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Culture of sheep poxvirus in a 3D Vero cells culture model

In the article the production of a three-dimensional culture of *Vero* cells using the methods of “hanging drop” and roller cultivation was described. The resulting spheroids remain viable for more than four days. For the formation of large spheroids with good proliferative activity, the inoculum concentration should be 200000 cells/ml. The additional study of the cell proliferation index in spheroids showed that cell growth was 3.5 times on the second day. A comparative study of the biological activity of the sheep poxvirus in 3D cell culture was carried out in comparison with traditional 2D cultivation. After infection of the spheroids with sheep poxvirus in the first passage, the virus titer was 5.33 ± 0.04 lg TCID₅₀/ml. At the second and third passages, the biological activity of the virus increased to 6.33 ± 0.04 and 6.41 ± 0.04 lg TCID₅₀/ml, respectively. Considering these data, three-dimensional culture allows for obtaining much cell biomass with high biological activity compared to the traditional culture method.

Keywords: 3D cell culture, Vero cell line, spheroids, sheep poxvirus.

Introduction

Viral infections are a significant global concern, with notable outbreaks caused by coronaviruses such as SARS-CoV, MERS-CoV, and SARS-CoV-2 [1-3]. Virology research has successfully utilized cell cultures as a crucial tool for researchers in various fields. This model provides a means to analyze biological properties and processes that cannot be observed at the level of a living organism.

In vitro cell culture has long been a reliable method for growing and studying viruses for research and diagnostic purposes. However, it is crucial to note that there are significant differences between cells in two-dimensional (2D) culture and cells in the body. These differences can impact how viruses interact with their host and how susceptible the cells are to certain viruses. Inadequate cell differentiation in 2D cultures can prevent some viruses from maturing properly and can result in inaccurate diagnostic results. However, even with successful infection, conventional cultures of cells on a solid surface can give results that differ from the results if the virus had infected cells in natural tissues. As a result, scientists are turning to three-dimensional (3D) cell cultures, which more closely resemble natural tissue and offer a promising alternative for studying infections. Although cell biology in 3D cultures is more complex, the numerous benefits make it a worthwhile investment in the long run [4-8].

The traditional method for cultivating Sheep poxvirus has been through 2D cell culture. This virus is crucial in developing a vaccine against sheep pox, with an annual demand for tens of millions of doses in Kazakhstan alone. To ensure maximum yield from the host system and efficient vaccine production, it is vital to establish optimal culture conditions that stimulate the growth of various viral strains. Therefore, a robust system is needed to facilitate the cultivation of different viruses in large quantities while providing the necessary optimal conditions for growth.

In this regard, scientific work aims to obtain spheroids of Vero cell lines by three-dimensional cultivation to study the biological activity of the Sheep poxvirus.

Experimental

The study utilized a three-dimensional cell culture Vero (3D) to investigate sheep pox virus biology. 3D cultures were obtained by two techniques.

The first method was a “hanging drop” of the cell suspension on the inside of the Petri dish lid, which was then flipped over and covered with the dish. The cells formed clusters at the interface between air and liquid due to surface tension. To prevent dehydration, a phosphate buffer was added to the dish. The droplet size did not exceed 40 µl, and long-term cultivation was impossible. After unearthing, the cups were placed in a CO₂ incubator at +37°C, and spheroid formation was observed daily under a microscope [9-10].

We seeded cells into multiple flasks with varying seed concentrations using the second method. After installing the cell culture vessel into a rotating bioreactor and leaving it in a room at +37°C with a maximum rotation speed of 600 rpm, we counted the number of spheroids formed within three days. We then determined their viability and proliferation index (PI).

To count the three-dimensional spheroid cultures, we first disaggregated the 3D cell culture and collected all spheroids with a serological pipette into centrifuge tubes. We centrifuged them at 200 x g for 5 minutes and poured out the excess fluid. We added 500 µl of medium per well of a 6-well plate to the sediment. Using a marker with a fine tip, we divided one well of a 96-well plate into squares and drew a plus sign on it. These squares helped us keep track of the spheroids we counted. We then added 50 µl of the suspension to the well and examined the spheroids under a microscope. We carefully counted the spheroids in each square [11–14].

