ISSN (Print) 1225-9918
ISSN (Online) 2287-3406
Journal of Life Science 2016 Vol. 26. No. 1. 23~33 DOI : http://dx.doi.org/10.5352/JLS.2016.26.1.23

Effect of NaCl on the Stability of Oncolytic Vaccinia Virus

Seong-Geun Kim[1], Gui Shao Ran[2], Hyuk-Chan Kwon[3] and Tae-Ho Hwang[3,4]*

[1]*Department of Internal Medicine, Pusan National University Yangsan Hospital, Yangsan 50612, Korea*
[2]*Department of Pharmacology, College of Medicine, Dong-A University, Busan 49201, Korea*
[3]*Gene and Cell Therapy Research Center for Vessel-associated Diseases, Pusan National University, Yangsan 50612, Korea*
[4]*Department of Pharmacology, School of Medicine, Pusan National University, Yangsan 50612, Korea*

Received October 27, 2015 / Revised December 18, 2015 / Accepted December 23, 2015

Pexa-Vec (JX-594) is a specific cancer-targeted oncolytic and immunotherapeutic vaccinia virus. The purpose of this study was to develop methods to maximize the stability of Pexa-Vec. In short-term instability testing, viral activity was rapidly decreased both at 4°C and at room temperature (RT), but it was completely restored after sonication followed by vortex. Long-term stability testing of Pexa-Vec in the following liquid formulations was performed: (A) 30 mM Tris/pH 7.6, (B) 30 mM Tris/pH 8.6, (C) 30 mM Tris/pH 7.6, 150 mM NaCl, 15% sucrose, (D) 30 mM Tris/pH 7.6, 15% sucrose, and (E) 30 mM Tris/pH 8.6, 15% sucrose. Viral activity decreased less than 2 log10 at 4°C, and RT was observed in 3 days in B, while viral activity was not decreased even after 4 - 8 weeks at 4°C and at 1 week in RT in A, suggesting that neutral pH may be essential to maintain virus stability. The addition of 15% sucrose into A (D) significantly increased viral stability at -20°C, 4°C, or RT, and it was also observed at pH 8.6 (E). The addition of 150 mM NaCl into D (C) significantly increased viral stability in addition to the sucrose effect at 4°C or RT. Accordingly, the viral activity in formulation C was maintained for 1.5 years at 4°C, and for 1-2 weeks in RT. In conclusion, we propose that formulation C can provide the most adequate condition for the proper storage of vaccinia oncolytic virus.

Key words : Formulation, NaCl, oncolytic virus, stability, viral activity

Introduction

Oncolytic viruses (OVs) mediate tumor regression through selective replication in and lysis of tumor cells and induction of systemic anti-tumor immunity without damage to normal cells [7]. At present dozens of natural or genetically engineered viruses are being investigated for the treatment of solid malignancies and there is increasing clinical evidence supporting their safety and efficacy, both as a monotherapy and in combination with other treat modalities [1].

Pexa-Vec (*pexastimogene devacirepvec*; JX-594) is a targeted and armed oncolytic and immunotherapeutic vaccinia virus engineered to express human granulocyte-macrophage colony-stimulating factor (GM-CSF) and β-galactosidase transgenes, and has multiple mechanisms of action to destroy cancer cells [6]. We also demonstrated that Pexa-Vec induced polyclonal antibody-mediated complement-dependent cytotoxicity (CDC) against multiple tumor antigens both in rabbits and in patients with diverse solid tumor types [9]. Pexa-Vec induces objective tumor responses in previous clinical trials [2, 11].

During these clinical trials, Pexa-Vec in liquid formulation should be used, therefore viral stability in liquid formulation is extremely important to meet qualified clinical study. It has shown that short term viral instability was involved in viral aggregation. Poliovirus and reovirus aggregate into clumps of up to several hundred particles when diluted into distilled water or in buffers at low pH and this was observation was also shown in adenovirus [3]. More than 90% of adenoviral infectivity was lost due to viral aggregation in less than pH 5.

The goal of the ultimate gene or viral delivery system is to produce viral system with maximum safety, effectiveness, and reliability. Among many different factors, the physical and chemical stability of the viral vectors may significantly affect for final goal of oncolytic viral therapy. As the viral vector degrades and/or aggregates, infection (or transduction) efficiency drugs and the reliability and effec-

***Corresponding author**
Tel : +82-55-360-1732, Fax : +82-55-382-7554
E-mail : thhwang@pusan.ac.kr
This is an Open-Access article distributed under the terms of the Creative Commons Attribution Non-Commercial License (http://creativecommons.org/licenses/by-nc/3.0) which permits unrestricted non-commercial use, distribution, and reproduction in any medium, provided the original work is properly cited.

tiveness of the preparation diminishes. If the degradation or aggregation products are toxic or immunogenic, the safety of the preparation is also compromised. Optimization of formulations to enhance chemical and physical stability and to prevent degradation and/or aggregation of viral vector system over time will secure the reliability of the drug preparation.

In the early phase of Pexa-Vec clinical trial, dose escalation study of intratumoral injections needed unanticipated time consuming procedure, and decrease of short term viral activity was controlled very carefully in the sites of clinical trial [11]. Historically, viral vector formulations have considered of glycerol (10-50%) in phosphate or Tris buffers with additions of other excipients such as salts, sugars, and bovine serum albumin [3]. The initial observation of short term viral stability, the viral instability may be via physical viral aggregation. Therefore, two different factors which can affect viral stability especially viral aggregation; ion strength (addition of NaCl) and pH [5]. Furthermore sucrose which may be essential for cryoprotectants was used to prepare different formulations [3]. In this study, viral activity both in terms of short and long term stability could be controlled significantly by liquid formulations. Accordingly we decided to find the best formulation to keep viral activity both short and long term period.

Materials and Methods

Virus

Pexa-Vec is a Wyeth vaccinia vaccine (Dryvax®, Wyeth Laboratories) derived oncolytic virus engineered to inactivate virus encoding thymidine kinase (TK) gene, and to express the human granulocyte-monocyte colony stimulating factor (hGM-CSF) and Eshcherichia coli beta-galactosidase transgenes.

Cell lines

Human cervical cancer cell line, Hela (CCL-2) were obtained from American Type Culture Collection (ATCC). Human ovarian cancer cell line A2780 (93112519) were purchased from European Collection of Cell Cultures (ECACC).

