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Original Article

Estimation of the Cultured Cells' Volume and Surface Area: Application of Stereological Methods on Vero Cells Infected by Rubella Virus

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What Known

- In the traditional assay, which is cell culture, rubella virus produces no or inconspicuous cytopathic effect in most of the cell lines.
- However, it takes a few days for cell changes to be observable. The stereological methods include invariator, nucleator, or surf actor techniques. These were applied to estimate the cell and nucleus volume and surfaces of the infected and non-infected Vero cells to detect early signs of virus infection.

What's New

- The Vero cell volume was decreased by 15-24%, 48 hours after the infection with rubella virus in comparison to the non-infected cells.
- Besides, the cell surface area was decreased by 13%, 48 hours after the infection.
- Molecular genetic analysis could be used for early and correct diagnosis of FMF in children with suspected manifestations
- No changes were detected in the nuclei.
- The values of the standard deviation and coefficient of variation of the cells estimated by invariator were lower compared to those measured by the nucleator or surfactor.

Abstract

Background: Morphological changes of the cells infected with rubella virus cannot be observed easily. Estimation of the size of the cultured cells can be a valuable parameter in this condition. This study was conducted to find answers to the following questions:

- How much time after infection with rubella virus, the volume and surface area of the Vero cells and their nuclei get started to change?
- How is it possible to apply stereological methods to estimate the volume and surface area of the cultured cells using the invariator, nucleator, and surfactor techniques?

Methods: The cultured Vero cells were infected with rubella virus. The cells of the control and experimental groups were harvested at 2, 4, 8, 24, and 48 hours following the incubation period. The cells were processed and embedded in paraffin. Invariator, nucleator, and surfactor were applied to estimate the size of the Vero cells and their nuclei.

Results: The cell volume was decreased by 15-24%, 48 hours after the infection in comparison to the non-infected cells. Besides, the cell surface area was decreased by 13%, 48 hours after the infection. However, no changes were detected in the nuclei. The values of the standard deviation and coefficient of variation of the cells, estimated by invariator, were lower compared to those measured by the nucleator or surfactor.

Conclusion: In this study, the volume and surface area of the Vero cells were reduced by rubella virus 48 hours after infection. Invariator is a more precise method compared to nucleator or surfactor.

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Keywords ● Vero cells ● Rubella virus ● Histology

Introduction

Viruses are the important causes of serious illnesses in humans. One of these viruses is rubella, the only member of the Rubivirus genus, which belongs to Togaviridae family. Rubella virus infects only humans and has no other reservoirs.[1,2] It causes a relatively benign illness, sometimes called German measles, with a rash and is one of the typical childhood diseases. The virus sets up a long-lived infection in the fetus that often causes developmental abnormalities by retarding the rate of cell division. In the traditional

"gold standard" assay, which is cell culture, rubella virus produces no or inconspicuous cytopathic effect (CPE) in most of the cell lines. Of course, there are some cell culture systems in which rubella virus will grow and produce plaque, however, it takes a few days. Using cells cultured in shell vials, viral antigens can be detected by immunofluorescence 3-4 days post-inoculation.[3,4]

It is important to be able to study faster methods to detect the rubella virus growth in the cell culture. Previous studies have shown that CPE were detectable using stereological methods.[5-7] The present research was conducted to evaluate the alterations in the surface area and volume of the cultured Vero cells using the mentioned methods. It attempts to find CPE changes in the early stages of the contamination with rubella virus. However, no CPE has been reported for the cells infected with rubella virus with the usual qualitative evaluation.[3-5] Therefore, detection of morphological changes of the cell or nuclei induced by the virus before observing any noticeable alterations afterward in the contamination or late production of obvious CPE can be beneficial for the antigen preparation or serological testing of specific rubella antibodies.

