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Stress Factors Affecting the Cryopreservation of Biological Components of Seed Virus for Avian Influenza Vaccine Production

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Abstract

In the past, many studies have been done to cryopreserve biological materials for future vaccine production. Scientists have been using different chemicals as cryoprotectants to preserve their cell lines on which desired viruses can be cultivated. Researchers have always been in search for better molecules to avoid cryoinjury during process of cryopreservation. In the present study, different molecules were evaluated for cryo-potency in preserving master seeds of "Vero cell" line and "Avian Influenza viruses". Cryoprotectants such as (Dimethyl sulfoxide) DMSO and Glycerol were used in different concentration and evaluated at -80^{o}C and -196^{o}C for different time interval. After 15 days and 30 days of cryopreservation the percentage viability of preserved cells were almost equal at both temperatures whereas, after 60 days, 90 days and 120 days the higher percentage of viability was recorded at -196^{o}C for both Vero cells and Avian Influenza virus. Different pH levels were set for both samples separately with same time interval and found slightly acidic pH (pH5.5) optimum for cryopreservation of Vero cell line and Neutral pH (pH7) for Avian Influenza virus. DMSO (20%) and Glycerol (40%) showed optimum percentage viability when cryopreserved for 120 days without any ill effect.

Keywords: Cryopreservation, Dimethyl sulfoxide (DMSO), Vero cell line, Avian influenza Virus (AIV)

1. Introduction

Cryopreservation is a scientific procedure to store living tissues, organs, cells and even organisms for long time by providing regulated cooling treatment down to freezing levels of temperature which get equal to −80 ℃ (solid CO_2) or −196 ℃ (liquid N_2) (Jhang et al., 2017). In 1949 cryobiology gained real motivation when the glycerol was reported as protectant against freezing injury. Since that discovery the liquid nitrogen (-196^{o}C) is being used in cryobiology, at this temperature the viability of the cells is independent of the period of storage and biological systems remain genetically stable. Cryopreservation of the uniform cells suspends all the physiochemical processes and preserve the 3D architecture. It would be important for food production in those areas where crops can never be cultivated due to cold stress. In polar and alpine regions the cultivation of crops can be increased from 40-45% by understanding the nature of freezing during injury.

Table 1. The Temperature range encountered in Cryobiology

Temperature (℃)	State
0^{o}C	Freezing point water
-20^{o}C	Domestic deep freeze
-21.8^{o}C	Eutectic temperature NaCl: water
-79^{o}C	Dry Ice (Solid carbon dioxide)
-88.7^{o}C	Lowest recorded environmental temperature
-139^{o}C	Limit for ice crystal growth
-196^{o}C	Boiling point nitrogen
-213^{o}C	Melting nitrogen
-273^{o}C	Absolute zero

Lyophilization (freeze-drying) is being used widely to preserve prokaryotes. In this technique the culture is frozen and then the water is extracted out directly by reducing pressure. Dried culture is then sealed under vacuum and at this state it can be stored at room temperature and resurrected simply upon addition of media. Unfortunately this method is only valid for prokaryotes not for eukaryotes because the eukaryotic cells are highly organized so in experiments the survival rate of these cells was very low as <0.1%, that's why the lyopholization is said to be mutagenic (Morris et al., 1981). The uses of cryo-preservation have been categorized into many fields and some of them include : a) Biology (molecular) and biochemistry, b) cryo-surgery, c) cryopreservation of cells (living) or organs, e) food related sciences, e) ecology and plant physiology and many medical applications such as :f) blood transfusion, g) transplantation of bone marrow, AI (artificial insemination) and IVF (*invitro* fertilization).

Table 2. Applications of cryopreservation in different processes

Cell Type	Applications	Advantages of cryo-preservation
Platelets, Red blood cells	Transfusion	Cryopreserved blood cells can be used for transfusion after years of storage
Bone marrow	Transplantation	It can be tissue typed and preserved, it becomes available when required
Mammalian Embryos	Implementation into foster mothers	Frozen embryos are easy to transport
Spermatozoa	Artificial insemination	Used in veterinary sciences for long term improvement of stock
Seeds	Conservation of plant genetic resources	Some seeds e.g. oil palm and coffee lose their viability using conventional methods (e.g. Freeze-drying).
Plant shoot-tip cells	Used to clone cells	These cells have the potential to regenerate into whole plant.
Suspension culture of bacteria, yeasts and protists	Starter cultures for industrial processes	In cryopreserved cells the genetic drift is avoided

Many uses of the cryopreservation technique exist in clinical and basic research and some limitations exist too. At very low temperatures no metabolism of cells remains active which may cause irreversible changes in proteins and lipids resulting in impairment cellular structure. If the CPA does not have any side effect at any concentration then the cells can be cryopreserved perfectly. When the cells are on ultra-low temperatures, they are biologically inactive and can be preserved for long period of times. Factors affecting the viability of cells may include growth conditions before harvesting, cell density, physiological state of cells, and choice of cryo-protectant and handling techniques. Cells yield the highest percentage of viability when they are harvested from a late logarithmic to early stationary phase while freezing. The desired concentration of viable cells after harvesting is between 10^6 to 10^7 cells/ml. To get the desired percentage of viability post-thaw, there is a need to make cell suspension at a concentration twice the desired cell concentration so, the equal volume (2X) of cryoprotectant can be added. Gentle handling techniques enhance the post thaw percentage viability of cells Genetic resource banks (GRBs) use cryopreservation technique to conserve the threatened, endangered and valuable species. Sperm cryopreservation is the first strategy to establish a germplasm bank for the conservation of this species. Difficulty in establishing a GRB is the lack of knowledge on specific traits of sperm, which is mandatory to design an adequate protocol design (Anel et al., 2010). But there could be physiological changes in samples with respect to time period for which it is cryopreserved. The ultra-structural changes in membranes due to freezing stresses are diverse with respect to different membrane systems (Fujikawa et al., 1985). Enzymatic and chemical activities which can be the cause of damage to the cell efficiently stop working at ultra-low temperatures so, the biological material remains intact for decades; whenever the biological material is required it can be regain by "thawing". Cryoprotectants are additional chemicals which round the pointed ends of crystals to avoid damage.