Virus manufacturing and formulations

Two batches of Pexa-Vec of clinical trial materials and one batch of laboratory purified virus were used for this study. Two clinical trial batches were produced by different GMP facilities using different cell lines, purification processes, and formulations. The first clinical trial material (CTM) of Pexa-Vec was manufactured in Novavax Inc. (Rockville, MD, USA). In Novavax batch, virus was amplified in Vero cell line, purified by sucrose cushion method described in detail below and stored in the liquid formulation of 10% glycerol in PBS. By limitation of CTM virus, the initial acute viral stability test was done using laboratory grade virus which were amplified in HeLa cells and purified and stored with the same method used in Novavax. And, finally we used different formulation in IDT Biologik GmbH virus which was manufacture as described below.

The compositions of formulation buffer

For all studies, 500 μl aliquots were added to sterilized glass vials (Afton scientific corp, US) and 1×10^9 pfu/ml or 3×10^8 pfu/ml Pexa-Vec were spiked in formulation buffers in Table 1, and the timing of the measurement of virus activity in different buffers was determined as indicated in Table 2. Aliquots of Pexa-Vec in different formulations were stored at -70℃, -20℃ or 4℃ for two years, room temperature (RT) for one month, or 37℃ for one week. Viral activity (infectious titer) in each aliquot was determined at indicated time in A2780 cells.

Measurement of virus activity; Plaque assay for Titration

A2780 cells were plated at a density of 3×10^5 or 4×10^5 cells per well in six well plates for the Pexa-Vec plaque assay. This 6-well plates were incubated at 37℃ and 5%

Table 1. Formulations of Pexa-Vec stored at 37℃, room temperature, 4℃, -20℃ and -70℃

	1×10^9 pfu/ml	3×10^8 pfu/ml
A	30 mM Tris at pH 7.6	30 mM Tris at pH 7.6
B	30 mM Tris at pH 8.6	30 mM Tris at pH 8.6
C	30 mM Tris pH 7.6, 150 mM NaCl and 15% sucrose	30 mM Tris pH 7.6, 150 mM NaCl and 15% sucrose
D	30 mM Tris, pH 7.6, 15% sucrose	30 mM Tris, pH 7.6, 15% sucrose
E	30 mM Tris, pH 8.6, 15% sucrose	

pfu: plaque forming unit

Table 2. The schedule of stability test measured by plaque titration in A2780 cell lines

	Time point										
37℃	3 d	7 d									
RT	3 d	7 d	2 w	1 m							
4℃			2 w	1 m	2 m	3 m	6 m	9 m	12 m	18 m	24 m
-20℃			2 w	1 m	2 m	3 m	6 m	9 m	12 m	18 m	24 m
-70℃					2 m	3 m	6 m	9 m	12 m	18 m	24 m

RT, room temperature; d, days; w, weeks; m, months

CO_2 for 16-20 hr before inoculation. After aspiration of the medium from the 6-well plates; 1 ml of dilution sample was added to each well and incubated for 2 hr ± 15 minutes at 37℃ and 5% CO_2. At the end of the inoculation period, inoculums were aspirated from the wells, and then it was replaced with 3 ml/well of the Carboxymethyl cellulose Solution (CMC) Overlay. After incubation for 3 days (72 ± 6 hr) at 37℃, 5% CO_2, the overlay from the wells were transferred by approximately 2 ml of 0.1% Crystal Violet Solution to each well, and then it was incubated at RT for 1-1.5 hr. After removal the crystal violet solution, the plates were inverted to dry for count plaques. After virus plaques were counted, the count number was adjusted to pfu/ml, and the average titer was calculated for each vial and time point. To estimate the rate of decay in titer, a linear regression model was used in which the dependent variable was the logarithm of the viral titer. Fig. 1 is the representative titering photos for measurement of Pexa-Vec activity done in A2780 cells.

Measurement of virus activity: cytotoxicity assay

Cells were cultured in DMEM medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin (Invitrogen) and 100 μg/ml streptomycin (Invitrogen) at 37℃ in a 5% CO_2 incubator. For the cytotoxicity measurement, A2780 cells were seeded in 96-well plates 20,000 cells in 100 ul per well. After overnight incubation for cell attachment, media was removed and growth media containing serially diluted virus. After 1-3 days of further incubation, viable cell concentrations were determined using Cell Counting Kit-8 (CCK-8; Dojindo, Japan). 10 μl of CCK-8 per well was added and incubated for 2 hr at 37℃ in a CO_2 incubator. The absorbance at 450 nm was measured using an EL808 microplate reader (Biotek, Winooski, VT, USA). Wells containing cells without virus were used as positive controls and wells incubated with media only were used as background value of cell viability calculation.

Percent cell viabilities were calculated by multiplying 100 to the ratio of average A450 values of each treatment wells subtracted with A450 values of background wells over aver-

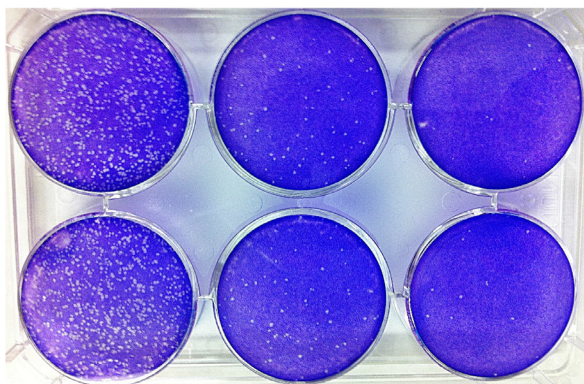

Fig. 1. Plaque assay of virus activity. A2780 cells were used for measuring the activity of Pexa-Vec. Pexa-Vec of diluted in to the range from 10^{-5}-10^{-7} fold from original dose 1×10^{9} pfu/ml produce from 10 to 1,000 plaque, then crystal violet stain. The plaques were counted, and the titer was expressed as the number of plaque forming units per milliliter (pfu/ml)

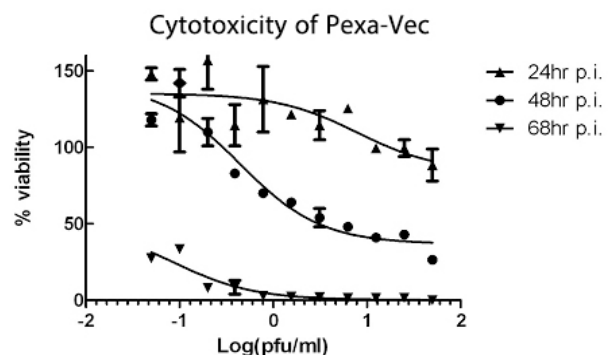

Fig. 2. Cytotoxicity assay of Pexa-Vec. A2780 cells were used for measuring the viral viability, serially diluted virus diluents of 10^{-2}-10^{-5} range (expected titer of 1×10^{4} pfu/ml - 1×10^{7} pfu/ml) determined 24 hr, 48 hr, and 68 hr. The absorbance at 450 nm was measured using an EL808 microplate reader. Viral activity was recalculated by percentile scale. % viability = $(A_{450sample} - A_{450blank}) / (A_{450cell\ only} - A_{450blank})$ x 100(%), p.i. : post infection

age A450 values of positive wells subtracted with average A450 values of background wells. % viability = ($A_{450sample}$ - $A_{450blank}$) / ($A_{450cell\ only}$ - $A_{450blank}$) ×100(%)

Statistical analysis

The differences in degree of virus viability were compared with the unpaired *t* test. The statistical analysis for the rate of decay in titer was performed with a linear regression model in GraphPad Prism 5 software.