Estimation of the size of the cultured cells can be a valuable parameter in the in vitro culture studies. Up to now, a limited number of studies have focused on the quantitative aspects of the cultured cells. However, the following studies have improved the methodology using quantitative evaluations. Chand and Legge (2011), studied the developing mouse ovarian follicles in an in vitro culture system using stereological methods.[8] Besides, Marićet et al. (2007) analyzed rat fetuses in an in vitro culture model.[9] Green and Day (2013) assessed the in vitro grown blastocysts using quantitative methods.[10] Pierettiet et al. (2014) used quantitative methods to analyze in vitro model of alveologenesis.[11] Tapias et al. (2013) evaluated the morphological properties of the soma and neurites using a computerized imaging system.[12] Although the volume and surface area can provide useful data about changes in the cultured cells, estimation of these parameters has not received adequate attention. Therefore, this research aims to present the methods of processing, embedding, sectioning, and sampling of the cells and estimate the volume and surface area of the Vero cells (and its nucleus) after in vitro experiment. In addition, different aspects of the methods are compared when applied on the Vero cell line. These presented methods can also be applied for the stereological evaluation of other cells through cultured experiments. The present study quantified the mean surface area and volume of the lineages of cells (Vero cells) and their nuclei using stereological methods, including invariator, nucleator, and surfactor.[13,14] Invariator is the method of estimating the volume and surface area of the local particles. Yet, nucleator has been the method for volume estimation and surfactor is the technique for surface area estimation.

Briefly, this study was conducted to find answers to the following questions:

- How much time after infection with rubella virus, the volume and surface area of the Vero cells and their nuclei get started to change?
- The stereological methods, including invariator, nucleator and surfactor has been employed in the *in vivo* studies. How is it possible to apply these methods to estimate the volume and surface area of the cultured cells?

Materials and Methods

Vero Cell Cultures

The Vero cell culture obtained from the cell bank of Pasteur Institute (Tehran, Iran) was grown in Dulbecco minimal essential medium in 25 cm^2 flasks (Nunc, Denmark) under 5% CO_2 at 36ºC for 48 hours. The culture media were supplemented with 5% FBS (fetal bovine serum GIBCO). For the maintenance, the medium was supplemented with 2% FBS. The mentioned media included penicillin and streptomycin sulphate (100 IU/mL and 100 µg/mL), respectively.[5]

Virus Stock

Standard rubella virus (live vaccine) was obtained from the Razi Institute, Tehran, Iran. As the virus could not produce CPE in Vero cells, for confirmation of rubella virus the infected Vero cells cultured on cover slips were fixed in cold acetone and stained by indirect fluorescent antibody test using polyclonal antibody (DAKO, Denmark) against rubella virus and fluorescein isothiocyanate (FITC) conjugated polyvalent rabbit anti-human immunoglobulin according to the manufacturer's instructions. Then, they were observed under a fluorescence microscope (Nikon, Japan) with blue filter.

Vero Cell Contamination with Rubella Virus

Confluent Vero cell cultures developed under 5% CO_2 at 36°C were infected with 100 microliter of re-suspended standard rubella Razi® vaccine virus with the titer of 10 TCID50/mL followed by 30 minutes adsorption at 22°C before adding the medium to the single cell layer at 37°C under

5% CO_2 for 3 days. Then, they were examined daily for the presence of CPE under an inverted phase contrast microscope. At 2, 4, 8, 24, and 48 hours after the incubation time, the cells included in triplicate well plates of the both control and experimental groups were harvested by scraping the cells with a cell scraper (Nunc, Denmark), collected in 1.5 ml Eppendorf tubes, centrifuged at 3000 rpm for ten minutes, and placed in neutral buffered formalin for 24 hours. The control cells were uninfected or normal Vero cells, which were collected at the same time as mentioned above.

Stereological Study

The cells were prepared for microscopic study by pouring ascending grades of ethanol (70%, 80%, 96%, and two steps of 100% ethanol) into a small tube containing the cells. Then xylol and warm paraffin were then added into the tube. Every step took 30 minutes. After placing the tube in a refrigerator, the paraffin block was pulled out from the tube. To quantify the volume and surface area of cell cytoplasm and nucleus, the cells should be sectioned according to the isotropic uniform random (IUR) protocol. According to ‘‘isector’’, the paraffin block was carved to make a spherical shape and randomly rotated and embedded in a new block.[15] The block was sectioned and four sections with 15 µm thickness were obtained from each block and stained with Heidenhain’s azan. In total, 8-10 microscopic fields were selected in each section in a systematic random manner. By changing the position of the microscope stage in an equal interval along the X- and Y-direction of the stage, it was possible to select the fields systematically random. The fields were sampled by manual moving of the stage in equal intervals using the ruler of the microscope stage. An oil immersion lens 100× with numerical aperture of 1.4 was used. Quantifications were completed at the final magnification of 3800×. The surface area and volume of the Vero cell were measured using invariator technique.[13,16] In addition, the volume and surface area of the cytoplasm and its nucleus were estimated using the nucleator and surfactor techniques.[14-17]

