Improvement of Incubation Conditions, Buffer System and the Effect of Airflow on Kinetic Growth Rate of *Theileria annulata* Schizont Infected Bovine Myeloid Cell

Short Communication

Hosseini Vafa*, N., Esmaeil-Nia, K., Abdigoudarzi, M. and Hashemi Fasharki, R.

Protozoology Dept., Razi Vaccine & Serum Research Institute,

P.O.Box 11365-1558, Tehran, Iran

Received 24 Dec 2003; accepted 27 Mar 2004

Summary

Kinetic growth rate of the *Theileria annulata* (*T.annualata*) schizont infected monocyte-macrophage cell (SIMC) using modified Stoker medium in different incubation conditions was studied. In this regard 28 Roux flasks were divided into four groups each of 7. Two groups (one with HEPES and the other without HEPES) were incubated with carbon dioxide flow. The reminder two groups were incubated under conventional condition. All bottles were observed for viability test and pH changes daily up to 7 days. To study the effect of airflow on cell growth rate some glass tubes with different diameter were inserted through stopper of Roux bottles containing cultured SIMC. The results indicate that carbon dioxide flow together with HEPES have an increasing effect on cell quantity moreover, the specific growth rate index of all groups were similar. The different amounts of airflow doesn't have any effect on oxygen consumption, therefore the routine culture method using the trapped air in bottles, is the most suitable method for making a suspension culture of *T.annulata* SIMCs.

Key words: *Theileria annulata*, kinetic growth rate, modified Stoker medium

Introduction

The most important factor affecting on animal cell culture is to select a proper medium. In discontinuous cultures, cells are inoculated in culture medium and as

*Author for correspondence. E-mail:noshin hosseini@yahoo.com

they grow, the nutrients will be absorbed and different metabolites accumulated in medium. So, the environment changes permanently, which will affect cell metabolism (Freshney 1987). Ideally, pH of culture medium should be in a range of 7.2-7.4, which is suitable for most of the cell lines grow. However, different cells grow in different pHs. The proper pH for each cell line should be detected by experiments. Different agents such as carbon dioxide, lactic acid and pyruvic acid accumulate in the medium, which resulted in changes of pH and threat the life cycle of cells. pH changes would be controlled by compounds acting as strong buffers such as sodium bicarbonate, however there are some disadvantages using it: 1) PKa of $NaHCO_3$ is 6.3 at 37°C which is lower than desired physiological pH (7.3) for most animal cell lines. 2) $NaHCO_3$ is deionized through the following reaction:

$$NaHCO_3+H_2O=Na+HCO_3^-+H_2O=Na+H_2CO_3+OH^-=Na+H_2O+CO_2$$

As a result CO_2 enters to the atmosphere so, the reaction tends to the right. The concentration of hydroxide ion is increased and the pH risen. Therefore to get an optimized pH, a suitable amount of CO_2 should be inserted to the medium, using a kind of biological zwiterion buffer such as HEPES. HEPES could be used together with sodium bicarbonate or as an alternative of it. It is chemically stable and inhibits the pH changes in cell culture medium (Doran 1995). One other important factor affecting the growth of animal cell lines is oxygen. The rate of oxygen consumption for animal cell lines is less than that of microbial cultures. In large-scale cultures of animal cells oxygen deprivation will has negative effect on cell growth (Taylor & Baker 1987). This study, aimed at the selecting the most proper conditions for growth of *T.annulata* SIMCs. The roles of HEPES in cell growth and ventilation effect using specific cell line in culture media were discussed.

Materials and Methods

Cell line and inoculation. *Theileria annulata* (*T.annulata*) schizont infected myeloid cell (SIMC) line, which is used for production of a live attenuated vaccine against bovine theileriosis (Razi Institute, Karaj), was used. Primary culture was

prepared from seed cells cry-preserved in liquid nitrogen. Cells were thawed and cultured in a volume of 12 ml culture media in 90-ml flasks and incubated at 37°C. When the cell population increased up to 7-8×10^5cell/ml they were sub-passaged in a volume of 100ml of culture medium in 1-L Roux flasks. A cell population as about 50,000cell/ml at the beginning of each sub-passage was used. Inoculation was done at the late log phase, which led to a continuous growth without significant latency. Latent phase was prolonged if inoculated cells were at the stationary phase.

Culture medium. A modified Stoker as a tissue culture medium was used. 10% bovine serum, 100IU/ml penicillin and 100mg/ml streptomycin were added. Medium was filtered through a nitrocellulose membrane (0.2μm pore size) and kept at 4°C until use.