Results

Selection of viral activity test method

To determine quantitative method for measuring viral activity to be used in stability study, plaque titration method and cytotoxicity assay method were compared. For plaque titration method, viral plaque forming activity was determined in A2780 cells lines. Pexa-Vec of diluted in to the range from 10^{-5} to 10^{-7} fold from original dose $1×10^9$ pfu/ml produce from 10 to 1,000 plaque (Fig. 1). Cellular cytotoxicity induced by virus was measured by adding two fold serially diluted virus diluents of 10^{-2} to 10^{-5} range (expected titer of $1×10^4$ pfu/ml – $1×10^7$ pfu/ml) determined 24 hr, 48 hr, 68 hr of the virus viability. Cell viability was decreased according to increased time of post infection (p<0.01). Both methods showed quantitative results in different virus titer ranges. But comparing with plaque titration method, cytotoxicity assay results was variable according to incubation time and need negative control of certified virus titer to measure virus activity reduction in long term stability test. Therefore plaque titration method selected for activity measure for this study.

The short term stability of Pexa-Vec in liquid formulation of PBS with 10% glycerol

Pexa-Vec clinical trial material for Phase 1 trial [11] was manufactured in Novavax Inc, and was in PBS with 10% glycerol. As injection of Pexa-Vec phase 1 dose escalation trial was designed to be done intratumor injection by ultrasonographic guide into hepatic mass, total time from withdrawal of Pexa-Vec from pharmacy to completion of injection procedure expected to take 1-3 hr depending on the experience of intervention radiologist and the accessibility into hepatic mass to be treated. Accordingly time dependent change of Pexa-Vec activity in RT or 4℃ was measured. As shown in Fig. 3A, Pexa-Vec viral activity was gradually decreased both in 4℃ and room temperature (20-25℃) and more rapid loss of viral infectivity was observed at RT storage condition (p<0.01). However, simple repetitive thawing-freezing cycle did not affect viral activity (Fig. 3B). In order to determine whether decrease of viral activity in RT or 4℃ is due to damage of viral genome and/or viral structural protein and/or simple aggregation of viruses, activity of Pexa-Vec incubated for 1 hr in RT was reaccessed and compared with Pexa-Vec stored at RT and undergone additional steps of vortexing, sonication, or vortexing followed by sonication or vice versa. As shown in Fig. 3C, decreased viral activity was fully recovered by adding serial vortexing and sonication step (p<0.05). But Pexa-Vec infectivity was not recovered fully by adding vortexing or sonication step alone. From this data we speculated that the major cause of rapid Pexa-Vec activity loss at RT exposure was physical aggregation of Pexa-Vec particles at current formulation.

Two Year Stability test of Pexa-Vec in same formulations of pH 7.6 or pH 8.6

As the complicated interventional procedure taking time can be used in new clinical trials and storage of drug under -70℃ considered not practical in clinical setting after commercialization of Pexa-Vec, it was essential to develop the liquid formulation retaining viral activity at preparation temperature (4℃ or RT) and during relatively long preparation time. For this purpose, five different formulation buffers which may affect virus stability were used to determine long term viral activity in different temperature and different period. Two different pH of liquid formulation was selected because pH may affect virus structural protein. Also, the concentration of NaCl was tested to test if ionic strength affects virus aggregation. And the effect of sucrose, wildly used to lessen physical stress at frozen condition, was tested. Using these three different parameters in combination, five kinds of formulation buffers were prepared (Table 1).

Firstly, two different concentration, high ($1×10^9$ pfu/ml) and low ($3×10^8$ pfu/ml), of the virus samples were spiked into formulation A (30 mM Tris, pH 7.6) and B (30 mM Tris, pH 8.6) to determine the effect of pH in formulation buffer. Virus aliquots prepared in two kinds of buffers were kept at -70℃ -20℃, 4℃, 37℃, and RT. Long term storage stability at -70℃, -20℃ and 4℃ was tested by measuring viral activity for 2 years at multiple time points. Stability test of 37℃ and RT stored samples was done for 1 months

at indicated time points (Table 2).

Virus activity was maintained through 2 years in -20℃ or -70℃ in both buffer condition, (Fig. 4). However, virus activity was detected only after two weeks storage at 4℃ with formulation A (30 mM Tris, pH 7.6) condition. Virus stability was reduced at increased temperature, but minor virus activity was detectible after day 3 with formulation A (30 mM Tris, pH 7.6) in RT storage (Fig. 4A), however no viral activity was detected at 37℃ in both buffer condition (data not shown). Though, virus concentration slightly affected virus stability, virus activity was detected only with low dose virus kept in formulation A (30 mM Tris, pH 7.6) at 4℃ and RT, suggesting viral aggregation may be an important factor for decrease of this temperature dependent decrease in viral activity. Overall, virus activity in pH 7.6 formulations is more stable than in pH 8.6 both in 4℃ or RT. The absence of points and lines in all graphs at indicated time points reflect complete inactivation of virus.

Two Year Stability test of Pexa-Vec in same formulations with or without 15% sucrose

Polysaccharide such as glycerol or disaccharide such as sucrose can be used for virus formulation as stabilizing additives. As sucrose is more acceptable for human use, the additional effect of 15% sucrose in formulation A and B which are designated as formulation D and E respectively were determined on viral stability.

There was no significant differences in viral stability in A2780 cell lines through all 2 years when stored at -20℃ or -70℃ regardless of buffer formulation D and E (Fig 5). However virus stability clearly increased at 4℃ and RT in the presence of 15% sucrose at pH 7.6 (Formulation D). Virus activity was detected even at 1 month in 4℃ and at 7days in RT although significant decrease at these time points (Fig. 6). These results suggest that 15% sucrose increased viral stability significantly in addition to pH 8.6 effect as shown in Fig. 7. No viral activity was detected at 37℃ in both buffer condition (data not shown).

