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High Vero Cell Density and Rabies Virus Proliferation on Fibracel Disks Versus Cytodex-1 in Spinner Flask

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Abstract: To achieve higher titer of rabies virus higher density of host cells will need. In this study, capability of FibraCel disks packed in 500 mL spinner basket versus Cytodex-1 in 500 mL spinner flask was investigated for propagation of Vero cells and PV rabies virus proliferation. Minimal Essential Medium (MEM) + 10% Foetal Calf Serum (FCS) and Virus Production- Serum Free Medium (VP-SFM) + 4 mM L-glutamine were used in growth phase and MEM+ 0.2% Bovine Serum Albumin (BSA) and VP-SFM were used in virus production phase. Adapted Vero cells grown in VP-SFM were used in all SFM experiments while batch and stepwise perfusion modes were applied and compared in growth stage. The highest Vero cell density were achieved in the trials with 10 g FibraCel disk in stepwise perfusion mode equal to 6.12×10^6 and 5.87×10^6 cells mL^{-1} in MEM and VP-SFM, respectively while with 2.73 g Cytodex-1 lower density equal to 4.2×10^6 and 4.0×10^6 cells mL^{-1} were achieved. The highest titer of rabies virus and overall virus production rate were resulted in VP-SFM and on 10 g disks equal to 2.9×10^7 Fluorescent Focus Unit (FFU) mL^{-1} and 0.14 FFU/Cell/h, respectively versus 1.7×10^7 FFU mL^{-1} and 0.08 FFU/cell/h on cytodex-1 in similar conditions. The second harvest of virus was also satisfactory in experiment with 10 g disks (1.7×10^7 FFU mL^{-1}) in compare to Cytodex-1 (0.51×10^7 FFU mL^{-1}). An equal surface area at 6600 and 12000 cm^{-2} were provided in all comparable trials with seeding density of 12.5×10^3 cells cm^{-2}. Adapted Vero cells grown in VP-SFM were used in all SFM experiments while batch and stepwise perfusion modes were applied and compared in growth stage.

Key words: Rabies virus production, fibracel disk, cytodex, vero cell, microcarrier

INTRODUCTION

Rabies has a long story as a fatal disease of the nervous system of mammals that cause by rabies virus and rabies vaccine is known as the only way to prevent human death due to rabies. Rabies occurs in more than 100 countries and territories with 55,000 annual deaths around the world and continues to be one of the major public health hazards in many developing countries (WHO, 2010; Knobel *et al*., 2005; Meslin *et al*., 1994; Martinzn, 2000; Nandi and Kumar, 2011). Although the efficacy and safety of modern cell culture vaccines have been recognized but some countries are still producing and using nervous tissue vaccines which are less effective and have the disadvantages of causing sever adverse reactions. Global programs to control rabies as the goal of WHO and investigation in field of economic production of safe rabies vaccine are being continued specially in infected developing countries (WHO, 2007; Rourou *et al*., 2009; Jagannathan *et al*., 2009).

The attachment-dependent cell (ADC) line that was first initiated and developed from a normal adult African velvet monkey (*Cercopithecus aethiops*) kidney tissue by Yasumura and Kawakita (1963) of Chiba University of Japan was named Vero. it has been demonstrated that low-passage number Vero is not presenting any threat to human health when used as a substrate for biological production authorized by WHO such as vaccines (Horaud, 1992; Montagnon *et al*., 1999; Vincent-Falquest *et al*., 1989). Vaccines produced by Vero cells are also more efficient and safer than animal nervous tissue culture and would be more economic than using human diploid cell strains such as MRC-5 (Kumar *et al*., 2002). Although, adapting Vero cells to grow as a free-cell suspension has reported by Paillet *et al*. (2009) and Daelli (2007) but Vero cells are still known as an attachment-

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Table 1: Characteristics of the carriers used for propagation of Vero cells and rabies virus

Carrier	Size, type and material	Used densities (g/500 mL)	Area ($cm^2 g^{-1}$)	Manufacture
Cytodex-1 Microcarrier	190 μm spherical, non-porous dextran matrix, positively charged, hydrophilic	1.5-2.75	4400	GE Healthcare, Sweden Cat# 17-0448-01,
FibraCel disks	6 mm porous disks, non-woven, polystyrene-polypropylene fabric.	5.5-10	1200	New Brunswick scientific, Edison, NJ (USA) Cat# M1292-0088

dependent cell line and therefore, absolutely requires high volumetric surface area to reach higher volumetric cell yields (Mendonca *et al*., 1993).

