Rabies virus viability after short-term cryopreservation using cryoprotectant agents

Viabilidade de vírus da Raiva após criopreservação de curto prazo com uso de agentes crioprotetores

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ABSTRACT

Rabies virus cryopreservation has been succinctly described in the scientific literature. The major researches about viral conservation emphasize the rabies diagnosis in decomposed samples. For now few information has been available concerning the use of cryoprotectants for rabies virus cryopreservation. This study aimed at assessing the viability of rabies virus after freezing/thawing procedures and investigating the effect of different concentrations of dimethyl sulphoxide (DMSO), glycerol (GLY), polyethyleneglycol (PEG) and sucrose (SUC) on rabies virus cryopreservation. Virus viability was assessed by virus isolation based on mouse inoculation test, titration and immunofluorescent antibody assay before and after 30 days of freezing procedures. The rabies virus samples after being exposed to cryopreservation without adding a cryoprotectant, its viability showed to be lower than that observed in samples exposed to other treatments. After 30 days of freezing procedure, the viability of cryopreserved samples using DMSO, GLY or PEG was lower than that observed in fresh samples. In addition, the use of sucrose at 10% or 68% concentrations induced positive effects on the viral particles viability after a short-term cryopreservation.

Keywords. challenge virus standard, rabies virus, cryoprotective agents, cryopreservation.

RESUMO

A criopreservação de vírus da raiva tem sido descrita de forma sucinta na literatura científica. Até o presente, poucas informações encontram-se disponíveis sobre o uso de agentes crioprotetores na conservação a frio de vírus da raiva. O objetivo deste trabalho foi de analisar a viabilidade de vírus da raiva expostos aos procedimentos de congelação/descongelação e de avaliar o efeito do dimetilsulfóxido (DMSO), do glicerol (GLI), do polietilenoglicol (PEG) e da sacarose (SAC), em diferentes concentrações, na criopreservação de vírus da raiva. A viabilidade viral foi testada por meio de isolamento viral utilizando-se testes de inoculação em camundongos, titulação viral e imunofluorescência direta antes e 30 dias após terem sido instituídos os protocolos de congelação. A viabilidade das amostras de vírus da raiva após criopreservação na ausência de agentes crioprotetores foi inferior àquela observada em outros tratamentos. Após 30 dias de congelação, a viabilidade das amostras criopreservadas com adição de DMSO, GLI e PEG foi mais baixa do que a observada em amostras frescas. Adicionalmente, o uso da sacarose nas concentrações de 10% ou 68% induziu efeitos positivos na viabilidade das partículas virais após criopreservação a curto prazo.

Palavras-chave. challenge virus standard, vírus da raiva, crioprotetores, criopreservação.

INTRODUCTION

The natural course of a life cycle dictates that biological materials will decay and die. The structure and function of the known organisms change and will be lost with time. Attempts to stop the biological clock have been chased by ancient and contemporaneous minds. At the center of many such schemes have been experiments with temperature and water content. Thereby, the development of cooling technologies provide a means of slowing the rate of deterioration of perishable goods and the use of much lower temperatures has offered a means of storing organisms in a state of suspended animation for extended, even indefinite, periods[1].

Cryopreservation means that materials are stored at low (from -20 to -80 °C in freezers) or ultra-low temperatures (-150 °C in liquid nitrogen containers); more recently, cryopreservation takes place in the liquid or in the vapor phase of nitrogen. However, the major drawback of cryopreservation consists in severe damage of the cells exposed to freezing. The cryoinjury is a lethal event, produced by the formation of intracellular ice, on the efflux of water outside the cell, and on an increase in the concentration of intracellular salts in the solution. The most critical range occurs between +15 and 0 °C, while minor events occur at temperatures below 0 °C, although thermal shock can occur between 0 °C and -80 °C. So, the modern era of cryobiology started with the discovery that some substances might protect eukaryotic cells against freeze damage; after this initial observation, several cryoprotectants agents were discovered and are now widely used[2,3].