Sucrose effect on viral stability can be observed more clearly by comparing virus activity in formulation A (30 mM Tris/pH 7.6 without 15% sucrose) and formulation D (30 mM Tris/pH 7.6 with 15% sucrose). However, virus

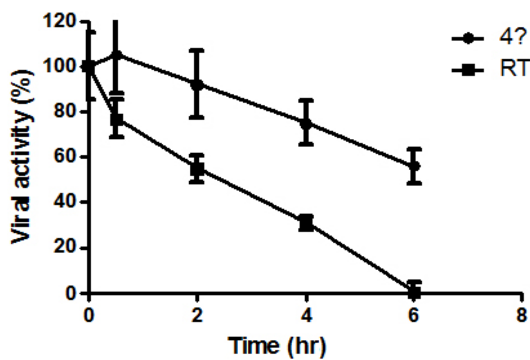

Fig. 3A. Vaccinia virus activity in RT or 4℃. Viral activity was measured by plaque titration method using A2780 cells. Initial Pexa-Vec solution was diluted using saline to make 3×10^3 pfu/ml. Virus was transferred to syringe and kept at room temperature or 4℃. After 0h, 0.5 hr, 2 hr, 4 hr and 6 hr, virus solution was used for titration. After 3 days of incubation, plaque number was counted and used for titer calculation. Viral activity % was calculated by dividing titer of each condition with 0 hr titer and multiplying 100. Viral activity (%) = (Titer after t hr incubation) / (Titer at 0 hr) ×100.

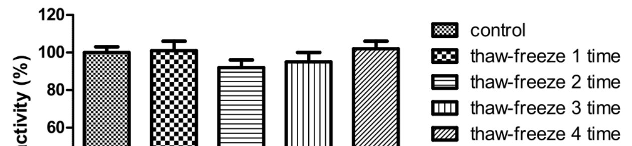

Fig. 3B. Vaccinia virus activity using thaw and freeze. Viral activity was measured by plaque titration method using A2780 cells. Virus (Pexa-Vec, 3×10^3 pfu/ml) went through 1-4 cycles of repetitive thawing in a 37 C water bath for 1 minute and freezing in the dry ice/ethanol bath for 3 minutes. Viral activity % was calculated by dividing titer of each condition with 0 hr titer and multiplying 100. Viral activity (%) = (Titer T-S) / (Titer at control) ×100.

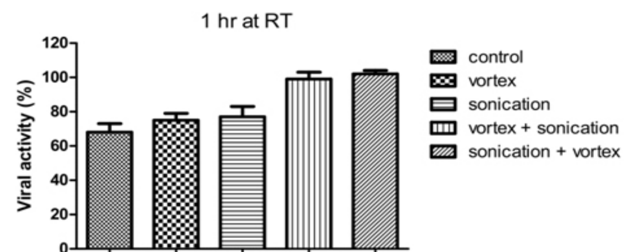

Fig. 3C. Vaccinia virus activity in RT after 1 hr. Viral activity was measured by plaque titration method using A2780 cells. Virus (Pexa-Vec, 3×10^3 pfu/ml) vortex – maximum 1 min. Sonication - amplitude 10%, 15 sec. Viral activity % was calculated by dividing titer of each condition with control titer and multiplying 100. Viral activity was recalculated by percentile scale.