The vaccine industry is rapidly moving away from traditional egg-based production to more rapid and cost-effective cell-based techniques. Microcarriers such as Cytodex for many years and newly Fibra-Cel disks are being used for suspension culture of bonded Vero cells. FibraCel made by New Brunswick Scientific (NBS) introduced in the early 1990s, have been in use for nearly two decades throughout the world for research and production. Fibra-Cel disks as a solid-support matrix provide an extremely high-surface area for growth of attachment-dependent cells designed for animal and insect cells, predominantly used for production of secreted products such as recombinant proteins and viruses. These disks composed of polyester non-woven fiber and polypropylene; treated electro statically to facilitate suspension cells adhering to the disks and becoming trapped in the fiber system (Table 1). The advantages and benefits of FibraCel disks over standard micro carrier technology would be: Low pressure drop across the bed, High surface-to-volume ratios, Less susceptibility of immobilized cells within the disks to shear forces from impeller blades and sparger gas bubbles, Higher mass transfer of nutrients and oxygen, Easy and efficient separation of biomass from secreted products (New Brunswick Scientific, 2006; De Oliveira Souza *et al*., 2005; Vyas and Cino, 2005).

Fibra-Cel disk as a porous macro carrier is being used in 0.5 to 300 L in form of packed-bed bioreactor, disposable bags, spinner and suspension culture that enhance the surface area available for attachment and multiplication of ADC such as Vero cells (Glaser and Bioreactor Fermentor Market Trends, 2009).

Classical cell culture vaccines commonly contained animal derived substances such as Fetal Calf Serum (FCS) in their production processing. Serum with having some essential nutrients and beneficial factors has properties of growth promoting for cells in cultures (Hesse and Wagner, 2000). Notwithstanding remarkable usefulness, using serum has also some disadvantages (Fishbein *et al*., 1993; Lubiniecki, 1999). Laboratories, for standardization of media composition and among some industrial-scale production units to facilitate downstream processing and reduce the risk of adventitious infectious agents, now prefer using serum-free media (Merten, 2000) although serum-free media are also not without disadvantages (Freshney, 2005). VP-SFM (Gibco) is a commercial Serum Free Media that is suitable for growth of Vero cells and producing virus such as rabies virus (Frazatti-Gallina *et al*., 2001).

Although rabies virus production by Vero cells grown in Serum Contained Media (SCM) and SFM were already repeatedly reported on micro carriers such as Cytodex-1 (Merten *et al*., 1999; Frazatti-Gallina *et al*., 2004) but there is not much data for production of rabies virus by Vero cells grown on disks.

In this study a laboratory investigation was presented in potential of FibraCel disks over against Cytodex-1 microcarriers in an equal surface area and in volume of 500 mL for cultivating of Vero cells and PV rabies virus production by the use of fed batch and stepwise perfusion modes in SCM and SFM.

MATERIALS AND METHODS

Cell line: Vero cells were obtained from cell bank of Pasteur Institute of Iran.

Virus strain: Fixed strain of Pasteur rabies Virus (PV) adapted to grow on Vero cells was prepared by WHO- collaborating and research rabies center of Pasteur Institute of Iran.

Sequential adaptation of vero cells to vp-sfm: This is Invitrogen's preferred method for adapting cells to Serum-Free Media (SFM), with a typical conversion being. In mid-logarithmic phase Vero cells (3.5×10^5 mL^{-1}) were first cultured in 75% serum contained MEM + 25% SFM in a 25 cm^{-2} T-flask at 37°C, 5% CO_2. When cell density reached to 2×10^6 mL^{-1}, they were subculture by density of 3×10^5 cells mL^{-1} in 50% SCM + 50% SFM again. The cultures of cells to being adapted were subcultured every 4 days for more 2 times with reduction of SCM to 25 and 10% and replacing the SFM. Cells were fully adapted when reach to 3×10^6 mL^{-1} with more than 90% viability in SFM. As described by Rourou *et al*. (2007) working cell bank of adapted Vero cells was established.

