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Original Article

The Effect of Methylene Blue in Combination with Red Visible Light on Model Virus Inactivation Fresh Frozen Plasma and Coagulation Factors

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Abstract

Background and Aims: Fresh Frozen Plasma (FFP) is one of the blood components. The risk of transmission of viruses from blood components regardless selection of blood donors and screening donated blood still remains as a problem. There are several methods for viral inactivation. In this study methylene blue (MB) photo inactivation process was used for inactivating viruses.

Materials and Methods: In this study Methylene Blue(MB) was used in final concentration of 1µM. Infected Fresh Frozen Plasma (FFP) illuminated by 143Pieces (PCs) of 1 w red Light Emitting Diodes (LEDs) from two side for 10,15 and 30 minutes and shacked 30 cycle in minutes. The central wavelength of these LED is 627 nm with 20 nm Full Width at Half Maximum (FWHM).Herpes simplex virus-1(HSV-1) and vesicular stomatitis virus (VSV) were used as model viruses to evaluated illumination effects on viral inactivation. Level of fresh frozen plasma (FFP) coagulation factors such as fibrinogen, FV, FVIII, protein C, antitrombin measured pre and post illumination.

Results: Initial HSV-1 and VSV titer were calculated to be 10^7 and $10^{6.5}$ $TCID_{50}$/ml, respectively .The level of viral inactivation was expressed as log-reduction. Titer reduction of HSV for 10, 15 and 30 minutes irradiation with shaking was>6, ≥7 and ≥ 7 logs, respectively. The ratio of coagulation factors activity remaining unchanged after pathogen inactivation with MB calculated. Illumination had major effects on the mean levels of fibrinogen and FVIII. Significant differences between level of factors before and after illumination were evaluated with a t test for paired samples. No significant differences were seen in the FFP coagulation factors before and after illumination (P>0.05).

Conclusion: As results show the optimum time for viral inactivation were adjusted to be 15 minutes. Due to the reduction of virus titer at various times, agitation with illumination is effective.

Keywords: methylene–blue; light; virus inactivation; shacking

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Introduction

Viral contamination of biological products is of concern to public health officials. Pathogen inactivation raise the safety margin for blood products by inactivation pathogen that remained undetected during screening due to window periods on test error (1). It also provides as proactive approach

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to inactivation emerging and as-yet-unidentified pathogen before they enter the blood supply chain and before screening test have been performed.

Methylene Blue in combination visible light is one of method for inactivation of pathogen in fresh frozen plasma has been developed in clinical for 15 years (1, 2). This system was shown to inactive a broad range of different DNA and RNA viruses in plasma.

FFP is used to treat congenital coagulation factor deficiencies, for which specific concentrates are not accessible but Methylene Blue pathogen inactivation process appear to result in loss of coagulation factor leading to additional transfusion of plasma (2).

In this study we systemically investigated the inactivation HSV-1 model for HBV by methylene blue plus visible light treatment while agitate to assess the capacity of this procedure to prevent transfusion mediated HSV transmission. Also level of selected coagulation factors of plasma such as factor V, VIII, antitrombin activity ,protein C and fibrinogen before and after photoinactivatin evaluated.

Methods

Source of cells

Foreskin sample collected from three different 4-8 week old healthy boy. Tissue samples were incubated at clean room for 2hr in antibiotic incubation medium (AIM) consisting of DMEM, 1000µg/ml streptomycin 1000IU/ml penicillin. Following two additional washes in AIM (15 min/wash), tissue sample were minced with sterile instrument, and 20-30 tissue fragment were planted in 25-cm^2 Nunc tissue culture flask. In addition minced tissue fragments were digested with 0.25%trypsin at 30°C for 20 minute. Dissociated cells were then washed once with phosphate buffer saline (PBS) and approximately 1 to 2×10^6 cells were transferred into each of several 25 cm^2 Nunc tissue culture flasks. Primary cell culture were initially maintained in the Dulbecco's Eagle Minimum Essential (DMEM) medium supplemented with 8% fetal bovine serum (FBS) according to previously described method (9). Cell cultures were incubated at 37°C and when monolayers were confluent, cell were sub cultivated at a ratio of 1:4 every 3 to 5 days with 0.25% trypsin solution (fluka chemical co). Concentration of FBS was reduced by half after 10 h passage. After cloning and several sub passage cells were seeded into 32 oz bottles. Subsequent subcultures were made twice per week. As positive and negative control the other human cell line (MRC-5) and a Rabbit kidney (RK-13) used in this study .Cell culture were maintained either by sub culturing in 32 oz bottles or were preserved in the frozen state suspended in the Dulbecco's Eagle Minimum Essential Medium (DMEM) containing 10% dim ethyl-sulfoxide and 20% fetal calf serum. Preservation step made when the cells placed in 2ml cryotube, which completely sealed, gradually frozen and then stored in liquid nitrogen (9).