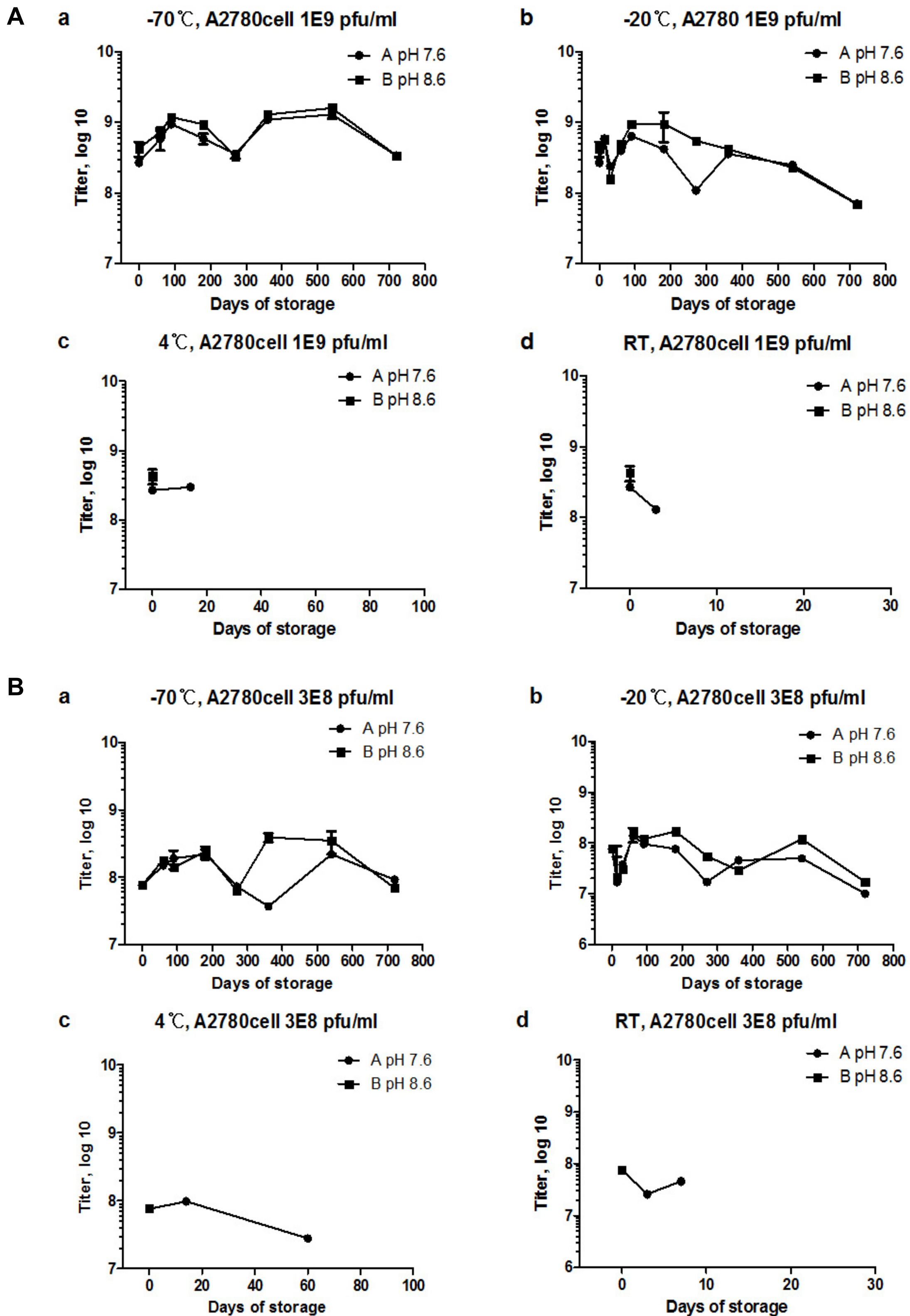

Fig. 4. Stability of formulation at pH 7.6 and 8.6(1×10^9 pfu/ml). A2780 cells were used for measuring the activity of Pexa-Vec (1×10^9 pfu/ml (A) or 3×10^8 pfu/ml(B)). To estimate the rate of decay in titer, a linear regression model was used in which the dependent variable was the logarithm of the viral titer. Aliquots of Pexa-Vec viruses in formulation A (30 mM Tris at pH 7.6) and B (30 mM Tris at pH 8.6) were stored for two years at -70℃, -20℃ and 4℃ storage condition. And stability of two formulation was tested short term; for one month at room temperature and for one week at 37℃.

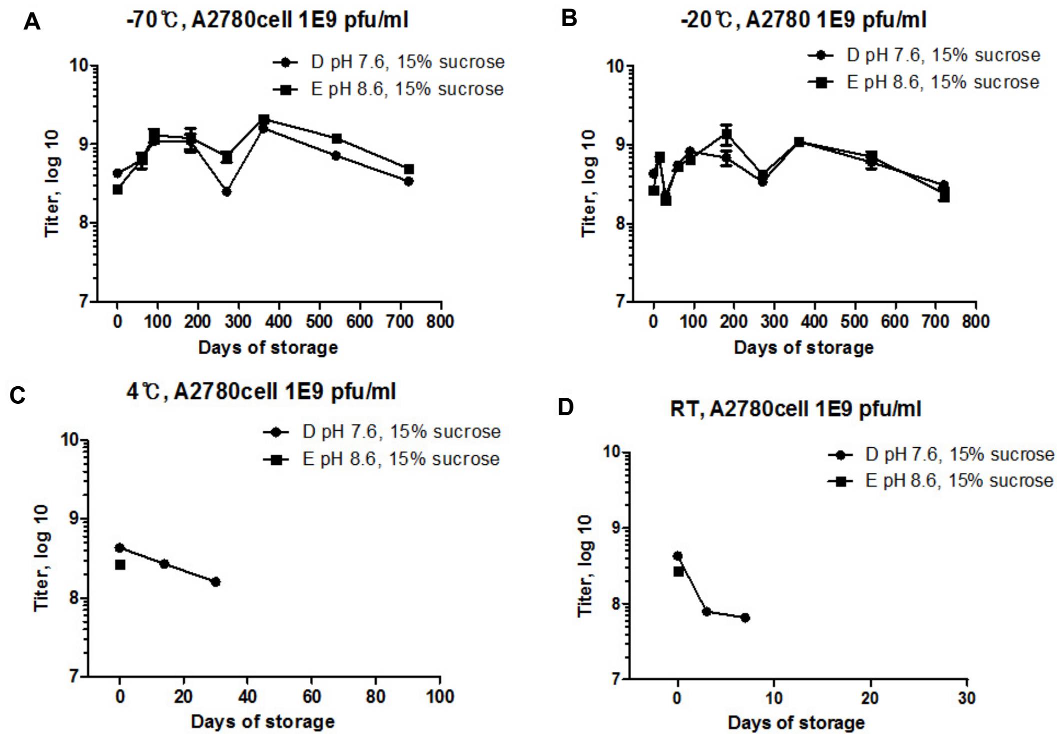

Fig. 5. Stability of formulation at 15% sucrose, pH 7.6 and 8.6. A2780 cells were used for measuring the activity of Pexa-Vec (1×10^9 pfu/ml). Aliquots of Pexa-Vec viruses in formulation D (30 mM Tris, pH 7.6, 15% sucrose) and E (30 mM Tris, pH 8.6, 15% sucrose) were stored for two years at -70℃, -20℃ and 4℃storage condition. And stability of two formulation was tested short term; for one month at room temperature and for one week at 37℃.

activity was maintained through for all 2 years in -70℃, but significant difference can be observed even in -20℃ in A2780 cells, and after spiking with low and high doses.

Two Year Stability test of Pexa-Vec in same formulations with or without 150 mM NaCl

As ionic strength can affect viral aggregation which decrease viral activity, we observed the effect of 150 mM NaCl in different formulation. For this purpose, we added 150 mM NaCl into formulation D (30 mM Tris/pH 7.6, 15% sucrose) which were designated as Formulation C (30 mM Tris/pH 7.6, 15% sucrose, 150 mM NaCl). Overall viral activity was not significantly different through all 2 years, but virus stability was significantly increased at 4℃ and RT compared with that in the absence of NaCl (formulation D). Viral activity was detected even at 18 months but not at 24 months in 4℃ in formulation C, and at 2 weeks in RT in formulation C (Fig. 8). This viral activity was same after spiking with low and high doses. Even though viral activity was significantly decreased at 18 months (in case of 4℃) and at 2 weeks (in case of RT), it is very striking increase of viral stability both at 4°C and RT (Fig. 8). No viral activity was detected at 37℃ in both buffer condition (data not shown).

Discussion

The poxviridae comprise a large family of complex DNA viruses that replicate in the cytoplasm of host cells. The causative agent of smallpox was the variola virus, a member of the genus Orhoproxvirus. Vaccinia virus, also a member of the genus Orthopoxvirus in the family of Poxviridae, was used as liver vaccine to immunize against smallpox. Successful worldwide vaccination with vaccinia virus culminated in the eradication of variola virus, thus global eradication of smallpox [9].

Numerous methods are known for making live virus preparations for vaccine and other purposes. Formulations and methods useful in freezing, lyophilizing, or otherwise storing viable virus to preserve their activity in laboratory or for vaccine use are also known. In the past, vaccinia virus have also been used to engineer viral vectors for recombinant gene expression and for the potential use as recombinant live vaccines [4, 8].