Culture media and supplements: Fetal Calf serum and VP-SFM (Cat# 12559-019) were supplied by Invitrogen

Table 2: Vero cells growth, rabies virus production and culture characteristics in the various trials

Trial No.	1	2	3	4	5	6	7	8	9	10	11	12
Culture media (500 mL)	MEM	SFM	MEM	SFM	MEM	SFM	MEM	SFM	MEM	SFM	MEM	SFM
Carrier	1.5 g CX	1.5 g CX	5.5 g FC	5.5 g FC	2.73 g CX	2.73 g CX	10 g FC	10 g FC	2.73 g CX	2.73 g CX	10 g FC	10 g FC
Culture mode	Batch	Batch	Batch	Batch	Batch	Batch	Batch	Batch	SWP	SWP	SWP	SWP

(Glasgow, UK). Lactate assay kit (Cat# k627-100) and Glucose assay kit (Cat # k606-100) were brought by BioVision (CA 94043 USA), Ammonia assay kit, MEM, non amino acids solution and all other chemicals were obtained from Sigma (St. Louis, USA).

Microcarrier preparation: Cytodex-1 was sterilized in Phosphate Buffer Saline (PBS) prior transfer into stirrer flasks according to manufacture instruction.

Fibra-cel disks preparation: Spinner basket was loaded with defined amount of Fibra-Cel disks using analytical balance and laminar air flow cabinet to minimize germ particles then was filled with appropriate volume of sterile PBS and autoclaved prior usage.

Preparation of cells in spinner cultures: MEM supplemented with 10% FCS and VP-SFM + 4 mM l-glutamine were used in SCM and SFM experiments, respectively.

Spinner basket containing FibraCel disks and PBS was initially autoclaved then PBS was replaced aseptically with 400 mL of supplemented media. With the autoclaved spinner flask, PBS was replaced with 400 mL of supplemented media containing Cytodex-1 aseptically.

Propagated Vero cells in T-flasks in SCM and in VP-SFM at 37°C in 5% CO_2 were treatment by trypsin and TrypLE Selec, t respectively as described by Rourou *et al.* (2007). Dissociated cells were used as inoculants with seeding density of 1.25×10^4 cell cm^{-2}. The final working volume in cultivation chamber adjusted 500 mL and headspace was inspired by 5% CO_2 atmosphere using an aquarium pump at 37°C incubator. Stirring speed was set at 25 rpm in beginning then continued at 40 rpm during cell growth and kept constant during sampling of stirrer flask to ensure a representative sampling.

Level of glucose and pH was measured and adjusted daily by adding of glucose (260 g L^{-1}) and $NaHCO_3$ (88 g L^{-1}) if necessary. In stepwise perfusion (draw-and-fill) mode, adjusting glucose and lactate concentration at suitable levels, was done by exchange of 75% of spent medium with an equal volume of fresh one daily at day 2 at rpm 0 in which microcarriers were allowed to settle. Large size of the disks (6 mm) helps the cell-free medium to be removed out from the spinner basket through a sterile tube by pumping. The pH 7.2 and glucose at not below 1g L^{-1} were daily fixed during propagation of cells. All experiments carried out triplicate as shown in Table 2.

Virus propagation phase: Once the high Vero cell density was achieved in each experiment (at day 4-9) media were removed completely then attached cells on carriers were washed two times by virus production medium (MEM + 0.2% BSA for serum contained and VP-SFM + 4 mM L-glutamine for serum free trials). After 3 h at 34°C, pH 7.4 and 5% CO_2; conditions that stimulate penetration of virus, virus inoculants with a Multiplicity of Infection (MOI) of 0.3 were transferred into the flasks to infect Vero cells. Infection phase continued for 4 days by daily sampling and adjusting pH at 7.4.