To assess the biological activity of the sheep poxvirus using a three-dimensional Vero cell culture system, we utilized an attenuated vaccine strain known as “NISKHI”. This strain had an infectious activity of 105.5 lg TCID₅₀/ml. A three-dimensional culture of Vero cells was infected with the sheep poxvirus at a dose of 0.1 TCD₅₀/cell. The collection of viral raw materials was carried out 92–96 hours after infection. We used an automated cell counter TC-20 (Biorad) with trypan blue dye to determine the viability of cells grown in the three-dimensional cell culture. Once cell viability dropped to 10%, we subjected the flasks to two freeze-thaw cycles ranging from minus 40°C to room temperature. We collected the virus-containing materials in sterile vials under aseptic conditions while simultaneously taking samples from each vial to determine the biological activity of the virus. The virus titer was calculated using the Reed I.J. and Muench H.A. method and was reported as lg TCID₅₀/ml [15–18].

Statistical processing of results. All experiments were conducted three times. The data was statistically processed using Excel and GraphPad ver. 8, which calculated the arithmetic mean (X) and standard error (m).

Results and Discussion

As a result of the studies, a 3D culture of Vero cells was obtained using two methods. Typically, spheroids formed by the hanging drop technique were observed through phase contrast microscopy. Meanwhile, the formation of cell spheroids in a drop on the first, third, and fifth days of cultivation was captured and examined using a fluorescent microscope (Fig. 1).

Figure 2 shows that cells gradually move towards each other on the first day after sowing. Small spheroids combine to form irregularly shaped cells, which merge into a three-dimensional cluster inside the hanging drop. Over the next two days, the cells reorganize and fuse together to form a spherical shape. By the fifth day, all the cells combine to form a single spheroid. The hanging drop method effectively produces uniform size and shape for 3D cell systems in Petri dishes. This method confirms that spheroids are formed on the fifth day of cultivation and can be of uniform size.

Innovative methods can be utilized to study and manipulate heterotypic cell lines within a 3D setting by forming hanging drop spheroids. This technique is adaptable to different cell types and provides valuable insights into the behaviour of cells in a three-dimensional environment.

Alternatively, a liquid medium can be utilized to cultivate cells in a shaker with constant stirring, producing spheroids on a larger scale. However, with this approach, cells cannot attach to the substrate and initiate aggregation and self-assembly (Fig. 3).

During the first day of cultivation, spheroids with varying sizes and shapes were obtained, which continued to increase in size as days passed. It was observed that these spheroids remained viable for over 4 days. This culture method should take into account that it is normal for some cell types to form a three-dimensional cluster of slightly irregular cells or “spheroids”, which may not be perfectly spherical in shape.