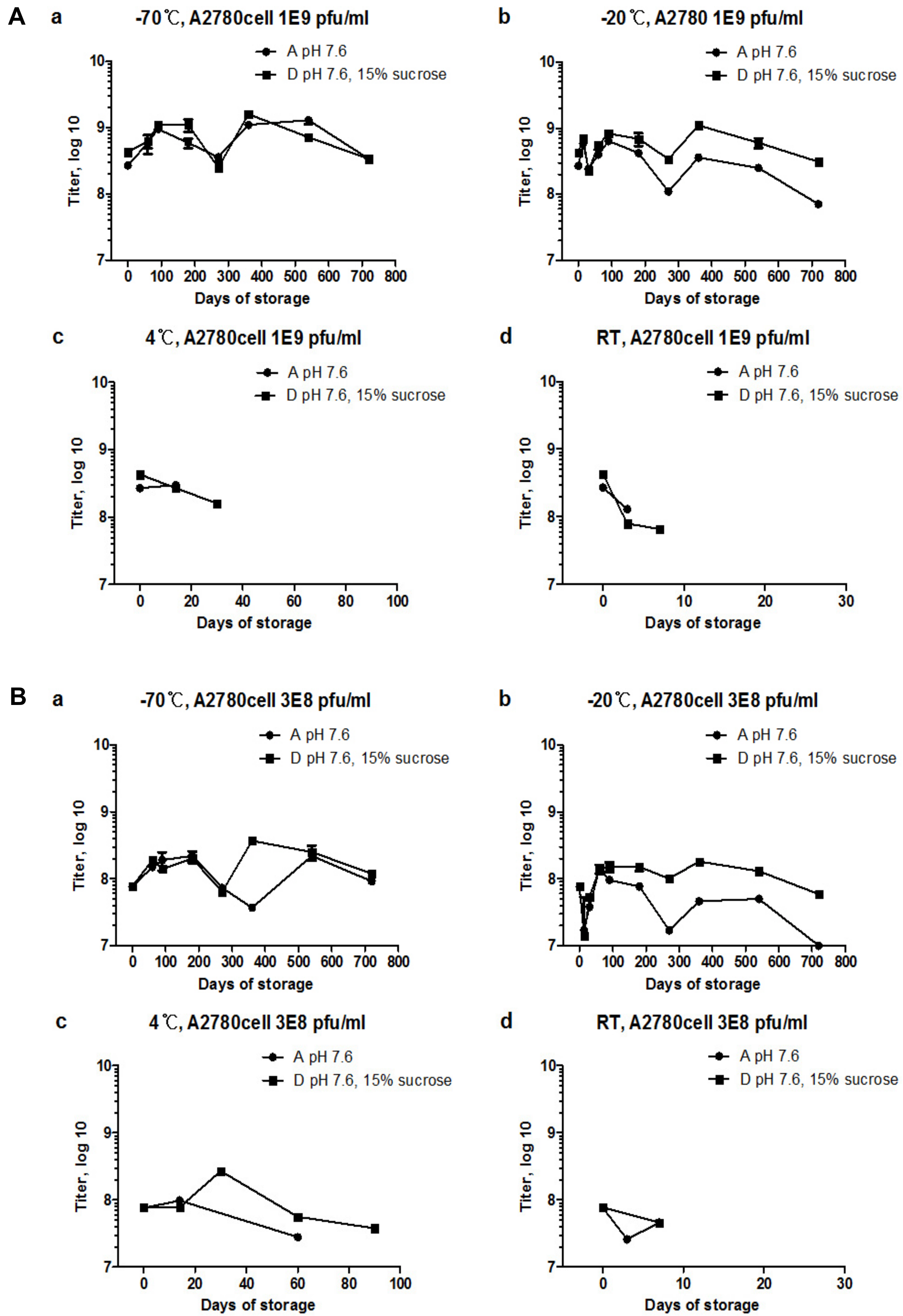

Fig. 6. Stability of formulation at pH 7.6, with or without 15% sucrose (1×10^9 pfu/ml). A2780 cells were used for measuring the activity of Pexa-Vec (1×10^9 pfu/ml (A) or 3×10^8 pfu/ml (B)). Aliquots of Pexa-Vec viruses in formulation A (30 mM Tris at pH 7.6) and D (30 mM Tris, pH 7.6, 15% sucrose) were stored for two years at -70℃, -20℃ and 4℃storage condition. And stability of two formulation was tested short term; for one month at room temperature, and for one week at 37℃.

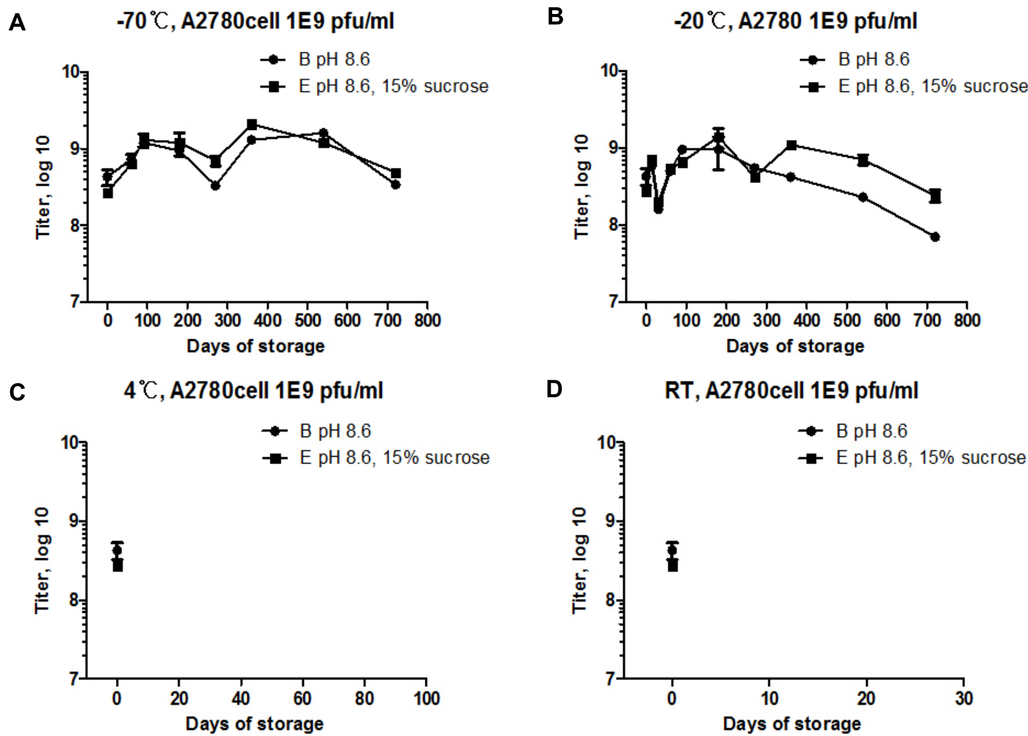

Fig. 7. Stability of formulation at pH 8.6, with or without 15% sucrose. A2780 cells were used for measuring the activity of Pexa-Vec (1×10^9 pfu/ml). Aliquots of Pexa-Vec viruses in formulation B (30 mM Tris at pH 8.6) and E (30 mM Tris, pH 8.6, 15% sucrose) were stored for two years at -70℃, -20℃ and 4℃storage condition. And stability of two formulation was tested short term; for one month at room temperature, and for one week at 37℃.