Off-line metabolite analysis methods: Growth and infection indexes including viable cell density, titer of virus, pH and concentration of glucose, lactate and ammonia were measured by daily sampling and using specific assay kits.

Cell counting: To estimate periodically the viable cell population during growth and infection phase, we used nuclei count method as described by Trabelsi *et al.* (2005). To estimate and visualize viable cells and to confirm cell density obtained already by nuclei count method during operation; defined number of disks were treated with PBS, followed by using a standard trypsinization buffer and incubation for 30 sec at 37°C, 5% CO_2, then the liquid was removed and 1 mL PBS was added and incubated at 37°C for 8 min (New Brunswick Scientific, 2006; Vyas and Cino, 2005). For detaching cells from cytodex-1 we followed standard trypsinization procedure (Trabelsi *et al.*, 2005). Detached cells were stained by Trypan blue and counted by hemacytometer slide (Doyle and Griffiths, 1998). Detached (suspended) cells in supernatant or free space on Cytodex-1 microcarriers will show the density of dead cells.

Rabies virus titration: Virus titer was estimated according to a modified Rapid Fluorescence Focus Inhibition Test (RFFIT) (Trabelsi *et al.*, 2006; Smith *et al.*, 1973) and expressed in Fluorescent Focus Units per mL (FFU mL^{-1}).

The overall virus productivity rate (vpr) was calculated as follows:

$$P = C/(X \text{ infection-x end})\times(1/\text{Infextion period})$$

where, C is the virus titer in FFU mL^{-1} obtained at the end of the culture. $X_{infection}$ and X_{end} stand for cell density level

at the time of infection and at the end of the culture, respectively. Infection period corresponds to the duration of the virus production phase in hours. Also monitoring of infected cells was done every day post infection based on count of carriers-attached viable cells and suspended dead cells. Also microscopic examination method was used to visualize infected cells. Trypsinized cells were dried on a microscopic slide and fixed by cold 80% acetone. Fixed cells then stained with fluorescein-labeled antibodies nucleocapsid immunoglobulins (Sanofi Diagnostic Pasteur, Marnes la Coquette, France, Cat. No. 72114).

RESULTS

In 12 trials (Table 2) a 500 mL spinner basket containing 5.5 and 10 g of Fibra-Cel disks versus a 500 mL spinner flask loaded with 1.5 and 2.75 g Cytodex-1 were used to evaluate Vero cells growth and rabies virus proliferation. The experiments were performed in batch and in stepwise perfusion mode for growth phase with initial inoculums of 1.25×10^4 cells cm^{-2} in SCM and VP-SFM, cells then reached at the highest density and were infected by PV strain (MOI of 0.3) in MEM + 0.2% BSA and in VP-SFM + 4 mM l-glutamine in batch mode.

Vero cells growth and metabolite profile: As shown in Fig. 1 more than 99% of the Vero cells of initial inoculums became strongly immobilized in the disks rapidly, within approximately 85 and 120 min in MEM and VP-SFM, respectively while with Cytodex-1 it was estimated about 120 and 320 min.

Comparison of growth curve (Fig. 2) of adapted and non-adapted Vero cells in VP-SFM in 25 cm^{-2} T-flask demonstrated that adapted cells grow well in SFM comparable with cells grown on SCM.

The highest surface cell density in amount of 255×10^3 cells cm^{-2} (Fig. 3) and equal to 6.12×10^6 cells mL^{-1} (Fig. 4) were achieved in trial 11 on 10 g disk (12000 cm^{-2}) and on SCM in stepwise perfusion mode during 8 days while in similar conditions on VP-SFM (trial 12) it was slightly lower in amount of 245×10^3 cells cm^{-2} (5.87×10^6 cells mL^{-1}). By the use of 2.73 g Cytodex-1 (12000 cm^{-2}) the highest cell density were estimated at 175×10^3 cells cm^{-2} (4.2×10^6 cells mL^{-1}) and 167×10^3 cells cm^{-2} (4.0×10^6 cells mL^{-1}) in SCM (trial 9) and SFM (trial 10), respectively. The lowest achieved surface cell density was seen in batch mode (trial 6) on 2.73 g Cytodex-1 in SFM equal to 130×10^3 cells cm^{-2} (3.11×10^6 cells mL^{-1}) while with lower Cytodex-1 (trial 2) higher cell density in amount of 175×10^3 cells cm^{-2} was achieved.

