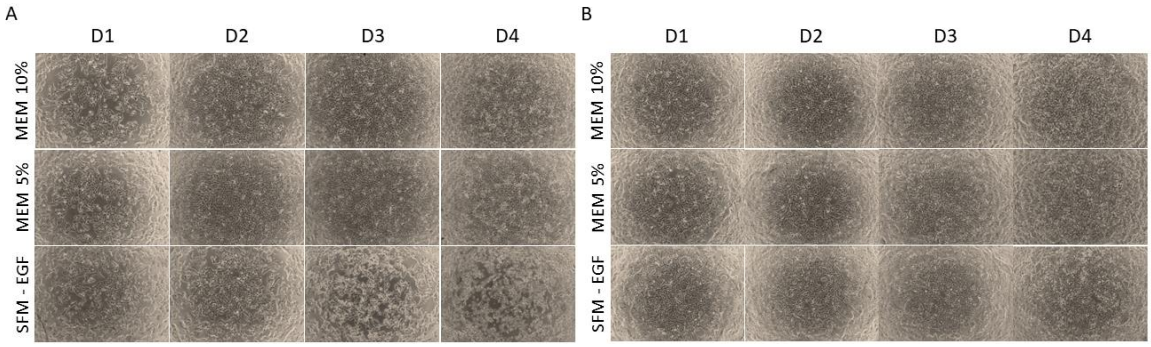

Figure 1. Microscopical observation of Vero E6 and Vero CCL-81 cells. No difference changes morphology cell based on microscopic examination using an inverted microscope (A) Vero E6 cell and (B) Vero CCL-81.

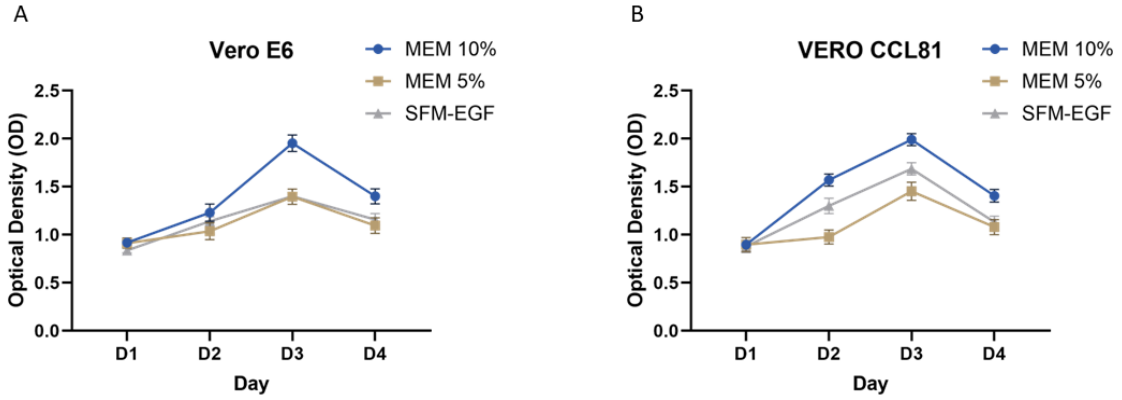

Figure 2. The graph of colorimetric-based proliferation assay. The colorimetric–based proliferation assay (MTT) showed that the proliferation of (A) Vero E6 and (B) Vero CCL-81 cells under SFM-EGF conditions was comparable to the serum-supplemented media.

Table 1. E6 and CCL-81Vero cell optical density (OD) value of proliferation assay

Media Treatment		MEM 10%	MEM 5%	SFM-EGF
Vero E6	**D1**	0.91 ± 0.09^{b}	0.91 ± 0.05^{b}	0.90 ± 0.02^{a}
	D2	1.23 ± 0.09^{b}	1.04 ± 0.09^{a}	1.14 ± 0.05^{ab}
	D3	1.95 ± 0.08^{a}	1.40 ± 0.08^{a}	1.40 ± 0.04^{a}
	D4	0.19 ± 0.08^{b}	1.09 ± 0.08^{a}	1.16 ± 0.06^{ab}
Vero CCL-81	**D1**	0.89 ± 0.05^{b}	0.89 ± 0.07^{b}	0.88 ± 0.07^{a}
	D2	1.57 ± 0.06^{b}	0.98 ± 0.07^{a}	1.30 ± 0.08^{ab}
	D3	1.99 ± 0.06^{a}	1.45 ± 0.09^{a}	1.69 ± 0.07^{a}
	D4	1.40 ± 0.07^{b}	1.08 ± 0.08^{a}	1.16 ± 0.06^{ab}

^{a,b}different superscripts in the same row showed significant differences ($p < 0.05$).