Invariator

To estimate the volume and the surface area of the Vero cell, a uniform random point grid of the fundamental area “*a*” was superimposed on the images, where “*a*=XY” (here 13.6 µm^2). A microscope connected to a computer was used. Using the stereological software (designed at Shiraz University of Medical Sciences), the suitable grids, including the point grid and unbiased counting frame were laid on the live images of the tissue sections observed on the computer monitor (Figure 1). The cells were sampled using optical disector in the 15 mm thick sections. Briefly, the stage was traveled downwards in the depth of the section (z-axis). If the nucleolus was in the focus at the beginning, the plane was disregarded. On the other hand, any nucleolus seen during downward movement of section was selected if it lay in the counting frame, contacts the acceptance (upper and right) borders, and did not contact the forbidden (lower and left) borders of the frame. The vertices were drawn from the nucleolus to all points of the grid, which were correlated with the sampled Vero cell. The test lines (intercept, chord, *L*) were drawn perpendicular to the vertices at the junction of the vertices and the points. The surface area and the volume of the cell were estimated using the following formulas.

$$\bar{V} = a\bar{L}$$

$$\bar{S}_V = 2a\bar{I}$$

Where "$\bar{I}$" is the mean number of intersections of the test lines with the cell (or nucleus) border and "$\bar{L}$" is the mean intercept length.[13]

Nucleator

The cells were sampled using optical disector as mentioned above. After sampling the nucleolus, a flat intercept was considered from the central point within the nucleolus. The lengths (intercept, ray, l_n) in the right and left directions out from the point to the borders of the nucleus (to estimate nuclear volume) and the cytoplasm wall (to estimate cytoplasm volume) were documented (Figure 1). 120–200 intercepts in each group were measured and finally the mean nucleus and cytoplasm volume in the number-weighted sampling was estimated using the following formula.[17]

$$V_N = \frac{4\pi}{3} \times \bar{l}_n^3$$

Surfactor

To estimate the mean cell volume, the isotropic intercept emanating from the nucleus to the border of the particle was measured on the sampled cells using disector principle (l_n) (Figure 1). The acute angle β ($0<\beta\leq\pi/2$) between the intercept length and the tangent to the boundary of the cell at the point of the intersection was also measured. The surface area was measured using the following formula.

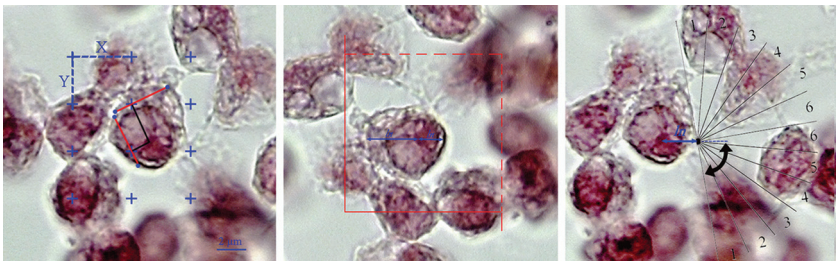

Figure 1: Shows estimation of the volume and the surface area of the Vero cell. Left: Invariator method, X and Y were used to estimate the area per point. The test lines are in red. Middle: Nucleator method, "l_n" indicates the intercept. Right: Surfactor method, curved arrow indicates the acute angle "β".