Table 3. Comparative study on the two different groups of cryoprotectants

Penetrating CPAs	**Non- Penetrating CPAs**	
Agents of Low Molecular Weight (< 100 Da)	Saccharides (<594 Da)	High Molecular weight agents (>1000 Da)
DMSO	Monosaccharides	Dextran
Ethylene glycol	Disaccharides, Trehalose, sucrose	Ficoll
Polyalcohols	Polysaccharides, Raffinose	Polyvinyl pyrrolidone
Propylene glycol		Hydroxyethyl starch
Formamide		Polyethylene glycol
Glycerol		

As the cryobiology allows to store biological materials for long times, it also needs to be understood that the choice of

reliable cryoprotectants and temperature is also very important. Glycerol works best -75^{o}C as cryoprotectant but the Dimethyl sulphoxide

(Kuleshova et al., 2007). DMSO is used widely due to its high penetration.

In the past few decades the work on cell lines has been done abundantly for the purpose of cryopreservation. Cell lines which are being cryopreserved for years, usually originate from mammalian source. Continuous cell lines from mammalian tissue serve as invaluable tool for biological sciences and also used as animal substrate for the production of pharmaceuticals of various types. Vero cells are mostly susceptible to various microbes and toxins having wide contribution in microbiology and human vaccine production. Vero cell line is continuous and aneuploid (differ in chromosome number from cell to cell). By cryopreserving the viruses, their infectivity will decrease by time, to revert the infectivity there is a need to put the virus into its host (e.g. bacteriophage). In case of Avian influenza virus Neuraminidase (NA) and hemagglutinin (HA) are the major structure. The presence of (HA) protein contains a bundle of information regarding virulence. HA is specific in its function and it has a unique capacity and proteolytic cleavage (PCS) site for amino acids (Miyata et al., 1975).

To cryopreserve a biological material (BM) there are some physiochemical factors which must be taken under control e.g. temperature, cooling rate, cryoprotectants, pH, media and holding temperature. The temperature must not be changed immediately otherwise the cell death may ensure due to cooling shock. For the preservation of biological material the use of proper media is necessary e.g. DMEM is used to preserve cells with higher fetal calf serum quantity than normally used to grow cells in lab. Before putting into final lowest temperature there is a need to hold the biological material at an optimum holding temperature for few hours or overnight, usually -20 ℃ is used as holding temperature. After that the biological material is preserved using cryofreezers (-150 ℃) or liquid nitrogen (-196 ℃) (Ivanova et al., 2015). The current study was undertaken to optimize physiochemical conditions for the cryopreservation of vero cell line and avian influenza virus for longer period of time and develop SOPs for the Seed Lot Management System (SLMS) in bio pharmaceuticals.

2. Materials & Methods

2.1 Experiment # 01: Physical Factors Affecting the Percentage Viability of Vero Cell Line During Cryopreservation

Effect of temperature during vero cells cryopreservation was evaluated using liquid nitrogen for different intervals of time. Total of six set of sterile cryopreservative ampules of one ml capacity were marked with specific code as shown in table # 01. Each of the ampules was filled with 0.5 ml of Vero cell line suspension having 85% viability. All the ampules were stored at -196^{o}C (liquid nitrogen) following descending temperature pattern of -20^{o}C for overnight. Ampule of each set having marking CT1, CT2, CT3, CT4, CT5 and CT6 (control) were pulled from the ultra-low temperature (-80^{o}C) to the ambient environment on 15DPS, 30DPS, 60DPS, 90DPS and 120DPS respectively. Other set of ampules stored at -196^{o}C were marked as CT7, CT8, CT9, CT10, CT11 and CT12 (control). Effect of pH on the viability of Vero cells during cryopreservation was evaluated by keeping them at-196^{o}C for different time intervals. Vero cell line was maintained at three different pH levels of pH 4.5, 5, 5.5 and 6.5. Control ampules for pH were CpH6, CpH12, CpH18 and CpH24 respectively.

2.2 Experiment # 02: Chemical Factors Affecting the Percentage Viability of Vero Cells During Cryopreservation

Effect of different cryoprotectants over Vero cell line was evaluated using ultra low temperature during cryopreservation. Total six sets each comprised of six sterile cryopreservative ampules of 1 ml capacity marked with specific code as shown in table # 02. In each ampule, 0.5 ml of Vero cell line suspension having 85% viability. All the ampules were stored at -196^{o}C following descending temperature pattern of -20^{o}C for overnight. The effect of DMSO and Glycerol during cryopreservation were evaluated. Ampules containing DMSO have been marked as CD1, CD2, CD3, CD4, CD5 and CD6 (control) and ampules containing glycerol were labelled as CG1, CG2, CG3, CG4, CG5 and CG6 (control) were exposed to ambient temperature on 15DPS, 30DPS, 60DPS, 90DPS and 120DPS respectively.

2.3 Experiment # 03: Physical Factors Affecting the Percentage Viability of Avian Influenza Virus During Cryopreservation

Effect of temperature over the viability of AIV was done by using different time intervals during cryopreservation. Total six set of sterile cryotubes of one ml capacity were marked with specific code as shown in table # 03. Each cryotubes was filled with 0.5 ml of AIV suspension having 85% viable cells. All cryotubes were stored at -196 ℃ (liquid nitrogen) following descending temperature pattern of -20 ℃ for overnight. Cryotubes of each set having marking VT1, VT2, VT3, VT4, VT5 and VT6 (control) were pulled from the ultra-low temperature to the ambient environment on 15DPS, 30DPS, 60DPS, 90DPS and 120DPS respectively. Other set of ampules stored at -196^{o}C were marked as VT7, VT8, VT9, VT10, VT11 and VT12 (control).