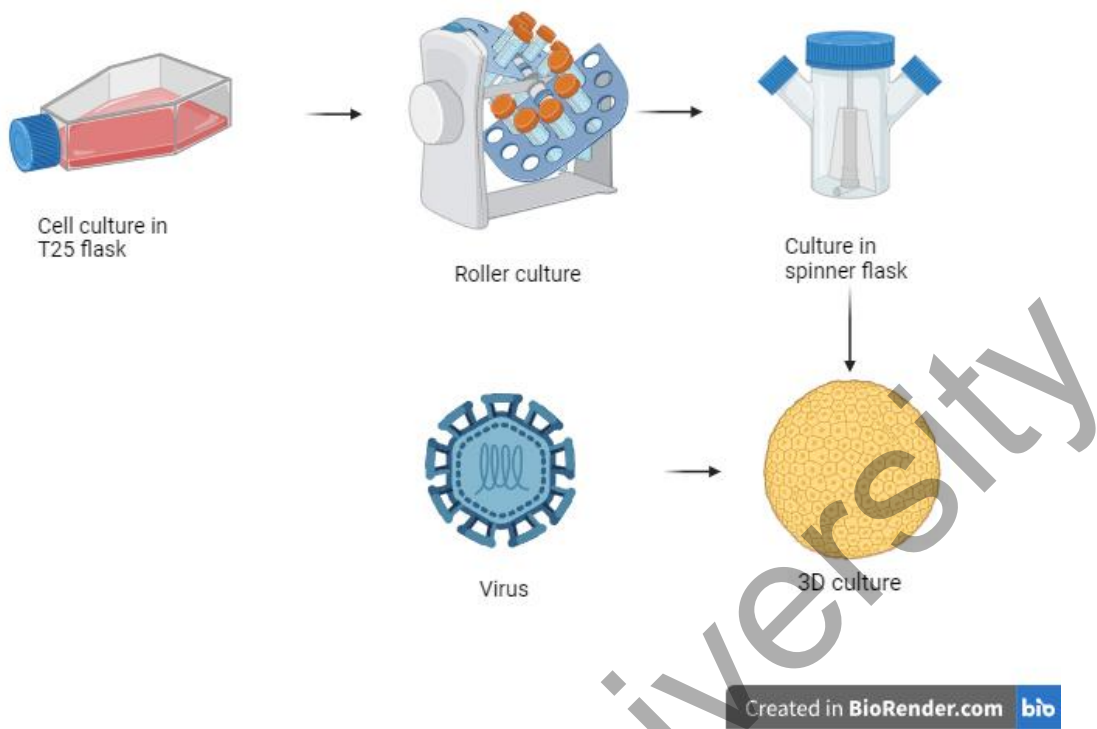
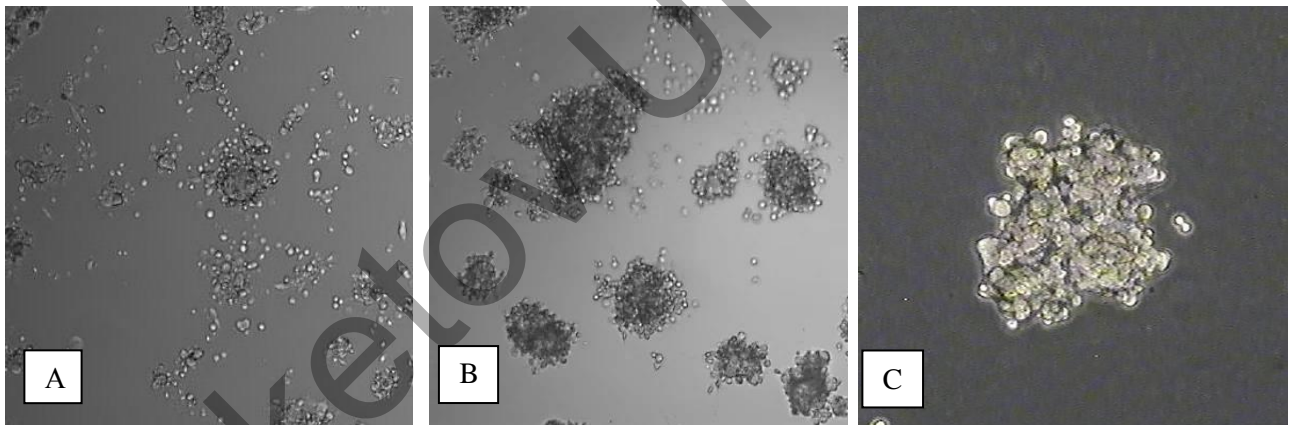
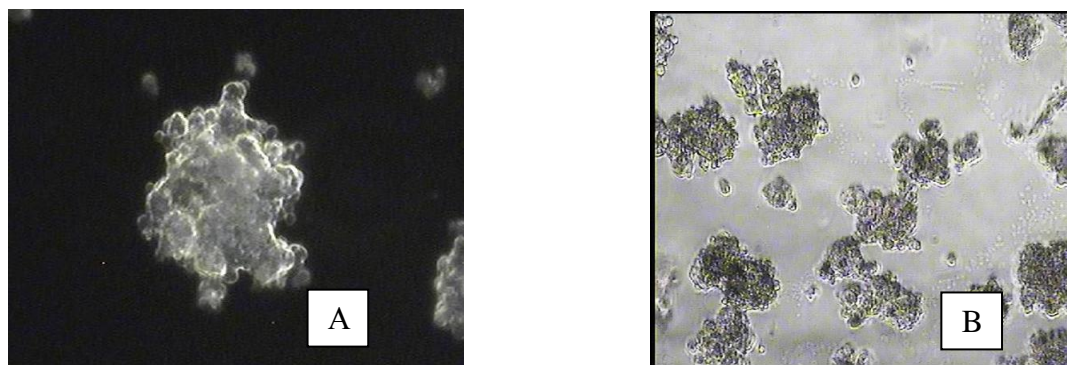