Experimental design. The combined effect of CO_2 flow and biological buffer HEPES as well as ventilation effect on growth rate of *T.annulata* SIMC were studied in two major parts.

1) Carbon dioxide flow and the role of HEPES. Two types of culture media one Stoker with HEPES (25μmol) and bicarbonate (1.4g/Lit) and other with only bicarbonate (1.4g/Lit) were prepared. Four series of flasks (each of 7) were sterilized. Groups 1 and 2 were inoculated with Stoker and groups 3 and 4 with Stoker+HEPES, and capped with the cotton. Groups 2 and 4 were incubated under conventional conditions and others under 5%CO_2 up to 7 days. The culture cells were examined for pH changes and cell count using vital dye (Erythrosein-B 0.4%) daily. This experiment repeated twice and the results were tested by analysis of variance (ANOVA) test.

2) Effect of ventilation on cell growth in flask. Glass tubes with different diameters were inserted through the stoppers of culture flasks. Diameters of the tubes were 5, 10 and 15mm for groups 1, 2 and 3, respectively. The tubes were capped by cotton and a control group was capped by rubber stopper. Each group contained 7 flasks, (one flask was cultured every day) then the flasks were kept in normal incubation condition (without CO_2). Daily control measurements including

viability test, pH control and dissolved oxygen level were carried out. This experiment repeated four times and the results were analyzed by one-way analysis of variance.

Measuring dissolved oxygen. Solomat 50 pH-meter was used for measuring dissolved oxygen that is equipped by special electrode for this ability. Using a magnetic stirrer at 37°C, the oxygen consumption rate was estimated and the rate was calculated by $DCA/Dt=\{(CA)_{t+e}-(CA)_{t-e}\}/2e$ formula.

Results and Discussion

The results are shown in figures 1 to 7. As shown in figure 1 growth rate in group 2 was very slow until day 4 and increased on day 5 then went to lethal phase by day 6. Cell production rate in this group was much more lower (325×10^3cell/ml) than other groups. The steady state time in stationary phase was very short. There may be a limiting factor for cell production process when the cell was harvested at the stationary phase. Analyzing of the pH changes curve (figure 2) is indicated that in primary step in culture process, pH of culture medium in group 2 was higher than other groups and this could affect cell growth process in the final stage.

In figure 3, the logarithm of cell number according to time for each tested group is shown. Using linear slope of the curve the specific growth rate could be estimated. The specific growth rate changes according to time are shown in figure 4. There was a decrease in specific growth rate at the beginning of culture, and then by day 3 the rate increased fast. The specific growth rate was 0.772 1/day and the doubling time (dt) is equal to 22h. The kinetic growth curve in other groups showed a similar pattern. Specific growth rate and dt were 0.710 1/day and 23h, 0.743 1/day and 22h and, 0.704 1/day and 23h for groups 1.3 and 4, respectively. There was no significant difference between specific growth rate and dt in experimental groups, but8 different incubation conditions and biological buffers c8ould affect cell growth rate. The maximum yield of cell production was seen in culture medium with

HEPES and incubation with CO_2 flow (1220×10^3cell/ml). In this condition, the peak of cell population was at days 5 and 6. This should be regarded as stationary phase then the cells went for the lethal (decline) phase.

Effect of the different diameters of ventilation tubes is shown in figure 5. As seen the cells were in latent phase for two days, and then they went into growth phase. The size of 5mm diameter resulted in the highest population number of cells (1475×10^3cell/ml). The peak of growth rate was on day 5. Growth changes curve showed non-desired view from the point of production rate. Using ventilation tubes of 10 and 15mm diameters the peak of growth rate was on day 5 and the numbers of cells were 1292.5×10^3 and 1091.2×10^3cell/ml, respectively. Also, the growth phase was longer in later so that there was more time for cell harvesting. Analysis of Variance test ($P<0.01$) of the results showed that there was no significant difference in cell populations between these experimental groups.

According to the pH changes (figure 6), it is concluded that rising pH at the beginning of growth process, directly depends on the diameter of ventilation tube, so when the ventilation tube diameter increases, there would be a pH elevation in early period of cell culture. This is the result of sodium bicarbonate dissociating and the production and release of CO_2. As shown in figure 7 oxygen consumption was almost similar pattern in all groups. Oxygen consumption rate was calculated 0.429ppm/day for all groups. It is concluded that the rate of oxygen consumption by *T.annulata* SIMC in culture is very low and the limited air in flask (9000ml air comprised 0.27gram oxygen) is enough for cell growth.