Media

Growth media consisted of DMEM plus 5-8% bovine serum. Maintenance medium was the same medium contains lower (2%) serum concentration.

Viruses Susceptibility

Viruses used in this study (Mumps, Measles, Rubella, Polio Vesicular stomatities virus) were part of the collection of Human Viral Vaccine department of Razi vaccine and serum research institute. Following adaptation of virus, inoculation (0.1 ml/25 cm^2 Nunc tissue culture flask or 16mm well in 96 well plastic micro plate) were done. The culture vessels were held for periods up to14 days at 33-35°C, when virus cytopathic effect (CPE) was observed supernatant fluid was harvested. The virus titer were determined by inoculating 10 fold dilution in a similar method and were expressed as $\log_{10}$ $TCID_{50}$/0.1ml. The final result calculated by the method of Read and Munch (2).

Chromosomal analysis

Chromosomal studies were done by treating cells with a hypotonic solution, fixation, flame spreading and Giemsa staining, according to the method of Moorhead et.al (3). A total of 500 selected metaphase spreads were counted

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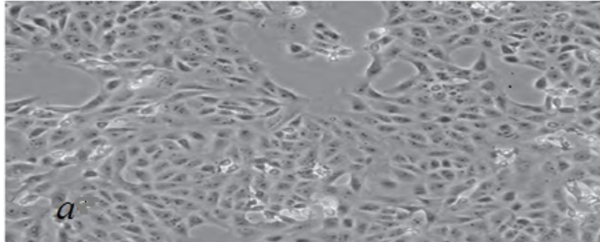
with phase-contrast microscopy. These procedures were repeated 3 times.



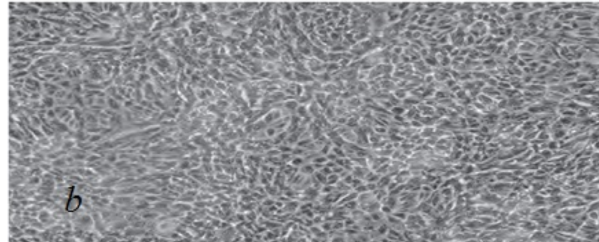


Fig. 1. Comparative picture of confluent (a) and sub confluent (b) RFSC-1 cell line.

Isoenzyme Assay

For detection of interspecies cross contamination Isoenzyme analysis method were used. In this method polymorphic enzymes can be visualized as electrophoresis variant. The pattern of electrophoresis giving rise specific for each species O'Brien (4), Bright (5), Stratton (6), Nims (7).

PCR-RFLP

A Polymerase Chain Reaction (PCR) Restriction Fragment Length Polymorphism (RFLP) assay was optimized, based on a pair of primers anneal to a portion of cytochrome *b* gene and a panel of six restriction enzymes. The derived pattern of RFLP was resolved on 3% agarose gel. Each species has specific pattern for restriction enzyme panel. By this pattern not only the species but also cross contamination between different cell lines can be distinguished. Losi FS, *et al* (8), Pun KM AC (9).

Adventitious agent testing

Verification the sterility of the cell line in order of different bacteria and viruses were made by the protocol of WHO (TRS: 842) (10).