Effect of pH over the viability of influenza virus was determined during cryopreservation by providing different time

intervals. Viruses were maintained at three different pH levels which are highly acidic (pH3), neutral (pH7) and highly basic (pH10). Control viruses in case of acidic, neutral and basic pH were VP6, VP12 and VP18 respectively.

2.4 Experiment # 04: Chemical Factors Affecting the Percentage Viability of Avian Influenza Virus (AIV) During Cryopreservation

Effect of different cryoprotectants over the viability of AIV was evaluated by using different time intervals during cryopreservation. Total six sets each comprised of six sterile cryopreservative ampules of 1 ml capacity marked with specific code as shown in table #04. In each cryotube, 0.5ml (256 HAU) of AIV suspension having 85% viability. All the cryotubes were stored at -196^{o}C following descending temperature pattern of -20^{o}C for overnight. The effect of DMSO and Glycerol during cryopreservation were evaluated. For this purpose DMSO and Glycerol were used with codes VD and VG respectively. Ampules containing DMSO have been marked as VD1, VD2, VD3, VD4, VD5 and VD6 (control) and ampules containing glycerol were labelled as VG1, VG2, VG3, VG4, VG5 and VG6 (control) were exposed to ambient temperature on 15DPS, 30DPS, 60DPS, 90DPS and 120DPS respectively.

2.5 Statistical Analysis

The data was analyzed by one-way analysis of variance (ANOVA) and subsequently by Duncan's multiple range test (DMR).

3. Result

Vero cell line containing ampules stored at -80^{o}C for 15 days, 30 days, 60 days, 90 days and 120 days showed mean standard deviation (M±SD) 90±0, 90±0, 89.5±1.12, 89.5±1.12 and 88.16±2.61 respectively in DMEM. The M±SD for percentage viability of cells involving five different time intervals at -80^{o}C was recorded as 89.43±0.97. Vero cell line containing ampules stored at -196^{o}C for 15 days, 30 days, 60 days, 90 days and 120 days showed M±SD 90±0, 90±0, 90±0, 89.83±0.37 and 89.66±0.74 respectively in DMEM. The M±SD for percentage viability of cells involving five different time intervals at -196^{o}C was recorded as 89.8±0.22 as shown in Table 04, Figure 1.

Vero cell line containing ampules stored at pH4.5 for 15 days, 30 days, 60 days, 90 days and 120 days showed M±SD 88.16±0.68, 88±0.57, 88±0.57, 87.66±0.94 and 87.33±1.10 respectively in DMEM. The M±SD for percentage viability of cells involving five different time intervals at pH4.5 was recorded as 87.83±0.77. Vero cell line containing ampules stored at pH5 for 15 days, 30 days, 60 days, 90 days and 120 days showed M±SD 90±0, 90±0, 89.83±0.37, 89.66±0.74 and 89.16±1.21 respectively in DMEM. The M±SD for percentage viability of cells involving five different time intervals at pH5 was recorded as 89.73±0.46. Vero cell line containing ampules stored at pH5.5 for 15 days, 30 days, 60 days, 90 days and 120 days showed M±SD 90±0, 90±0, 90±0, 89.83±0.37 and 89.66±0.74 respectively in DMEM. The M±SD for percentage viability of cells involving five different time intervals at pH5.5 was recorded as 89.8±0.22. Vero cell line containing ampules stored at pH6.5 for 15 days, 30 days, 60 days, 90 days and 120 days showed M±SD 90±0, 90±0, 90±0, 89.83±0.37 and 89.83±0.37 respectively in DMEM. The M±SD for percentage viability of cells involving five different time intervals at pH 6.5 was recorded as 89.9±0.14 as shown in Table 04, Figure 2.

Table 4. Physical factors affecting the percentage viability of vero cell line during cryopreservation

Parameter (Temperature, pH)	**Sample identification (n=6)**	**Post storage percentage viability of Vero cell line (M±SD)**						
		15 *DPS	**30 *DPS**	**60 *DPS**	**90 *DPS**	**120 *DPS**	**120 *DPS (control)**	**ANOVA P-Value (M±SD)**
-80^{o}C	CT1,CT2,CT3, CT4,CT5,CT6	90±0	90±0	89.5±1.12	89.5±1.12	88.16±2.61	90±0	**0.18 89.43±0.97**
P-value		N/A	N/A	0.16	0.16	0.16		
-196^{o}C	CT7,CT8,CT9, CT10,CT11,CT12	**90±0**	**90±0**	**90±0**	**89.83±0.37**	**89.66±0.74**	**90±0**	**0.5 89.8±0.22**
P-Value		N/A	N/A	N/A	0.162	0.161	N/A	
P- Value. -80^{o}C & -196^{o}C		N/A	N/A	0.17	0.27	0.12	N/A	

pH 4.5	CPH1,CPH2,CPH3, CPH4,CPH5,CPH6	**88.16±0.68**	**88±0.57**	**88±0.57**	**87.7±0.94**	**87.3±1.10**	**90±0**	**0.5 87.83±0.77**
P- Value		0.001	0.00019	0.00019	0.0009	0.001	N/A	
pH 5	CPH7,CPH8,CPH9, CPH10,CPH11, CPH12	**90±0**	**90±0**	**89.8±0.37**	**89.7±0.74**	**89.2±1.21**	**90±0**	**0.26 89.73±0.46**
P- Value		N/A	N/A	0.162	0.162	0.077	N/A	
pH 4.5 & 5		0.0009	0.00028	0.0001	0.0023	0.02		
pH 5.5	CPH13,CPH14,CPH15, CPH16,CPH17, CPH18	**90±0**	**90±0**	**90±0**	**89.8±0.37**	**89.6±0.74**	**90±0**	**0.5 89.8±0.22**
P- Value		N/A	N/A	N/A	0.162	0.162	N/A	
pH 6.5	CPH19,CPH20,CPH21,CPH22,CPH23, CPH24	**90±0**	**90±0**	**90±0**	**89.8±0.37**	**89.8±0.37**	**90±0**	**0.5 89.9±0.14**
P- Value		N/A	N/A	N/A	0.16	0.16	N/A	
pH 5.5 & 6.5		N/A	N/A	N/A	0.5	0.33	N/A	