The biological activity of EGF depends on specific binding to the cell membrane receptor Epidermal Growth Factor Receptor (EGFR) which then stimulates biochemical processes that initiate the cell cycle (Acosta *et al*., 2009; Hamid *et al*., 2019), proliferation, cell differentiation, cell migration, inhibit apoptosis and angiogenesis (Wee and Wang, 2017). EGF has a mitogenic effect which has been proven to stimulate mRNA synthesis (Acosta *et al*., 2009; Meybosch *et al*., 2019). EGF induces the expression mRNA of cyclins, cyclin A defines the control points of the cell cycle (Hamid *et al*., 2018). It binds both CDK2 and CDC2 giving rise to two distinct cyclin A kinase activities, one appearing in the S phase and the other one in the G2 phase of cell proliferation (Hyder *et al*., 2012).

MTT assay has become the basic method to measure cell proliferation based on metabolic activity cells. The principle of the MTT test was based on the conversion of MTT into formazan crystals by mitochondrial activity in living cells (Präbst *et al*., 2017). Culture media provides nutrients for cell growth, proliferation, and metabolism. Consequently, the composition of culture media, such as the presence or absence of serum, could influence the MTT assay measurements by influencing the cells' biological behavior such as the level of metabolic activity and thus MTT reduction (Ghasemi *et al*., 2021). Therefore, the proliferation of Vero E6 and Vero CCL-81 grown in media treatment such as SFM-EGF, MEM 10%, and MEM 5% FBS supplementation were evaluated using MTT for four days.

CONCLUSION

The results of this study showed that utilizing serum-free media with the addition of EGF was able to support the proliferation of Vero E6 cells and Vero CCL-81 cells, resulting in a comparable result compared to the serum supplementation media. Thus, their use can be an alternative medium to replace serum.

ACKNOWLEDGEMENTS

The authors express their gratitude to the Faculty of Veterinary Medicine, Universitas Airlangga, Surabaya, for supporting this study through Airlangga Research Fund (Penelitian Dasar Unggulan/PDU) Grant No: 1512/UN3.FKH/PT.01.03/2024.

AUTHORS' CONTRIBUTIONS

SK: Conceptualization and drafted the manuscript. DD, AA, HS, and AYW: Prepared cell culture. FKM and D: Validation, supervision, and formal analysis. WR, YD, and YP: Performed sample evaluation. HS, D, YP, and SK: Performed the statistical analysis and the preparation of table and figure. All authors have read, reviewed, and approved the final manuscript.

COMPETING INTERESTS

The authors declare that they have no competing interests.

REFERENCES

Acosta, B. J., Cowley, G. J., Saura, L. P., Lopez, G. T., Santana, C. M., Mola, L. E., Nieto, G. G., & Martinez, H. L. (2009). Epidermal growth factor in clinical practice – a review of its biological actions, clinical indications and safety implication. *International Wound Journal*, 6(5), 331–346.

Boosntra, J., Rijken, P., Humbel, B., Cremers, F., Verkleij, A., & Henegouwen, B. P. (1995). The epidermal growth factor. *Cell Biology International*, (19), 413–430.

Chruszcz, M., Mikolajczak, K., Mank, N., Mejorek, A. K., Porebski, P., & Mino, W. (2013). Serum Albumims-Unusual allergens. *Elsevier*, 1830(12), 5375–5381.

Freshney, I. R., & Davis, C. A. (2010). Culture of Animal Cells, A Manual of Basic Technique and Specialized Application, 8th Edition. Wiley-Blackwell.