Figure 1. The design of studies for obtaining a 3D cell culture and cultivating viruses within them



A — the first day; B — the third day; C — the 5th day (magn. $\times 10$)

Figure 2. The formation of cell spheroids of Vero cell culture



A — magn. ×40; B — magn. ×10

Figure 3. 3D model of Vero cell culture

The viability and amount of spheroid formation were studied at different seed concentrations, with cells being seeded at concentrations of 75.0×10^5 , 100.0×10^5 , 200.0×10^5 , and 300.0×10^5 cells/ml. The number of viable cells within the spheroids was determined on the first, second, and fourth days of cultivation. All studies were conducted in three technical repetitions, and the average value was calculated (Table 1).

Table 1

The formation and viability of Vero cell culture spheroids at various seeding concentrations ($X \pm m$, $n=3$)

Cell culture	Sowing concentration	Number of spheroids, days			Spheroid diameter (μm), days			% of viable cells in a spheroid, days		
		1	2	4	1	2	4	1	2	4
Vero	75×10^4	167	205	389	70	115	200	98	85	74
	100×10^5	175	310	179	100	170	190	99	85	72
	200×10^5	220	415	566	100–200	240	250–500	98	98	93
	300×10^5	410	620	1267	250	260	300	97	86	79

Based on the experimental data, the optimal seed concentration was selected to provide the maximum rate of cell proliferation in spheroids. As outlined in Table 1, the largest number of spheroids formed at an inoculation concentration of 200×10^5 and 300×10^5 , with a range of 566 to 1267. These spheroids were relatively large, measuring between 300 to 500 μm , and had a high percentage of viable cells ranging from 86 to 98% on the second day.

Our research showed that cells in a three-dimensional system tend to proliferate actively on the first and third days of cultivation, with a gradual decrease in the number of viable cells observed from the fourth day onwards. Specifically, on the fourth day of incubation, the percentage of viable cells in the spheroid ranged from 72% to 79% in samples with sowing concentrations of 75×10^4 , 100×10^5 , and 300×10^5 cells/ml.

Thus, it has been established that an inoculation concentration of 200 thousand cells/ml is required to form large spheroids with good proliferative activity. With the additional study of the cell proliferation index in spheroids, it was noted that cell growth was 3.5 times on the second day.

In this work, the sensitivity of the 3D Vero cell culture system to the sheep poxvirus was studied in comparison with 2D cultures. In these cell cultures, the virus caused destructive changes in cells by rounding, further separating the affected cells from each other with release into the medium and destruction of the monolayer. Manifestations of cytopathic action in cells are shown in Figure 4.