Table 1: The mean and coefficient of variation (CV) volume and surface area of the Vero cells, its nucleus of the infected, or non-infected with rubella virus using different methods

Indices	Groups	Non-infected (hours)					Infected (hours)				
		2	4	8	24	48	2	4	8	24	48
Invariator											
Cell	Volume	403 (0.41)	353 (0.39)	383 (0.27)	369 (0.25)	361 (0.34)	393 (0.33)	381 (0.49)	371 (0.35)	347 (0.30)	309 (0.33)*
	Surface area	298 (0.27)	244 (0.28)	270 (0.23)	254 (0.23)	260 (0.24)	248 (0.29)	232 (0.35)	246 (0.26)	250 (0.23)	226 (0.23)*
Nucleus	Volume	50 (0.40)	46 (0.44)	43 (0.31)	42 (0.34)	39 (0.26)	41 (0.22)	40 (0.20)	38 (0.21)	32 (0.16)	34 (0.15)
	Surface area	59 (0.28)	58 (0.26)	54 (0.02)	52 (0.18)	54 (0.02)	54 (0.02)	54 (0.02)	54 (0.02)	54 (0.02)	54 (0.02)
Nucleator											
Cell	Volume	424 (0.44)	330 (0.71)	400 (0.40)	380 (0.48)	383 (0.58)	364 (0.51)	355 (0.66)	352 (0.63)	345 (0.68)	291 (0.64)*
Nucleus	Volume	48 (0.61)	47 (0.58)	42 (0.53)	41 (0.56)	40 (0.48)	45 (0.85)	45 (0.89)	40 (0.45)	36 (0.5)	32 (0.85)
Surfactor											
Cell	Surface area	285 (0.33)	257 (0.43)	302 (0.40)	283 (0.40)	285 (0.33)	289 (0.32)	267 (0.36)	264 (0.48)	277 (0.46)	233 (0.48)*
Nucleus	Surface area	71 (0.41)	72 (0.62)	69 (0.45)	84 (0.54)	71 (0.45)	76 (0.49)	67 (0.39)	68 (0.56)	70 (0.55)	57 (0.57)

*P<0.019, Infected Vero cells vs. non-infected cells, 48 hours after initiation

$$S_v = 4\pi \times \overline{I_n^2} \times c(\beta), c(\beta) = 1 + \left(\frac{\pi}{2} - \beta\right) \times \cot\beta$$

For the sake of simplicity, the angles were classified by the transparent protractor overlaid on the images. The protractor was a half circle, which was divided into twelve 15º classes (Figure 1).[14]

Statistical Analysis

The cytoplasm and nucleus volume in the control and experimental groups were evaluated using *repeated measures ANOVA* and t-test. P<0.05 was judged as statistically significant. Coefficient of variation (SD/Mean) was also calculated.

Results

Table 1 shows the results of the study. The mean, standard deviation, and coefficient of variation of the volume and surface area of the cells and their nuclei in different groups are presented.

It takes ~4 hours to estimate the mean cell volume and surface area using invariator. It also takes ~4 hours to estimate the volume using nucleator and the surface area using surfactor. The values of the standard deviation and coefficient of variation of the cells estimated by invariator were lower than those measured by the nucleator or surfactor.

Evaluation of the data showed only 48 h after infection, the cell volume and surface area were changed and no significant changes were observed at the other time. The Vero cell volume decreased by 15-24% in comparison to the non-infected cells 48 h after infection (P<0.019). Besides, the cell surface area was decreased by 13% after the infection in comparison to the non-infected cells (P<0.019). However, no significant change was observed in the volume and surface area of the nucleus of the Vero cell after the infection.

Discussion

One aspect of this experiment was showing significant reductions of the volume and surface area of the Vero cells contacted by rubella virus become visible as early as 48 h following the contact. On the contrary to herpes simplex and measles viruses, which we reported to increase the surface areas and/or volume of the Vero cells as well as the cultured cells,[6,7] it is notable that rubella virus decreases such stereological parameters of the cultured Vero cells. It may be consistent with fact that rubella virus slows down the rate of in-vivo cell divisions.[4] Overall, marked morphological changes, known as CPE, in the rubella virus infected cell lines are scarce.[3-5] It has been rarely reported that few cell lines like RC-IAL are sensitive enough to show rubella virus growth 4 days after infection.[3]