*DPS- Day post storage

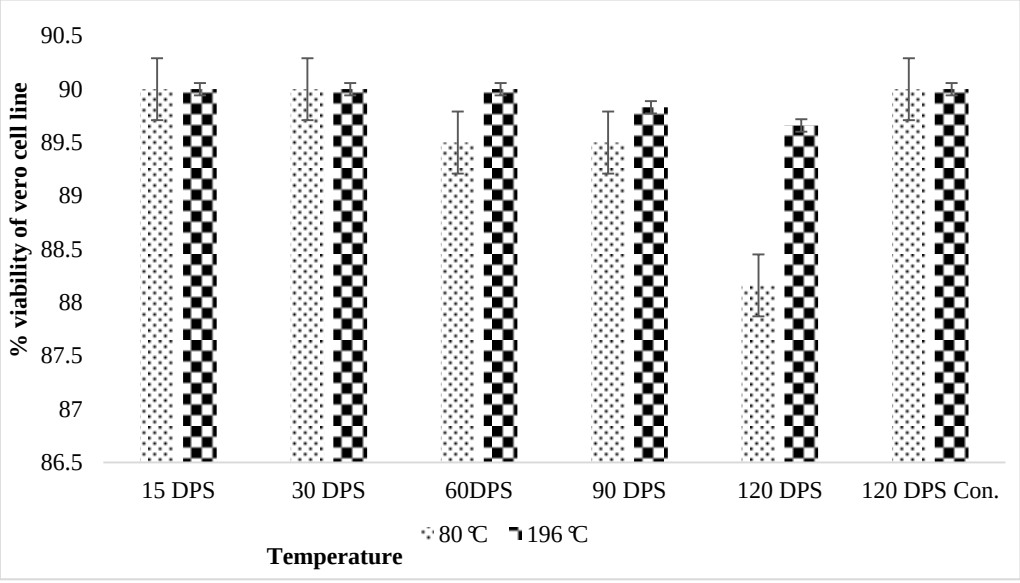

Figure 1. Effect of temperature on percentage viability of vero cell line during cryopreservation

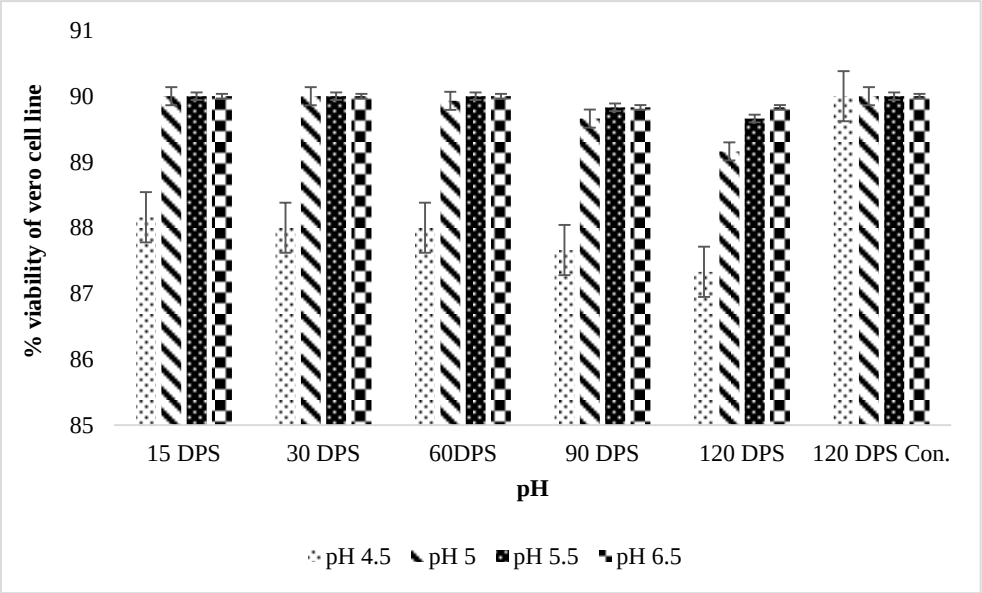

Figure 2. Effect of pH on percentage viability of vero cell line during cryopreservation

Vero cell line containing ampules having DMSO as cryoprotectant stored at -196^{o}C for 15 days, 30 days, 60 days, 90 days and 120 days showed M±SD 77±0, 77±0, 77±0, 76.66±0.69 and 76.5±1.03 respectively in DMEM. The M±SD for percentage viability of cells involving five different time intervals having DMSO as cryoprotectant was recorded as 76.8±0.34 (table: 10). Vero cell line containing ampules having Glycerol as cryoprotectant stored at -196^{o}C for 15 days, 30 days, 60 days, 90 days and 120 days showed M±SD 90±0, 90±0, 90±0, 89.6±0.74 and 89.5±1.11 respectively in DMEM. The M±SD for percentage viability of cells involving five different time intervals having Glycerol as cryoprotectant was recorded as 89.8±0.37 as shown in Table 05, Figure 3.