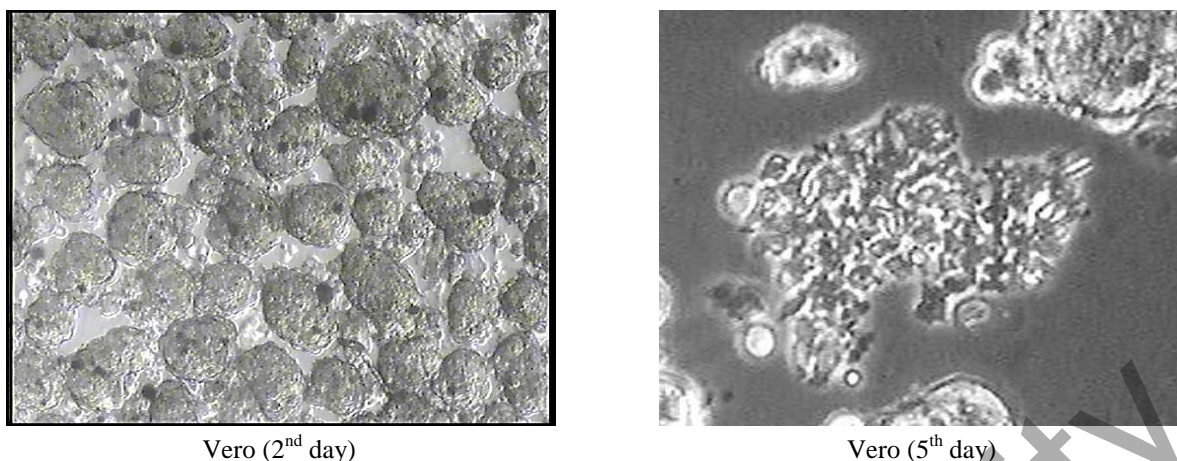


Figure 4. Sheep poxvirus reproduction in the 3D cell system

As shown in Figure 4, starting from the second day after infection, the first signs of cytopathic changes in cells visible during microscopy are observed. On days 5–7 of cultivation, complete destruction of the cellular system was detected.

Next, sheep poxvirus's viability and biological activity in 3D and 2D cell cultures were studied. Cell viability was determined using an automated cell counter TC-20 (Table 2).

Table 2

The viability of 3D and 2D Vero cell culture models after infection with sheep poxvirus

2D cell culture	Total number of cells, days			% of viable cells in the monolayer, days		
	1	2	4	1	2	4
	4.58×10^5	4.47×10^5	4.49×10^5	90	71	15
3D cell culture	Total number of spheroids, days			% of viable cells in the spheroids, days		
	1	2	4	1	2	4
	229	233	230	89	67	10

Table 2 illustrates that the virus has a cytopathic effect on cells, which is noticeable after one day of infection, regardless of whether the cell cultivation is done in 2D or 3D.

The results of assessing the biological activity of the sheep poxvirus in a three-dimensional culture are shown in Figure 5.

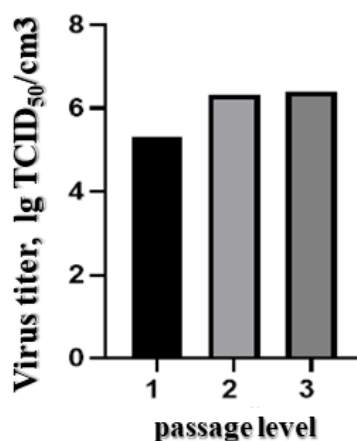


Figure 5. The biological activity of sheep poxvirus in 3D Vero cell culture

Figure 5 shows that the virus titer was 5.33 ± 0.04 lg TCID₅₀/ml in the first passage. And at the second and third passages, the virus's biological activity increased to 6.33 ± 0.04 and 6.41 ± 0.04 lg TCID₅₀/ml,

respectively. The results show that the sheep poxvirus's biological activity is not compromised in three-dimensional culture compared to 2D models.

Thus, culturing infected cells in 3D systems can more accurately mimic physiological conditions than in 2D monolayers. However, it is difficult to see a pronounced cytopathic effect of the virus in a three-dimensional culture. Because visualization of cells embedded in the matrix at different depths is more complex than in 2D monolayers and is limited to 40x magnification since the working distance of the high magnification objectives does not allow penetration into the depth of the matrix. Microscopic examination revealed changes in infected cell morphology and colour and decreased cell viability from 96% to 10%. Changing the morphology of infected cells in 3D culture to a more rounded and wrinkled shape makes microscopic observations more difficult than in monolayer cultures.

Conclusion

A 3D model can be created easily using hanging drop and cell culture in agitated flasks. The first method results in spheroid-shaped cells of the same size, while the second may have a slightly irregular shape. This technique is useful for the large-scale production of vaccines and medicines.

Research has shown that an inoculum concentration of 200×10^5 cells is necessary to form large, highly proliferative spheroids. The cell proliferation index in spheroids increased by 3.5 times, and the viability percentage of formed spheroids was up to 98% on the fourth day of cultivation.