The results of our study could be a plan to improve the conditions of bovine theileriosis vaccine production method, which is made to date by a suspension culture of *T.annulata* SIMCs. Although the results of groups 1, 3 and 4 were not significantly differ use of group 3 for better growth rate of monocyte-macrophage cells is suggested. In this way the cells would increase without any decrease in their biological properties.

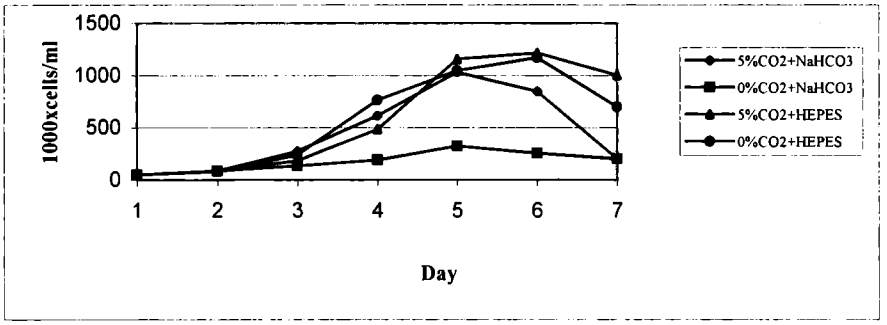

Figure 1. *Evaluation of buffer system and airflow on kinetic of T.annulata SIMCs growth rate*

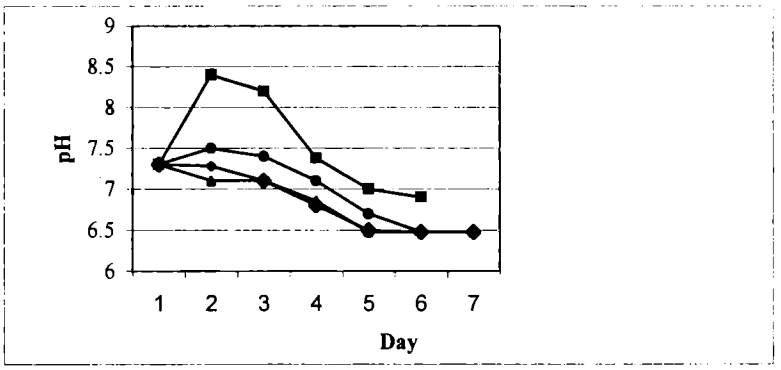

Figure 2. *pH changes on the evaluation of buffer system and airflow on kinetic of T.annulata SIMCs growth rate*

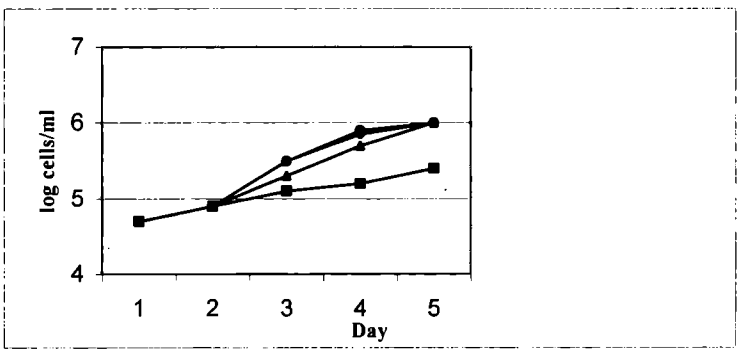

Figure 3. *The logarithm of T.annulata SIMCs number on the evaluation of buffer system and airflow on kinetic of the cells growth rate*

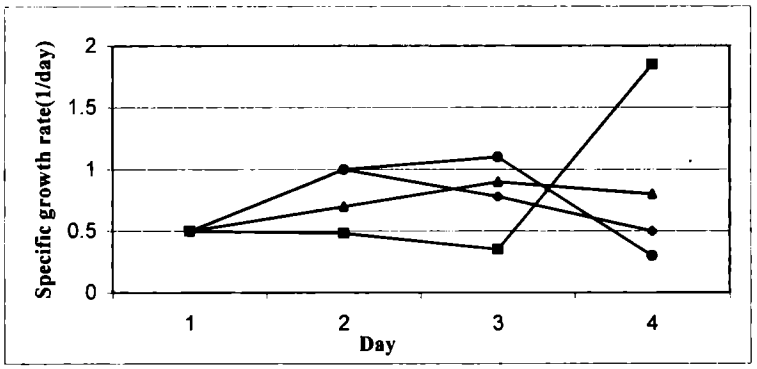

Figure 4. *Specific growth rate changes on the evaluation of buffer system and airflow on kinetic of T.annulata SIMCs growth rate*