Table 5. Chemical factors affecting the percentage viability of vero cell line during cryopreservation

Parameter Cryoprotectant	**Sample identification (n=6)**	**Post storage percentage viability of Vero cell line (M±SD)**						**ANOVA P-Value (M±SD)**
		15 *DPS	**30 *DPS**	**60 *DPS**	**90 *DPS**	**120 *DPS**	**120 *DPS (control)**	
DMSO	CD1,CD2,CD3,CD4,CD5, CD6	**77±0**	**77±0**	**77±0**	**76.7±0.69**	**76.5±1.03**	**90±0**	**0.55 76.84±0.34**
P- Value		N/A	N/A	N/A	0.000000039	0.00000028	N/A	
Glycerol	CG1,CG2,CG3,CG4,CG5, CG6	**90±0**	**90±0**	**90±0**	**89.6±0.74**	**89.5±0.11**	**90±0**	**0.55 89.82±0.17**
P- Value		N/A	N/A	N/A	0.16	0.16	N/A	

*DPS- Day post storage

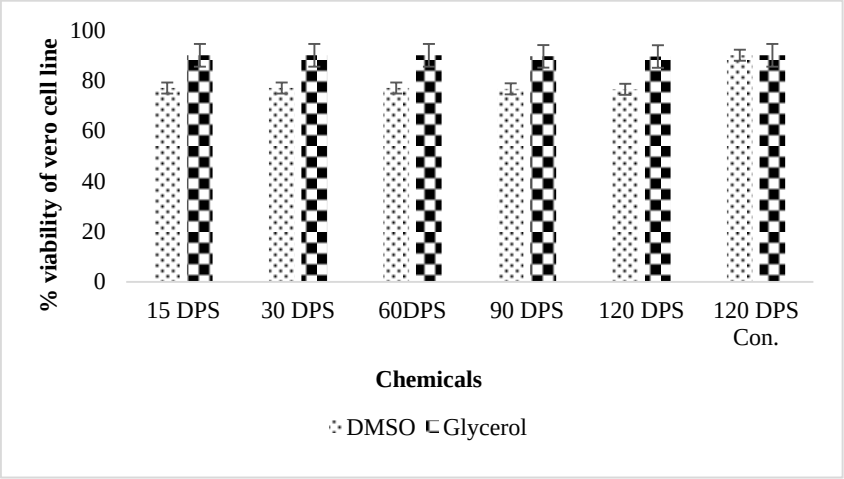

Figure 3. Effect of chemicals on percentage viability of vero cell line during cryopreservation

Avian influenza virus containing ampules stored at -80°C for 15 days, 30 days, 60 days, 90 days and 120 days showed M±SD 256±0, 256±0, 234.6±47.7, 234.6±47.7 and 202.7±77.6 respectively in DMEM. The M±SD for percentage viability of viruses involving five different time intervals at -80°C was recorded as 236.78±34.6. Avian influenza virus containing ampules stored at -196°C for 15 days, 30 days, 60 days, 90 days and 120 days showed M±SD 256±0, 256±0, 256±0, 256±0 and 234.7±41.16 respectively in DMEM. The M±SD for percentage viability of viruses involving five different time intervals at -196°C was recorded as 251.74±8.83 (Table 6, Figure 4). AIV containing ampules stored at pH3 for 15 days, 30 days, 60 days, 90 days and 120 days showed M±SD 0±0, 0±0, 0±0, 0±0 and 0±0 respectively in DMEM. The M±SD for percentage viability of AIV involving five different time intervals at pH3 was recorded as 0±0. AIV containing ampules stored at pH7 for 15 days, 30 days, 60 days, 90 days and 120 days showed M±SD 256±0, 256±0,256±0, 234.7±47.7 and 202.7±77.6 respectively in DMEM. The M±SD for percentage viability of viruses involving five different time intervals at pH7 was recorded as 241.08±25.06. AIV containing ampules stored at pH10 for 15 days, 30 days, 60 days, 90 days and 120 days showed M±SD 256±0, 234.6±47.7, 213.3±60.3, 192±64 and 106.6±30.1 respectively in DMEM. The M±SD for the percentage viability of viruses involving five different time intervals at pH10 was recorded as 200.5±40.4 as shown in table 6, Figure 5.

Table 6. Physical factors affecting the percentage viability of avian influenza virus during cryopreservation

Parameter (Temperature, pH)	**Sample identification (n=6)**	**Post storage percentage viability of AIV (HA Titers) (M±SD)**						**ANOVA P-Value (M±SD)**
		15 *DPS	**30 *DPS**	**60 *DPS**	**90 *DPS**	**120 *DPS**	**120 *DPS (control)**	
-80°C	VT1,VT2,VT3, VT4,VT5,VT6	**256±0**	**256±0**	**234.6±47.7**	**234.6±47.7**	**202.7±77.6**	**256±0**	**0.36 236.78±34.6**
P- Value		N/A	N/A	0.16	0.16	0.08	N/A	
-196°C	VT7,VT8, VT9,VT10,VT11, VT12	**256±0**	**256±0**	**256±0**	**256±0**	**234.7±44.16**	**256±0**	**0.42**
P- Value		N/A	N/A	N/A	N/A	0.14	N/A	**251.7±8.83**
P- Value of temp -80 ℃ & -196 ℃		N/A	N/A	0.18	0.18	0.22	N/A	
pH 3	VP1,VP2,VP3,VP4, P5, VP6	**0±0**	**0±0**	**0±0**	**0±0**	**0±0**	**256±0**	**0±0**
P- Value		N/A	N/A	N/A	N/A	N/A	N/A	
pH 7	VP7,VP8,VP9, VP10, VP11, VP12	**256±0**	**256±0**	**256±0**	**234.7±47.7**	**202.7±77.6**	**256±0**	**0.19 241.08±25.06**
P- Value		N/A	N/A	N/A	0.16	0.08	N/A	
pH 10	VP13,VP14, P15,VP16, VP17, VP18	**256±0**	**234.6±47.7**	**213.3±60.3**	**192±64**	**106.6±30.1**	**256±0**	**0.00038 200.5±40.4**
P- Value		N/A	0.16	0.07	0.03	0.000033	N/A	
P- Value of pH 7 & 10		N/A	0.17	0.07	0.12	0.013	N/A	