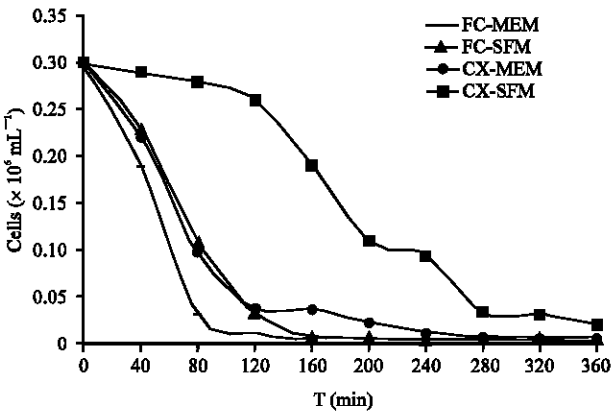

Fig. 1: Vero cell adhesion to the FibraCel disk (FC) and Cytodex-1 (CX) during the 6 hours of culture in MEM+10%FCS and VP-SFM

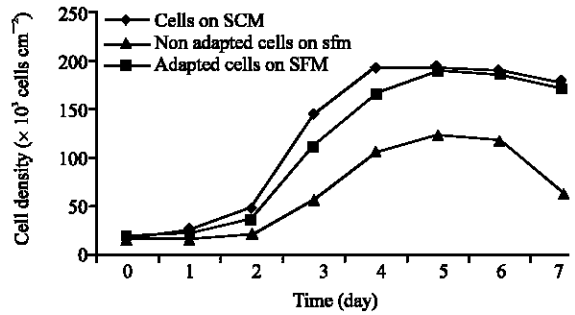

Fig. 2: The curves of Vero cells growth on SCM and comparison with adapted and non adapted Vero cells grown on SFM

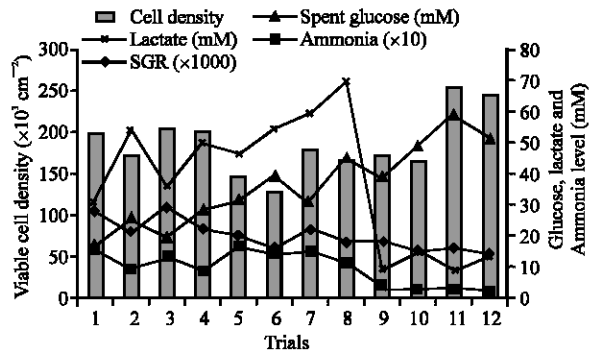

Fig. 3: Evaluation of Vero cell density, Specific Growth Rate (SGR), Glucose consumption, Lactate and Ammonia level achieved in batch (1-8) and stepwise perfusion trials (9-12)

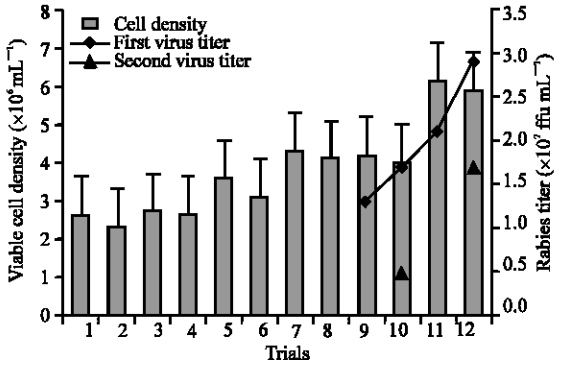

Fig. 4: Vero cell density and rabies virus titer achieved on FibraCel disks in spinner basket and on Cytodex-1 in stirrer flask Batch (1-8), stepwise perfusion (9-12), first rabies virus titer (9-12) and second titer (10,12) were shown

The highest and lowest specific growth rates were seen in trials 3 and 12, respectively.