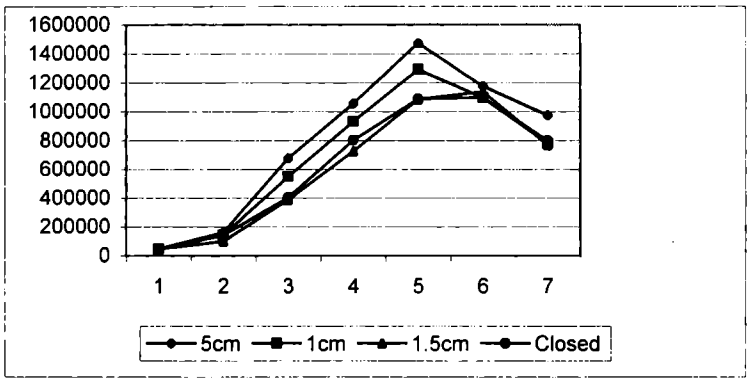

Figure 5. *Effect of ventilation on kinetic of T.annulata SIMCs growth rate*

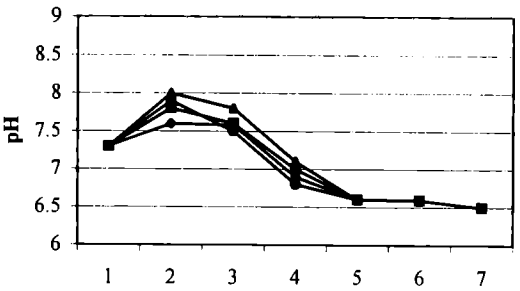

Figure 6. *pH changes on evaluation of ventilation effect on kinetic of T.annulata SIMCs growth rate*

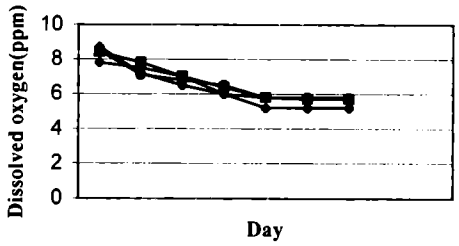

Figure 7. *Oxygen consumption rate in T.annulata SIMCs*

Acknowledgement

The authors wish to thank all the staff of Protozoology department of Razi Institute for their technical supports.

Referrences

Doran, P.M. (1995). *Bioprocess Engineering Principles*. Academic Press. USA.

Freshney, R.I. (1987). *Culture of Animal Cells: A Manual of Basic Technique* (2nd edn), Pp:1-3 & 57-86. A.R.Liss. New York.

Hashemi-Fesharki, R. (1988). Control of *Theileria annulata* in Iran. *Parasitology Today* 4:36-40.

Hashemi-Fesharki, R. (1986). *Bovine Theileriosis in Iran*. Pp. 126-133. Agricultural and Natural Resources Research, Iran (In Persian).

Hashemi-Fesharki, R., Shad-del, R. (1973). Vaccination of calves and milking cows with different strains of *Theileria annulata*. *American Journal of Veterinary Research* 34:1465-1467.

Jo, E., Kim, D. and Moon, M.H. (1993). Step-fortification of nutrients in mammalian cell culture. *Biotechnology and Bioengineering* 42:1218-1228.

Miller, W.M., Blanch, H.W. and Wilke, C.R. (1988). A kinetic analysis of hybridoma growth metabolism in batch and continuous suspension culture; effect of nutrient concentration, dilution rate, and pH. *Biotechnology and Bioengineering* 32:947-965.

Morgan, S.J., Darling, D.C. (1993). *Animal Cell Culture*. Pp:244-245. BIOS Scientific. London.

Parve, P., Wolfgang, U.F. and Sukatsch, D.A. (1987). *Fundamentals of Biotechnology*. Pp:153-177. VCH Verlagsgesellschaft. Germany.

Taylor, A.E.R., Baker, J.R. (1987). *In Vitro Methods for Parasite Cultivation*. Pp: 230-245. Academic Press. London.