Recently, a new anticancer product which is a vaccinia virus derived oncolytic virus, is on the edge of commercialization in cancer therapeutic fields [8]. Based on short term instability test results, Pexa-Vec drug preparation and ultrasonography guided injection was allowed to be done within two hr in phase 1 trial. In the initial short term instability test in RT or 4℃, viral activity was rapidly decreased both in 4℃ and RT but became stable by 1-4 cycles of repetitive freezing in the dry ice/ethanol bath for 3 minutes and thawing in a 37℃ water bath for 1 minute. The decrease of virus activity was completely restored after sonication followed by vortex and vice versa. Also, we showed that low dose virus is more stable than high dose virus. So the decrease of viral activity may be not due to genome dissociation or dysfunctional change of viral structural protein but physical aggregation which is reversible by sonication and vortex. However, in clinical setting, both sonication and vortex may not be a practical procedure. Accordingly, the proper liquid formulation which can maintain viral activity should be developed.

In this study, five different buffers with three different factors which may affect virus stability were used to determine liquid formulation. We showed that pH in formulation may affect virus structural protein both reversible or irreversibly, and subsequently affect viral activity. High dose of virus may be aggregated but may be inhibited in the presence of NaCl as ionic strength is increased. Finally sucrose may be helpful for minimizing physical stress. As it was reported that acidic pH significantly decreases viral activity in short time by increased viral aggregation [2], we used pH 7.6 or pH 8.6 in this study as choosing from either flank of pH 8.0 also maintains minimum viral aggregation. However the viral stability was less in pH 8.6 formulation, it may be speculated that structural protein of virus may be affected in pH 8.6 resulting in decrease of viral stability. The significant increase of viral stability in the presence of NaCl can be expected with increased ionic strength. Further increase of viral stability was observed in the presence of sucrose. All these results was originally expected when we design this study, however the degree of the increase in viral stability in 4℃ and RT was more than dramatic than was expected.

As measurement of viral activity in this study was observed by a cell based assay for 2 years, it is not surprising

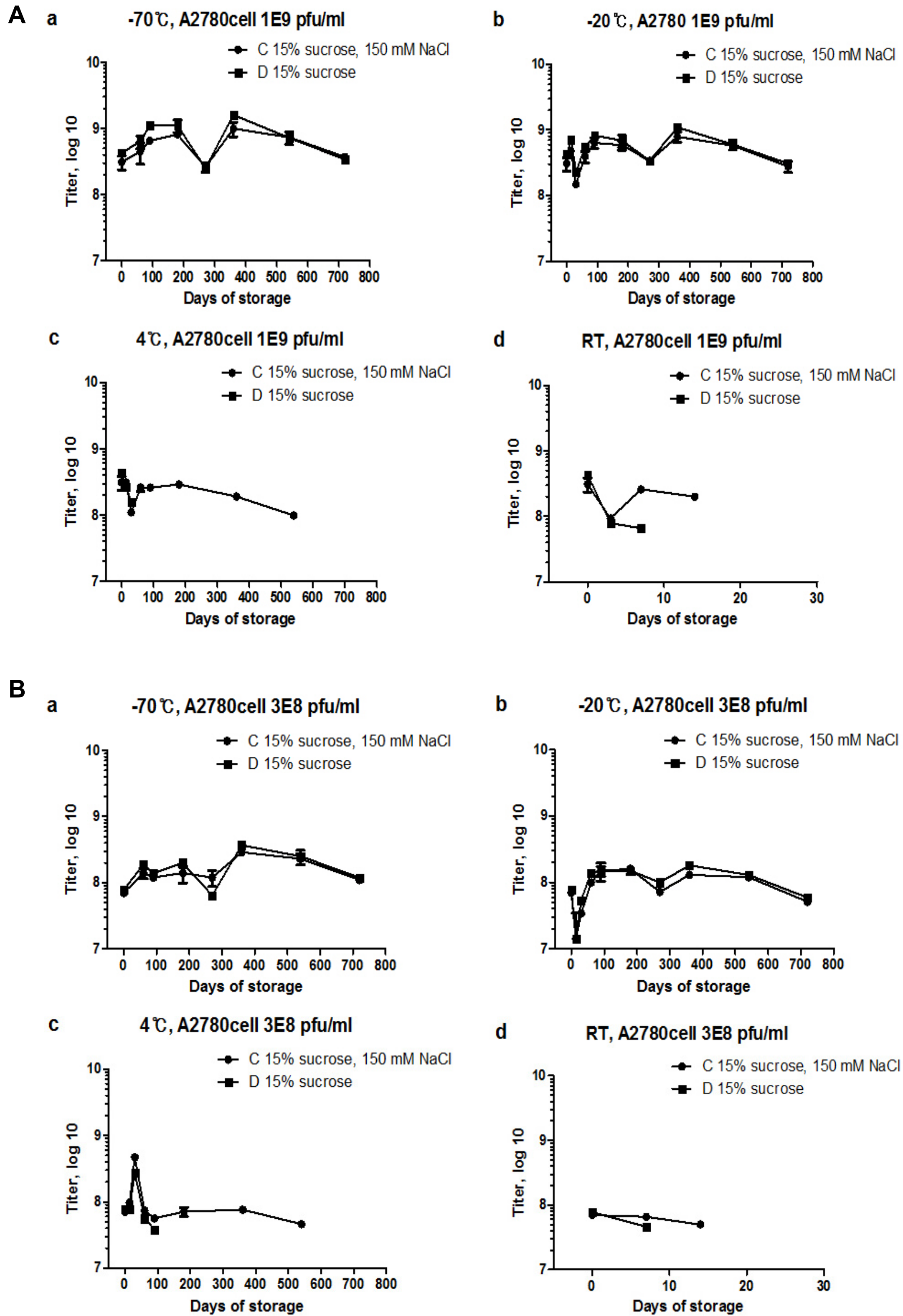

Fig. 8. Stability of formulation at pH 7.6, 15% sucrose, with or without 150 mM NaCl (1×10^9 pfu/ml). A2780 cells were used for measuring the activity of Pexa-Vec (1×10^9 pfu/ml (A) or 3×10^8 pfu/ml (B)). Aliquots of Pexa-Vec viruses in formulation C (30 mM Tris pH 7.6, 150 mM NaCl and 15% sucrose) and D (30 mM Tris, pH 7.6, 15% sucrose) were stored for two years at -70℃, -20℃ and 4℃ storage condition. And stability of two formulation was tested short term; for one month at room temperature and for one week at 37℃.

to see variability of inter assay. Therefore, we used two different cell lines and it should be noted that transient change of viral activity at one time point and unique deviation was disregarded. All data showing consistent and clear pattern change were collected for analysis. In conclusion, the liquid formulation composed of 30 mM Tris/pH 7.6, 150 mM NaCl and 15% sucrose is proper for maintaining stability of Peca-Vec.