Profile of produced Lactate, ammonia and spent glucose resulting in the growth phase also were shown in Fig. 3. The higher concentration of lactate were resulted in batch trials with SFM in compare with SCM especially in trials with disks, the while ammonia appeared at higher level in trials with MEM.

The highest level of lactate equal to 69.43 mM was measured in trial 8 with 10g FibraCel disks and in SFM while in MEM and in similar conditions, 59.12 mM lactate resulted. In stepwise perfusion mode (trial 9-12), lactate and ammonia concentration reduced to the lowest levels as shown in Fig. 3.

Rabies virus production: Achieved rabies virus titers in the trials 9-12 is shown in Fig. 4. The highest virus concentration was achieved in trail 12 with 10 g disk and in stepwise mode. Also, cell density at the time of infection and at day 3 post infection were estimated in amount of 5.87×10^6 and 3.0×10^6 cells mL^{-1}, initial and final titer of virus concentration equal to 0.18×10^7 and 2.9×10^7 FFU mL^{-1} and overall virus productivity at 0.14 FFU/Cell/h. In trial 11 in the same condition but on SCM, final virus concentration reached 2.1×10^7 FFU mL^{-1}. In trial 10 with 2.73 g Cytodex-1 on VP-SFM, virus proliferation while cell density and titer of virus at day 3 post infection reached 1.1×10^6 cells mL^{-1} and 0.12×10^7 FFU mL^{-1}, respectively. The second harvests in trial 10 and 12 in which spent SFM at day 3 post infection was completely exchanged with fresh one and kept for 3 next days, titer of virus was 0.51×10^7 FFU mL^{-1} and 1.7×10^7 FFU mL^{-1} for Cytodex-1 and disks, respectively.

DISCUSSION

Comparing the adhesion, Vero cells mighty anchored to the FibraCel disks faster than that occurred on Cytodex-1 that could be due to a safer situation for Vero cells inside the porous disks in beginning of culture letting the cells to trap and attach quickly on the inner fibers as well as their outer surface (negatively charged). Surface cell density achieved in SCM batch experiments on 13.2 cm^{-2} mL^{-1} of Cytodex-1 and FibraCel disks were almost closed together (±0.01 log). These results were also comparable with that obtained by Trabelsi *et al.* (2005) equal to 212×10^3 cells cm^{-2} (2.8×10^6 cells mL^{-1}). They used Vero cells grown in MEM+10% FCS+0.2 mM Serine+0.2 mM methionine and 3 g L^{-1} Cytodex-1 (13.2 cm^{-2} mL^{-1}) in 250 mL spinner flask. Present results on VP-SFM (trial 2, 4) were a little less than those we achieved in SCM and also those that has reported by Rourou *et al.* (2007) equal to 227×10^3 cells cm^{-2} (2×10^6 cells mL^{-1}) by the use of 2 g L^{-1} of Cytodex-1 in 250 mL spinner flask containing VP-SFM. Difference was bigger between trial 1 and 2 (±0.06 log) than between trial 3 and 4 (±0.01 log) and also between trial 2 and 4 then between trial 1 and 3 that represent efficiency of FibraCel disk when using SFM.

In increased volumetric available surface area (24 cm^{-2} mL^{-1}), achieved cell density on 10 g FibraCel disks were higher than those achieved on 2.73 g Cytodex-1 but altogether a relatively decrease in specific growth rate and surface cell density seen in trial 5-8 in compare to trials 1-4. These results are almost in agreement with Trabelsi *et al.* (2006) who reported a cell density equal to 212×10^3 cells cm^{-2} (2.22×10^6 cells mL^{-1}) and 155×10^3 cells cm^{-2} (4.1×10^6 cells mL^{-1}), respectively on 3 and 6 g L^{-1} Cytodex-1 in 250 mL spinner flasks. Relative decrement of surface cell density with 2.73 g versus 1.5 g Cytodex-1 might be due to a more cell injury in face to more collisions of beads and shear force in higher concentration of microcarriers. This relative decrement with 10 g versus 5.5 g disk was lower probably because of lower shear forces. Furthermore discrepancy between achieved cell density levels on FibraCel disks in VP-SFM and in MEM was less sensible than those achieved when using Cytodex-1. Apparently inside the porous disks, cells safeness was better established than on the surface of Cytodex-1 in particular when using SFM in which cells would be more sensitive to shear forces. Yokomizo *et al.* (2004) described that macroporous microcarriers have the ability to protect cells against detrimental hydrodynamic forces generated by collision of microcarriers.