*DPS- Day post storage

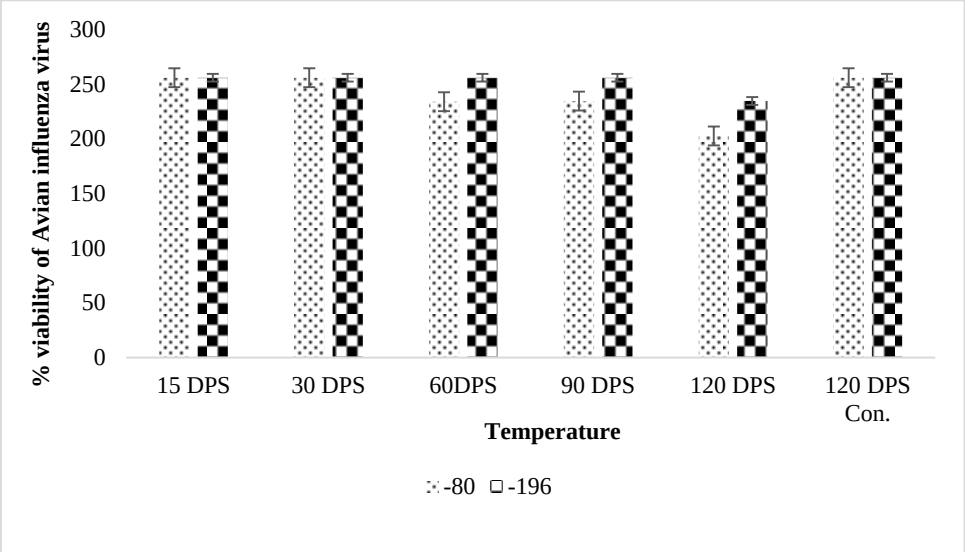

Figure 4. Effect of temperature on percentage viability of avian influenza virus during cryopreservation

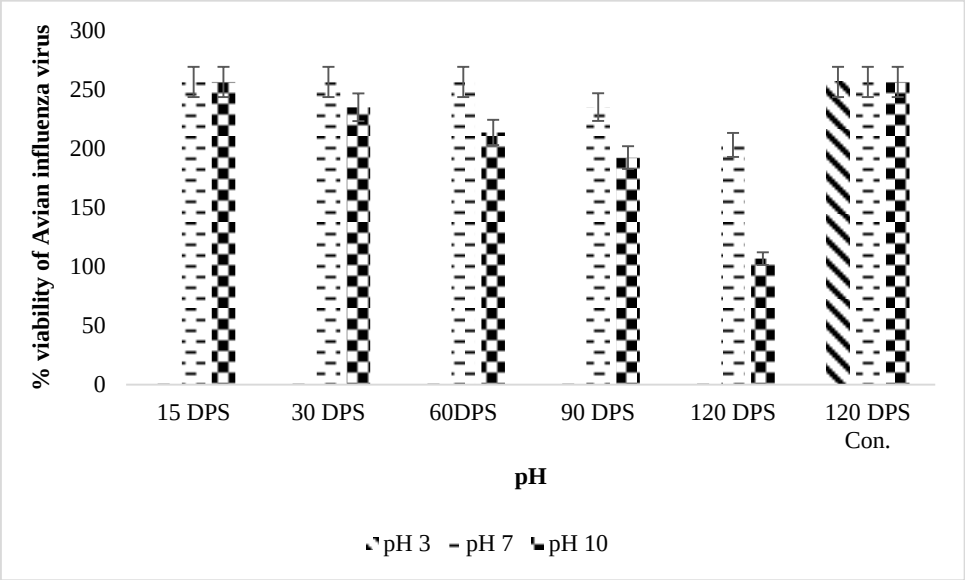

Figure 5. Effect of pH on percentage viability of avian influenza virus during cryopreservation

AIV containing ampules having DMSO as cryoprotectant stored at -196oC for 15 days, 30 days, 60 days, 90 days and 120 days showed M±SD 90±0, 90±0, 90±0, 90±0 and 89.5±0.76 respectively in DMEM. The M±SD for percentage viability of viruses involving five different time intervals having DMSO as cryoprotectant was recorded as 89.9±0.15. Avian Influenza virus containing ampules having Glycerol as cryoprotectant stored at -196oC for 15 days, 30 days, 60 days, 90 days and 120 days showed M±SD 45±0, 45±0, 45±0, 44.83±0.37 and 44.66±0.75 respectively in DMEM. The M±SD for percentage viability of viruses involving five different time intervals having Glycerol as cryoprotectant was recorded as 44.89±0.22 as shown in table 7, Figure 6.

Table 7. Chemical factors affecting the percentage viability of avian influenza virus during cryopreservation

Parameter Cryoprotectant	**Sample identification (n=6)**	**Post storage percentage viability of AIV (HA Titers) (M ±SD)**						**ANOVA P-Value (M ±SD)**
		15 *DPS	**30 *DPS**	**60 *DPS**	**90 *DPS**	**120 *DPS**	**120 *DPS (control)**	
DMSO	VD1,VD2,VD3,VD4, VD5, VD6	**90 ± 0**	**90 ± 0**	**90 ± 0**	**90 ±0**	**89.5 ±0.76**	**90 ±0**	**0.24 89.9 ±0.15**
P- Value		N/A	N/A	N/A	N/A	0.08	N/A	
Glycerol	VG1,VG2,VG3,VG4, VG5, VG6	**45 ± 0**	**45 ± 0**	**45 ± 0**	**44.8 ±0.37**	**44.7 ±0.75**	**50 ±0**	**0.5 .89 ±0.22**
P- Value		N/A	N/A	N/A	0.0000002082024	0.0000055423244	N/A	