Acknowledgement

This work was supported by a research grant of Research Institute for Convergence of Biomedical Science and Technology in Pusan National University Yangsan Hospital.

References

1. Breitbach, C. J., Parato, K., Burke, J., Hwang, T. H., Bell, J. C. and Kirn, D. H. 2015. Pexa-Vec double agent engineered vaccinia: oncolytic and active immunotherapeutic. *Curr. Opin. Virol.* **13**, 49-54.
2. Cruz, P. E., Silva, A. C., Roldão, A., Carmo, M., Carrondo, M. J. and Alves, P. M. 2006. Screening of novel excipients for improving the stability of retroviral and adenoviral vectors. *Biotechno.l Prog.* **22**, 568-576.
3. Donnelly, O. G., Errington-Mais, F., Prestwich, R., Harrington, K., Pandha, H., Vile, R. and Melcher, A. A. 2012. Recent clinical experience with oncolytic viruses. *Curr. Pharm. Biotechnol.* **13**, 1834-1841.
4. Galdiero, F. 1979. Adenovirus aggregation and preservation in extracellular environment. *Arch. Virol.* **59**, 99-105.
5. Heo, J., Reid, T., Ruo, L., Breitbach, C. J., Rose, S., Bloomston, M., Cho, M., Lim, H. Y., Chung, H. C., Kim, C. W., Burke, J., Lencioni, R., Hickman, T., Moon, A., Lee, Y. S., Kim, M. K., Daneshmand, M., Dubois, K., Longpre, L., Ngo, M., Rooney, C., Bell, J. C., Rhee, B. G., Patt, R., Hwang, T. H. and Kirn, D. H. 2013. Randomized dose-finding clinical trial of oncolytic immunotherapeutic vaccinia JX-594 in liver cancer. *Nat. Med.* **19**, 329-336.
6. Kaufman, H. L., Kohlhapp, F. J. and Zloza, A. 2015. Oncolytic viruses: a new class of immunotherapy drugs. *Nat Rev. Drug Discov.* **14**, 642-662.
7. Kim, M. K., Breitbach, C. J., Moon, A., Heo, J., Lee, Y. K., Cho, M., Lee, J. W., Kim, S. G., Kang, D. H., Bell, J. C., Park, B. H., Kirn, D. H. and Hwang, T. H. 2013. Oncolytic and immunotherapeutic vaccinia induces antibody-mediated complement-dependent cancer cell lysis in humans. *Sci. Transl. Med.* **5**, 185ra63
8. Mackett, M., Smith, G. L. and Moss, B. 1982. Vaccinia virus: A selectable eukaryotic cloning and expression vector. *Proc. Natl. Acad. Sci. USA* **79**, 7,415-7,419.
9. Park, B. H., Hwang, T., Liu, T. C., Sze, D. Y., Kim, J. S., Kwon, H. C., Oh, S. Y., Han, S. Y., Yoon, J. H., Hong, S. H., Moon, A., Speth, K., Park, C., Ahn, Y. J., Daneshmand, M., Rhee, B. G., Pinedo, H. M., Bell, J. C. and Kirn, D. H. 2008. Use of a targeted oncolytic poxvirus, JX-594, in patients with refractory primary or metastatic liver cancer: a phase I trial. *Lancet Oncol.* **9**, 533-542.
10. Smith, G. L., Mackett, M. and Moss, B. 1984. Recombinant vaccinia viruses as new live vaccines. *Biotechnol. Genet. Eng. Rev.* **2**, 383-407.
11. Verardi, P. H., Titong, A. and Hagen, C. J. 2012. A vaccinia virus renaissance: new vaccine and immunotherapeutic uses after smallpox eradication. *Hum. Vaccin. Immunother.* **8**, 961-970.

초록 : 항암 백시니아 바이러스의 안전성에 대한 염화나트륨의 효과

김성근[1] · 계소연[2] · 권혁찬[3] · 황태호[3,4]*
([1]양산부산대학교병원 혈액종양내과, [2]동아대학교 의과대학 약리학교실, [3]부산대학교 혈관질환 유전자세포치료 연구센터, [4]부산대학교 의과대학 약리학교실)

Pexa-Vec (JX-594)은 암특이적 암용해 면역치료제인 백시니아 바이러스이다. 본 연구의 목적은 Pexa-Vec의 안정성을 극대화하기 위한 방법을 개발하는 것이다. 단기안정성 실험에서 바이러스의 활성은 4℃와 실온에서 감소하였으나, 초음파처리와 회전처리로 완전히 회복되었다. Pexa-Vec의 장기안정성 시험은 (A) 30 mM Tris/pH 7.6, (B) 30 mM Tris/pH 8.6, (C) 30 mM Tris/pH 7.6, 150 mM NaCl, 15% sucrose, (D) 30 mM Tris/pH 7.6, 15% sucrose, (E) 30 mM Tris/pH 8.6, 15% sucrose 조건 하에서 수행하였다. 제형 A는 4℃에서 4-8주 후, 실온에서 1주일 후에 2로그 이하로 바이러스활성이 감소되었다. 반면 제형 B의 경우 4℃와 실온에서 바이러스 활성이3일 후 감소되는 것으로 관찰되어 중성 산도가 바이러스 안정성을 유지하는데 필수적이다. 제형A에 15%의 슈크로즈 수크로오스를 추가했을 때(제형D), -20℃, 4℃와 실온에서 바이러스성 안정성이 크게 증가 하였고, 제형 E (pH 7.6)에서 다시 한번 확인되었다. 제형 D (pH 7.6)에 150 mM 염화나트륨을 추가한 제형 C에서 바이러스 안전성을 증가시키는 슈크로즈 수크로오스 효과를 더욱 향상시켜, 4℃와 실온에서 바이러스 활성이 각각 1.5년과 1-2주 동안 유지되는 결과를 보였다. 결론적으로, 우리는 제형C가 항암 백시니아 바이러스를 적절히 저장하기 위한 충분한 조건을 제공할 수 있다고 제안한다.