Results

Cell morphology

RFSC-1 cell line was established from fore skin tissues of 4-8 week old healthy boy. This cell line has been passages more than 135 times since its initiation based on the methods of Hay flick and Moorhead (1961). The methods we used for clonally isolation of RFSC-1 have been previously reported by Fereshney (11, 12). In primary cloned cultures both fibroblastic and epithelial cell shape was obtained. Then fibroblastoid clones grew more rapidly and takes dominant in all of cultures flask. So during next cultures RFSC-1 showed fibroblast like appearance in its morphology although derived from skin (Fig. 1). Growth medium was Dulbecco's Eagles Basal Medium (DMEM) supplemented with 5-8% serum. A 4-ozflask was seeded each with 6ml of cells at concentration of 10^5cells per milli liter. After 3 days incubation in 37°C a confluent sheet of fibroblastoid cells (Fig. 1) was formed. Cell were sub-cultured every 3 days, split ratio was 1:4 .Up to 135th subculture this features were constant. When the passage number reaches to 5, cells were harvested and frozen in the presence of preservative (10% dimethyl sulfoxide in DMEM). Attempt was made for every 5 passage stocking and producing enough working seed from this cell line.

Viral susceptibility

Viruses used for evaluation the RFSC-1 susceptibility was vesicular stomatites virus (New jersey serotype), Measles virus (AIK strain) Mumps virus (Hoshino&RS#12 strain), Rubella (Takahashi strain). Two different Subcultures were selected for these studies. Different stock viruses were first adopted by one or two passages and then were seeded in 4-oz flasks containing a confluent sheet of RFSC-1. The supernatant fluid of cultures were harvested when cytopathic effect (CPE) were sufficiently progressed and potency test were made in sensitive cells depending on the type of virus. Results are reflected in (Table 1).

The virus sensitivity spectrum of this cell line show a good sensitivity for mumps virus strain (RS#12) this sensitivity is as compare as MRC-5 cell line. This sensitivity was positive also during Heamadsorption against Guinea pig RBC.

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Chromosomal analysis

The RFSC-1 Chromosomal analysis is shown in (Fig. 2). This analysis was performed at population doubling level 57. At each population doubling 500 cells spread were examined and counted. This procedure was repeated independently 3 times (Fig. 2). The chromosomes were arranged according to decreasing size for pair 1to 19, although the number of chromosomes increases more than three. The sex chromosomes were placed at the end of the Karyotype as normal shape in compare of other species.

Iso enzyme and PCR-RFLP Assay

The result of isoenzyme assay and RFLP-PCR were shown in (Table 2 & Fig. 3 & 4). The results of isoenzyme assay test show that RFSC-1 cell line has the human origin. In this test as positive and negative control used from MRC-5 (Human fetal lung) and RK-13 (Rabbit Kidney). Also the result of RFLP-PCR (Fig. 4) showed that there is not any cross contamination with another cell line.

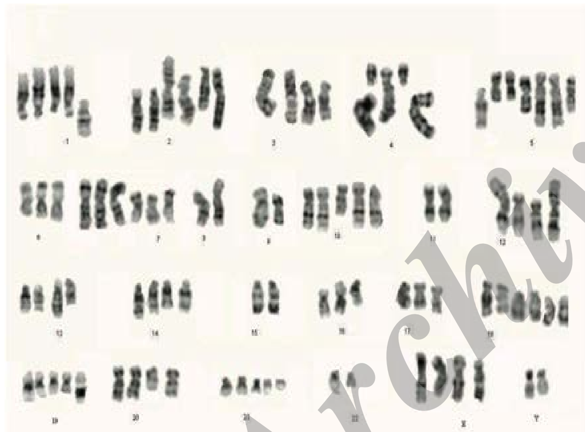

Fig. 2. Haploid karyotype of RFSC-1 shows more than one pair for each chromosome. The number of chromosome has variation during each passage.

Table 1: Taxonomic distribution of questions		
Cell passage	RFSC-1/MUMPS	MRC-5/MUMPS
17	5.5-6*	5.5-6
45	5.5-6	5.5-6

Adventitious agent testing

Adventitious agent testing of RFSC-1 showed there are not any cross contamination to other viruses and this cell line is completely sterile.