*DPS- Day post storage

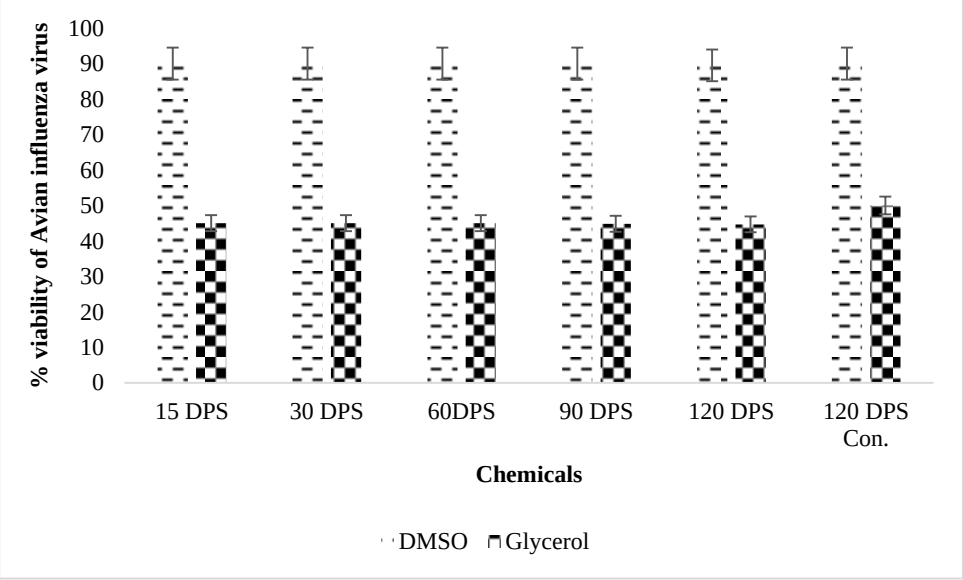

Figure 6. Effect of chemicals on percentage viability of avian influenza virus during cryopreservation

4. Discussion

Cryopreservation is the technique of storing biological material at ultra-low temperatures (-196 ℃) in order to maintain their viability for long period of time. Biological materials can also be stored at -80^{o}C but for less time than the temperature of liquid nitrogen (Grout et al., 1990). Cryopreservation technique involves the slow reducing temperature of cells to -20 ℃ to -60 ℃ followed by transfer to temperatures below -130 ℃. At temperatures between 0 ℃ and −25°C, the enzymatic activity of cells is only slowed but remains active, while below −40°C physiochemical exchanges are frozen. The damage to biological material is mainly due to about a 95% loss of intracellular water, a considerable increase of electrolyte concentrations in both intra and extracellular media and possible ice crystal formation in the intracellular spaces that deform and compress cells and even destroy intracellular structures (Siddiqui et al., 2016). The choice of reliable cryoprotssecting agents (CPAs) for long term – is highly necessary however, in some cases CPAs themselves can be damaging to cells, especially when used in high concentrations. For example, high concentration of DMSO may alter chromosome stability, which can lead to a risk of tumor formation. In this study the effect of two different chemicals (CPAs) has been evaluated for cryopreservation of Vero cell line and Avian Influenza virus at different temperatures for

percentage viabil time intervals and the percentage of viability has been monitored. While cryopreserving these both samples the choice of CPAs and physical factors was made according to their biological nature to avoid damage because of crystal formation. The whole discussion will be based on the rejection and acceptance of "null hypothesis" and "alternative hypothesis" according to M±SD values. As the "null hypothesis" states that there is no significant difference between the mean standard deviations of two or more variables in question so, if the "P<0.05" then the "null hypothesis" will be rejected and "alternative hypothesis" will be accepted which states that there is a significant difference between the mean standard deviations of subjected variables. In other words if the "P>0.05" then the null hypothesis will be accepted and if the "P<0.05" then the alternative hypothesis will be accepted. In experiment number "01" of this study the temperature was maintained at -80^{o}C and -196^{o}C separately and six "Vero cell line" containing cryo-vials were stored for 15 days, 30 days, 60 days, 90 days and 120 days at each temperature. All the "MSD" & "P" values were calculated for every above mentioned time interval individually and the final "P" values were found as "P=0.18" and "P=0.5" for -80^{o}C and -196^{o}C respectively. Because the "P" value was greater than 0.05 (P>0.05) for both temperatures then the "null hypothesis" was accepted and the "alternative hypothesis" was rejected. In the same experiment the pH was kept at 4.5, 5, 5.5 and 6.5 providing all the same above mentioned conditions and found their "P" values as "P=0.5", "P=0.26", "P=0.52" & "P=0.54" respectively. Here "P" value for all of observations was greater than 0.05 (P>0.05) so, the "null hypothesis" was accepted and the "alternative hypothesis" was rejected, which means that there is no significant difference between the resulting values of this study and the results of "control group". Vero cell line stored at -80^{o}C for 15 days and 30 days showed significantly higher viability of Vero cells as compared to 60 days, 90 days and 120 days and also the Vero cells stored at -196^{o}C for 15 days, 30 days and 60 days showed higher cryoprotective response as compared to 90 days and 120 days. Moreover, the Vero cells stored at -196^{o}C showed comparatively high percentage of viability as compared to the Vero cells stored at -80^{o}C for all time intervals. In the case of pH levels to cryopreserve Vero cells, the best cryoprotective response was recorded at pH5.5 and pH 6.5 which showed high percentage of viability as compared to the pH4.5 and pH5. The mean standard deviations and percentage of viability results of this experiment are very close to the research findings of Siddiqui who revealed the higher percentage of viability of Vero cells by cryopreservating them at -196^{o}C for six months (Bakhach et al., 2009). The findings of this experiment are also in accordance with Murakami which reported that the pH5.2 and pH5.4 is better to cryopreserve Vero cells for long intervals of time.