In stepwise perfusion mode cell density improved on FibraCel disks in higher level than those obtained on Cytodex-1 and higher than those discussed above.

Mendonca and Periera (1995) reported a high cell density comparable to present results and equal to 136,636 cells cm^{-2} (6.0×10^{6} cells mL^{-1}) on 10 g L^{-1} Cytodex-1 using perfusion culture mode. Their result supported ours and indicates higher cell density could be achieved with available higher surface. Specific growth rate shows a decrease in the experiments with SFM and disk that is because of need to longer incubation time to reach maximum cell density.

The fecundity of fed-batch mode will be limited while nutrients are being depleted and toxic metabolites, mostly Lactate and ammonia arisen (Hinterkorner *et al*., 2007; Wentz and Schiiger, 1992) more than 20-30 mM and as low as 2-3 mM, respectively (Genari *et al*., 1998; Hassell *et al*., 1991). This is why a lesser cell density was achieved in the batch trials of this work. In stepwise perfusion mode, we maintained glucose at 6-15 mM, lactate and ammonia at non toxic level that resulting improvement in Vero cells fertility and specific growth rate on disks more than on beads. Although, Quesney *et al*. (2001) have mentioned lactate and ammonium ions did not reach toxic levels in SFM for Vero cells it seems however in present of higher toxic metabolites, cells on Cytodex-1 are more fragile in the face of harmfulness whereas immobilized cells in packed disks would be in lesser danger of these physical and chemical harms. Merten *et al*. (1994) have showed that in Vero cells submitted to nutritional stress conditions, the pattern of growth and adhesion will be altered. Growth inside the FibraCel disks far from stress might be another reason of higher growth rate of Vero cells seen with disks in this study.

In this study, the highest titer of rabies virus and overall virus productivity was achieved on FibraCel disk. Also virus productivity of VP-SFM was higher than MEM+ 0.2% BSA. We harvested the highest level of virus on 10 g FibraCel disks and on SFM, about 0.2 log higher than that reached on 2.73 g Cytodex-1 with higher overall virus productivity rate in similar conditions. Count of detached cells three days post infection indicated a higher reduction rate of infected cells on Cytodex-1 probably due to more fragility of infected cells in collisions of beads that could be considered as a reason for lesser virus productivity. In this respect, second virus harvest from FibraCel disks was higher than on Cytodex-1 because of lower rate of infected cell mortality. There is no former report on rabies virus production by the use of Vero cells on FibraCel disks Gumusderelioglu *et al*. (2001) have reported production of rabies virus using BHK-An66 in 5 g L^{-1} non-woven polyester fabric (NWPF) packed-bed reactor with virus titer of 2.2×10^{5} FFU mL^{-1}. They also mentioned to NWPF packed reactors can be considered as a suitable system for the large-scale production of rabies virus. Namdev and Lio (2000) used FibraCel disks in a disposable-bag wave action system to cultivate CHO cells for production of recombinant protein and have reported that FibraCel disks are better option than Cytodex-3 and CultiSpher-S to support cell growth with viability over a long time about 11 days.

CONCLUSION

This study showed that the level of Vero cells density and titer of rabies virus could reach higher on FibraCel disk than on equal surface area provided by Cytodex-1 in MEM and VP-SFM, in batch or stepwise perfusion mode either. Our results showed a good operational capability of FibraCel disk to use as an alternative solid support for Vero cells distinctively when using SFM.

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