Discussion

Today the entire virological laboratories are in urgent need to have cell bank from different cell line. This need comes from different degree of sensitivity that a virus shows to different cell substrate. Although tissue culture was started at the beginning of the twentieth century (13, 14) as a technique for studying the behavior of animal cells, but at present is the most important tool in production of antigen (15) biological product, vaccine (16), isolation and detection of viruses (17, 18). The primary explants of tissue dominated the field of 'tissue culture'' for more than 50 years (19, 20). Despite the fact that most of the explosive

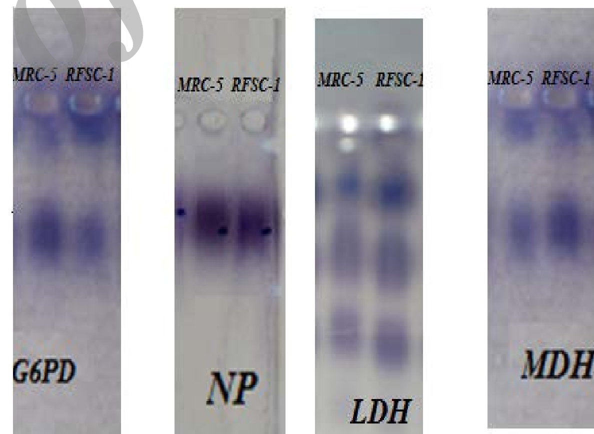

Fig. 3. Distribution of parvovirus B19 IgG antibody means (IU/mL) showed in vertical axis according to age groups of women showed in horizontal axis.

expansion in the field of virology, was made possible by the use of dispersed cells in the second half of the twentieth century. These cells taken from original tissue as primary culture, cell line or cell strain, following enzymatic, mechanical, or chemical disaggregation (21). Dulbecco's was the first who demonstrated the outgrowth disaggregation of explanted cells and subsequent plating out of the dispersed cells. Although the term of cell strains generated by

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Fischer, Carrel, and others often using for that cell line that access by surgical subdivision of the culture. The first cloned cell strain, isolated by capillary cloning from mouse L-cells was L929 (22). It was not until the 1950s that trypsin became more generally used for subculture, following procedures described by Dulbecco to obtain passaged monolayer cultures for viral plaque assays (23) and generation of a single cell suspension by trypsinization, which facilitated the further development of single cell cloning. Establishment a foreskin line (RFSC-1) derived will greatly enhance attempts to isolate and characterize the Mumps viruses. This cell line replicated well under the described condition, was easy to maintain, and has high viability following storage in liquid nitrogen. Razi vaccine and serum research institute has the history of more than 90 years of biological and about 50 years in human viral vaccine production.

Establishment of cell substrates from human tissue as a part of our project on human cell line may be accomplished with relative ease. Outgrowths in continuous culture vary and much of the effort in developing selective conditions has been aimed at suppressing fibroblastic overgrowth, which not only dominates other cells in the culture but may actively inhibit them (24). A foreskin line (RFSC-1) derived during this study and was maintained more than 135 sub passages using 1-2 split twice weekly**.** The virus sensitivity spectrum of this line showed not only in susceptibility but in titer there is a closer relationship between MRC5 and RFSC-1. The RFSC-1, like their human counterpart MRC-5 supported the propagation of Mumps virus strain. Also of importance was the ability of (RFSC-1) to continue *in vitro* for a greater period of time than MRC-5 cells .These cells could be sub-passaged 138 till now in contrast with the 40-50 passage seen with human lung