In experiment number "02" of this research study two chemicals DMSO & Glycerol were used as cryoprotectants separately. In this experiment six "Vero cell line" containing cryo-vials were stored for 15 days, 30 days, 60 days, 90 days and 120 days containing each of these cryoprotectants discretely. All the "MSD" & "P" values were calculated for every above mentioned time interval individually and the final "P" values for both of these chemicals were found as "P=0.54" & "P=0.55" for DMSO and Glycerol respectively. As the "P>0.05" for both observations so, the "null hypothesis" was accepted and the "alternative hypothesis" was rejected. Mean standard deviation values for percentage viability of cells having DMSO and Glycerol as cryoprotectant were "76.84±0.34" & "89.82±0.17" respectively. The Vero cells containing cryo-vials having Glycerol as cryoprotectant stored for 15 days, 30 days and 60 days showed comparatively higher percentage of viability as compared to DMSO which showed lower viability percentage cryopreserved for the same intervals of time. These results are closely related to findings of Hammerstedt who have done a related experiment using DMSO and Glycerol as cryoprotectants and publicized that the Vero cells gave higher cryoprotective response with Glycerol than DMSO to cryopreserve Vero cells (Hammerstedt et al., 1990).

In experiment number "03" of this work, the temperature was maintained at -80^{o}C and -196^{o}C separately and six "AIV" containing cryo-vials were stored for 15 days, 30 days, 60 days, 90 days and 120 days at each temperature, respectively. All the "MSD" and "P" values were calculated for every above mentioned time interval discretely and the final "P" values were found as "P=0.36" and "P=0.42" at -80^{o}C and -196^{o}C for at -80^{o}C and -196^{o}C respectively. Because the "P" value was greater than 0.05 (P>0.05) for both temperatures then the "null hypothesis" was accepted and the "alternative hypothesis" was rejected. In the same experiment the pH was kept at 3, 7 and 10 providing all the same above mentioned conditions. At pH3 no viability was seen after any time interval so no MSD & "P" value was calculated. For pH7 and pH10 the "P" values were found as "P=0.19" and "P=0.00038" respectively. Here "P" value for pH7 was greater than 0.05 (P>0.05) so, the "null hypothesis" was accepted and the "alternative hypothesis" was rejected for this observation. The "P<0.05" in case of pH10 that's why the null hypothesis was rejected. Avian Influenza virus stored at -80^{o}C for 15 days and 30 days showed significantly higher viability of viruses as compared to 60 days, 90 days and 120 days, also the AIVs stored at -196^{o}C for 15 days, 30 days and 60 days showed higher cryoprotective response as compared to 90 days and 120 days too. Moreover, the AIVs stored at -196^{o}C showed comparatively high percentage of viability as compared to the viruses stored at -80^{o}C for all similar time intervals. In the case of pH levels to cryopreserve AIVs, the best cryoprotective response was recorded at pH7 which showed high percentage of viability as compared to the pH3 which showed no viability percentage and pH10 which showed relatively lower percentage of viability as compared to pH7. The mean standard deviation and percentage of viability results of this experiment are close to the research findings of Shahid who reported that acidic pH levels are virucidal and should not be used to cryopreserve cells for long times and revealed that

the best storing temperature without injury is -196^{o}C (Shahid et al., 2020).

In experiment number "04" of this work two chemicals DMSO & Glycerol were used as cryoprotectants separately. In this experiment six "AIV" containing cryo-vials were stored for 15 days, 30 days, 60 days, 90 days and 120 days containing each of these cryoprotectants separately. All the "MSD" & "P" values were calculated for every above mentioned time interval individually and the final "P" values for both of these chemicals were found as "P=0.24" & "P=0.5" for DMSO and Glycerol, respectively. As the "P>0.05" for both observations so, the "null hypothesis" was accepted and the "alternative hypothesis" was rejected. Mean standard deviation values for percentage viability of cells having DMSO and Glycerol as cryoprotectant were "89.9±0.15" & "44.89±0.22" respectively. Avian Influenza Virus containing cryo-vials having Glycerol as cryoprotectant stored for 15 days, 30 days and 60 days showed comparatively lower percentage of viability as compared to DMSO which showed higher percentage of viability cryopreserved for the same intervals of time These results are closely related to findings of Nagasaki who reported that the percentage of viability is higher with cryo-protectants and better one is DMSO for long term storage of Avian Influenza viruses (Nagasaki et al., 1999).

Cryovials are placed in a Styrofoam rack at -80^{o}C temperature for 2-3 hours which results in non-uniform cooling rate but close to -1^{o}C/min and suitable for a variety of cells. After that the transfer is made to the storage temperature. Mainly Glycerol, Dimethyle sulphaoxide (DMSO), Proline and sugars are used as cryoprotectants (Meryman et al., 2007). Another function of cryoprotectants is to slow down the rapidity of freezing temperature to avoid shock. Novel methods are being investigated to remove the cryoprotectants from the procedure. Glycerol and DMSO are usually used for "Vero cell lines" and "avian influenza viruses"(Heszky et al., 1990). Like other cells Vero cells consist of cell membrane, nucleus, cytoplasm and hundreds of organelles inside it. Vero cells were obtained from African green monkey in 1962 by two scientists Y. Kawasaki and Y. Yasumura at Chiba University, Japan. Since then many Vero cell lines have been developed which ultimately belong to just one source. These developed cell lines are named as Vero, Vero E6 and Vero 76. These cell lines are being used throughout the world usually in the fields of Virology. While culturing cell lines, it is necessary to store the stock in case of experiment failure and to save time and risk of contamination (Wang et al., 2007).

5. Conclusion

The purpose of this research work is the long term cryopreservation of "AIVs" and "Vero cells" for vaccine production in future or whenever it is needed. In this dissertation, different physiochemical factors are used to check the best cryoprotective response by "Vero cell line" and "Avian Influenza virus" against different time intervals. From the physical factors, the best cryoprotective response by both samples is observed at temperature -196^{o}C whereas, the different optimum pH levels are recorded for both samples. As the "Vero cells" showed best cryoprotective response at a slightly acidic pH while neutral pH was observed as optimum level for the long term cryopreservation of "Avian Influenza virus". From the both chemicals used as cryoprotectants the higher post thaw percentage of viability of "Vero cells" is recorded with "Glycerol" while the better cryoprotective response by Avian Influenza Virus is observed with "DMSO".

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