Ghasemi, M., Turnbull, T., Sebastian, S., & Kempson, I. (2021). The MTT Assay: Utility, Limitations, Pitfalls, and Interpretation in Bulk and Single-Cell Analysis. *International Journal of Molecular Sciences*, (22), 1–30.

Hamid, I. S., Ekowati, J., & Purnama, M. T. E. (2019). *Kaempferia galanga* L. Inhibiting Effect on Vascular Endothelial Growth Factor (VEGF) and Cyclooxygenase-2 (Cox-2) Expression on Endothelium of Chorioallantoic Membrane. *Indian Veterinary Journal*, 96(9), 80–82.

Hamid, I. S., Aksono, E. B., Sukmanadi, M., & Purnama, M. T. E. (2018). Antiangiogenesis activity test of tin leaf (*Ficus carica* L.) on the number of blood vessels and VEGF expression of chorioallantoic membrane of embryonated chicken eggs. *European Journal of Oncology Pharmacy*, 1(4), e00007.

Hashim, H. Z. Y., Mel, M., Salleh, M. H., Nor, A. Y., Othman, H. S., & Razak, A. H. Y. W. (2012). Serum in Mammalian Cell Culture: Weighing the Challenges of Bioprocessing, Ethics and Animal Welfare. *Advances in Natural Applied Sciences*, 6(5), 596–600.

Hyder, A., Ehnert, S., Hinz, H., Nussler, K. A., Fandrich, F., & Ungefroren. (2017). EGF and HB-EGF enhance the proliferation of programmable cells of monocytic origin (PCMO) through activation MEK/ERK signaling and improve differentiation of PCMO-derived hepatocyte-like cells. Biomed Central.

Jayaraman, S., Dave, M., Rajagopal, P., Rangsamy, B., Manjunathan, R., & Raktim, M. (2022). Chapter 3 Role of Animal Cell Culture in Vaccine Development. Cell Diagnostic and Research Center, pp: 29–44.

Jung, K., Kang, B. K., Kim, J. Y., Shin, K. S., Lee, C. S., & Song, D. S. (2008). Effects of epidermal growth factor on atrophic enteritis in piglets induced by experimental porcine epidemic diarrhea virus. *The Veterinary Journal*, 177, 231–235.

Kiesslich, S., & Kamen, A. A., (2020). Vero cell upstream bioprocess development for the production of viral vector and vaccines. *Biotechnology Advances*, 1.

Konishi, K., Yamaji, T., Sakuma, C., Kasai, F., Endo, T., Kohara, A., Hanada, K., & Osada, N. (2022). Whole-Genome Sequencing of Vero E6 (Vero C1008) and Comparative Analysis of Four Vero Cell Sublines. *Frontiers*, (13), 1–9.

Lee, K. D., Park, J., & Seo, W. D. (2020). Suspension culture of Vero cells for the production of adenovirus type 5. *Clinical and Experimental Vaccine Research*, 9(1), 48–55.

Meybosch, S., Monie, D. A., Anne, C., Bruyndonckx, J. A., Winter, D. Y. B., Trouet, D., & Ledeganck, J. K. (2019). Epidermal growth factor and its influencing variables in healthy children and adult. *Plos One*, 14(1), 1–13.

Petch, A. L., Harris, J., Raymond, W. V., Blasband, A., Lee, C. D., & Earp, S. H. (1990). Molecular and Cellular Biology, 2973–2982.

Posung, M., Promkhatkaew, D., Borg, J., & Tongta, A. (2021). Development of a modified serum free medium for Vero cell cultures: effect of protein hydrolysates, L-glutamine and SITE liquid media supplement on cell growth. *Cytotechnology*, (73), 683–695.

Shen, F. C., Guilbault C., Li X., Elahi M. S., Ansore S., Kamen A., & Gilbert R. (2019). Development of suspension adapted Vero cell culture process technology for production of viral vaccines. *Elsevier*, 37(47), 6996–7002.

Verma, A., Verma, M., & Singh, A. (2020). Animal tissue culture principles and applications. *Animal Biotechnology*, 14, 269–293.

Wee, P., & Wang, Z. (2017). Epidermal Growth Factor Receptor Cell Proliferation Signaling Pathways. *Cancers*, (9), 1–45.

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