A cell fusion assay was also reported to be able to detect rubella virus, at about 7 days post infection.[5] This indicates that such measurement of the stereological parameters is a good criterion for making a faster identification of Vero cells contacted with rubella virus when showing no gross CPE microscopically. There are some methods for rapid detection of viruses, for example, indirect immunofluorescence (IF), electron microscopy, and nucleic acid amplification techniques.[5] However, the most sensitive and traditional gold standard method for the detection of virus growth is the cell culture systems.[3-5] In the present research, quantitative parameters were applied to detect rubella virus growth in Vero cell culture. This is a cheaper method for detecting rubella virus growth in the early stages of infection of the Vero cells, as the cells which do not occasionally show gross morphological changes. It can also be useful for subsequent viral antigen preparation or serological testing of specific rubella antibodies followed by stereological confirmation of rubella virus growth in the Vero cell line.

The other aspect of the present study was the application of different methods on the cultured cells. The techniques include the estimators of the volume and surface area of the cultured cells. The results of cell size comparison showed a lower variance of the invariator methods. This finding is in accordance with the results obtained by Cruz-Orive (2008).[18] He showed that at least for the unit ball particle model considered here with a nucleolus at a distance *k* from the centre: (i) one-ray invariator, estimator of the surface area, is simpler and more efficient than the surfactor, and (ii) one-intercept invariator, estimator of the volume, is more efficient than the one-ray nucleator. Cruz-Orive described the comparisons as follows.When a one-ray nucleator and a one-chord invariator are defined on a pivotal section, the CV of the second estimator does not depend on the position of the pivotal point. It is likely to be more precise than nucleator unless the pivotal point is rather central in the cell. Cruz-Orive (2012) also explained the integrated form of the estimations.[19] He described that if the integrate over all the rays emanating from the pivotal point from 0 to 2π (nucleator) and the integrate over all the chords corresponding to all possible uniform random points (invariator) are considered on a pivotal section, nucleator and invariator are identical. It is also true in case of surface area estimation. The analogous of one-ray nucleator is the one-ray surfactor. Whereas the analogous to the volume estimation the one-cord invariator is likely to be more precise than one-ray surfactor.

The present research has focused on two local particle estimators. It should be mentioned that if the particles were large enough to be sectioned into 8-12 sections, Cavalieri technique could be applied. Another technique to estimate the local particle volume is estimating the total number and volume of the particles. Then, the total volume could be divided by the number to obtain the mean particle volume. Marićet et al. (2007) showed that quantitative research of growth processes in cultivated fetuses can accurately be done by combining classic methods of measuring whole fetus diameters and stereological methods.[9] The present study utilized the stereological techniques for estimating the Vero cells infected with rubella virus. In the first step, it was explained how it was possible to correctly sample the cells according to their number. In the second step, the measurement method was explained. Sampling the cells according to their numerical density has been called the number-weighted volume (not according to their volume, surface, or height). In doing so, the cells were transferred from the culture media to a small tube. It enables us to make a collection of the cells to be sampled using the optical disector. To estimate the mean volume or surface area, IUR sectioning is necessary. Isector method is the simplest way to make this type of sectioning. Finally, two methods of measurement were applied in this study. Standard deviation and coefficient of variation of the estimated data obtained with invariator were lower compared to the values measured through nucleator. The low dispersion shows that the measurements are more alike and mostly like the mean. On the other hand, higher dispersion or greater standard deviation

shows that there are higher values all over the map. In other words, the nucleator curve with a large standard deviation is more flat with fewer values at the mean.

The present study had some limitations. One of the limitations was the embedding of the cells in paraffin mold. Shrinkage of the tissue is higher in paraffin compared to resin molds.[20] Nonetheless, paraffin was used as a usual embedding media and showed the possibility of the method.

Conclusion

The Vero cell volume and surface area can be decreased 48 hours after the rubella virus infection determined using invariator, nucleator, and surfactor techniques. The values of the standard deviation and coefficient of variation of the cells estimated by invariator were lower compared to those measured by the nucleator or surfactor.

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