Based on existing literature, it is commonly understood that 3D cultures more closely match the structure and microenvironment of tissues and organs. In addition, the 3D model allows for more representative results that are closer to real biological systems. Viruses often target specific tissues or cell types within the body, and 3D models provide a closer representation of these target cells. On the other hand, viruses may have different mechanisms for entering host cells, and these mechanisms may be better reproduced in 3D cultures. Cells in the 3D model have increased expression of surface receptors that facilitate virus attachment and entry, resulting in a higher rate of viral infection. The understanding of the mechanisms of virus spread, cellular tropism and pathogenesis is most realistic when using a three-dimensional culture model [19–22].

With its versatility, this cell culture technology can be used to study various viruses, providing more accurate modelling of a virus's behavior in an animal system.

This 3D culture system is also invaluable in studying the replication of herpes simplex viruses, varicella zoster viruses and other viruses such as poxviruses, adenoviruses, and parvoviruses [23–24]. It also allows for evaluating antiviral drugs' efficacy [25–26].

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Vero жасуша өсіндісінің 3D моделінде қой шешегі вирусын өсіру

Мақалада «аспалы тамшы» және роллерлік жасанды жағдайда өсіру әдістерін пайдалана отырып, 3D өлшемді Vero жасушасының өсіндісін алу сипатталған. Алынған сфероидтар 4 күннен астам өміршеңдігін сақтайды. Жаксы пролиферативті белсенділігі бар үлкен сфероидтарды қалыптастыру

үшін тұқым концентрациясы 200 000 жасуша/мл болуы керек. Сфероидтардағы жасуша пролиферациясының индексін қосымша зерттегенде екінші тәулікте жасушаның өсуі 3,5 есе болғаны байқалды. 3D жасуша өсіндісіндегі қой шешегі вирусының биологиялық белсенділігін дәстүрлі 2D жасанды жағдайдағы өсірумен салыстырмалы зерттеу жүргізілді. Сфероидтардың қой шешегі вирусын жұқтырғаннан кейін бірінші пассажда вирус титрі $5,33 \pm 0,04 \lg \text{ТЦД}_{50}/\text{см}^3$ болды. Екінші және үшінші пассажда вирустың биологиялық белсенділігі сәйкесінше $6,33 \pm 0,04$ және $6,41 \pm 0,04 \lg \text{ТЦД}_{50}/\text{см}^3$ дейін өсті. Осы мәліметтерді ескере отырып, 3D өлшемді өсінді дәстүрлі өсіру әдісімен салыстырғанда жоғары биологиялық белсенділікке ие жасуша биомассасының үлкен мөлшерін алуға мүмкіндік береді.

Кілт сөздер: 3D жасушалар өсінділері, Vero жасуша өсіндісі, сфероидтар, қой шешегі вирусы.

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Культивирование вируса оспы овец в 3D модели культуры клеток Vero

В статье описано получение трехмерной культуры клеток Vero с использованием методов «виская капля» и роллерного культивирования. Полученные сфероиды сохраняют жизнеспособность более 4-х суток. Для формирования крупных сфероидов с хорошей пролиферативной активностью посевная концентрация должна составлять 200 тыс. кл/мл. При дополнительном изучении индекса пролиферации клеток в сфероидах было отмечено, что на вторые сутки прирост клеток составил 3,5 раза. Проводилось сравнительное изучение биологической активности вируса оспы овец в 3D культуре клеток по сравнению с традиционным 2D культивированием. После заражения сфероидов вирусом оспы овец в первом пассаже титр вируса составил $5,33 \pm 0,04 \lg \text{ТЦД}_{50}/\text{см}^3$. На втором и третьем пассажах биологическая активность вируса повышалась до $6,33 \pm 0,04$ и $6,41 \pm 0,04 \lg \text{ТЦД}_{50}/\text{см}^3$ соответственно. Учитывая эти данные, трехмерная культура позволяет получить большое количество клеточной биомассы с высокой биологической активностью по сравнению с традиционным методом культивирования.

Ключевые слова: 3D культура клеток, клеточная линия Vero, сфероиды, вирус оспы овец.

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