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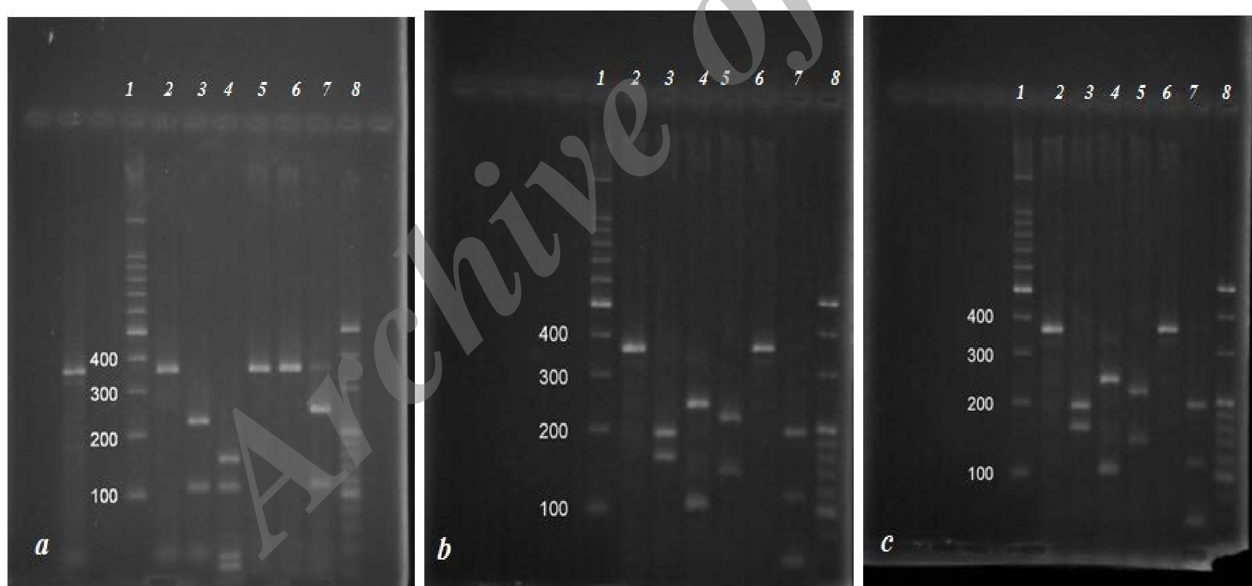

Fig. 4. Electophoretic profile on 3%agarose gel of PCR-RFLP on DNA from (a)RK-13 as negative control (b)MRC-5 as positive control(c)RFSC-1 the new human skin cell line. For each agarose gel shown, the loading order of restrictions was *AluI, HinfIII, HaeIIi,TagI, RsaI, MboI*. Molecular weight marker were 100 -lane1 and 20bp lane 8.

Table 2: Bioinformatic restriction profile of cell culture belonging to RFSC-1 in compare of negative (RK-13) and positive (MRC-5) control

Species	*AluI*	*HinfI*	*HaeIII*	*TaqI*	*RsaI*	*MboI*
RFSC-1	358	198 160	231,106,21	217,141	358	192 115 51
MRC	358	198 160	231,106,21	217,141	358	192 115 51
RK-13	358	236,122	153,128,45, 32	358	358	243,115

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cells MRC-5. The results of chromosome analyses of RFSC-1 are presented in fig. 3. The RFSC-1 remained heteroploid for over138 passage and also an extra-large metacentric chromosome observed in these cells. These feature demonstrated developing of a new continuous cell line. In the 1950s and 1960s many continuous lines were unknowingly cross contaminated with other cell lines including Hella cells. In 1970s and 1980s as many as one in three cell lines deposited in cell banks were contaminated (25).

Comprehensive testing regimens for detection of adventitious agents in vaccine cell substrate are designed to minimize the risk of virus and cell to cell cross contamination in vaccines and assure product safety. This contamination comes from different material such as viruses', bacteria, and other cell line. There are different test for detection of cross contamination such as Using standardized STR or DNA bar-coding (26). In this way, it is important to retain tissue or DNA from the donor and to do comparative DNA STR profiling or DNA bar-coding (27, 28) between the new cell line and its presumed source as soon as it becomes established. In this paper two test isoenzyme assays and RFLP-PCR used for safety verification of RFSC-1. The result of two test conducted suggest that this new cell line belong to human origin and also had no any cross contamination to other cell line or any other pathogens.

The authors describe briefly studies on development of continuous cell line from human tissue .The main criteria to be used for evaluation of RFSC-1 establishment were its infinite life potential, it's maintenance in cell culture medium as determined by cytogenetic analysis ,it's sensitivity to human virus . Related criteria included the testing of a qualifying line for proof of species of origin, for freedom from any type of contamination, and the capability of storage in frozen state or liquid nitrogen.

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