Cryoprotective additives may provide protection by being intracellular (penetrating agents) or extracellular (nonpenetrating agents). Penetrating agents create the environment for the reduction of cell water content at low temperatures, they bind intracellular water colligatively which prevents excessive dehydration, reduces salt toxicity and prevents the formation of large ice crystals within the cell and hyperosmotic injury. On the other way, nonpenetrating agents may promote osmotic efflux of cell water during the cooling process, reducing the intracellular ice formation[3].

In virology, a range of procedures exists for maintaining virus stocks and these depend to some extent on the peculiar properties of the particular viruses. They are noncellular structures, much smaller and less biochemically complex than the simplest unicellular organisms. This relative simplicity, diminutive size and the absence of free water are largely responsible for the stability of viruses in diverse temperatures and their fairly easy preservation. Many viruses can be kept for months at refrigerator temperatures and stored for years at very low temperatures without the need for special preservatives or carefully regulated slow freezing techniques. Nevertheless, RNA viruses are less stable than DNA viruses and those with lipid envelopes, like rabies virus, are often less stable than non-enveloped viruses at ambient temperatures[4].

For the last century, rabies virus has been studied in many research institutes around the world due to its crucial importance in public health. However, its cryopreservation has been briefly described in the scientific literature. In cases where the sample is required only as an antigen for routine diagnostic and the retention of virus infectivity is not essential, the virus samples can be stored without loss of antigenic activity, even though the infectivity might be significantly reduced[4].

On the other hand, in many research situations there is only a small window of opportunity after which the study subject may no longer be available or conditions may have changed. Thus, these precious samples must be handled and stored carefully; allowing archiving of material, repeated experiments from the same specimen source and, by allowing distribution of stored samples, facilitates research collaboration. Storage or banking of the samples is, in itself, a central issue especially for long-term studies and for sample use in new studies in the future. Storage can affect the quality of the samples and determines whether their future use is possible[5].

The infectivity of many viruses decrease during transport and storage, resulting in isolation failures from clinical specimens and diminished titers in virus stocks. This phenomenon occurs more rapidly at 20 °C and 4 °C than at ultralow temperatures. Since the former requires equipment that is not commonly found in most clinical and diagnostic laboratories, many virologists find it useful combining infected samples with a cryoprotectant agent. Glycerol, dimethyl sulphoxide, albumin, balanced salt solutions plus gelatin, animal sera, skim milk, polyethyleneglycol, sucrose and sorbitol are among the more commonly used cryoprotectants[6].

Until now little information is available concerning the cryopreservation of rabies virus and the use of cryoprotectant agents in such samples. Thus, the aim of this work was to evaluate the viability of rabies virus after freezing/thawing procedures and assess the

effect of dimethyl sulphoxide (DMSO), glycerol (GLY), polyethyleneglycol (PEG) and sucrose (SUC), in different concentrations, on rabies virus cryopreservation.

MATERIAL AND METHODS

Experimental animals

Swiss albino mice (*Mus musculus*), three-four weeks old, weighting 10-16g were used. The probability sampling was utilized in order to have a random selection method that assures that the different units in the population have equal probabilities of being chosen.

All experiments were performed according to the Committee of Animal Handling and Ethical Regulation (protocol number: 08185881-7). The mice were anesthetized individually before every invasive procedure using inhalation anesthetics (halothane or isoflurane, depending on availability). Inhalant anesthetics were either used to euthanatize the mice in terminal state of the disease. The animal was placed in a closed receptacle containing cotton or gauze soaked with an appropriate amount of the anesthetic. Vapors were inhaled until respiration ceases and death ensues, associated to a longer induction time.

Virus strain

The mice were infected with the murine-adapted Challenge Virus Standard (CVS) strain of rabies virus. The CVS strain induced clinical signs which are undoubtedly those of rabies, resulting from predictable (fixed) properties in terms of incubation period, pathological and clinical effects. In addition, it represents a more homogeneous viral population. The work strain CVS-31 (lyophilized specimen) was ceded by Dr. Phyllis Catharina Romijn (PESAGRO-RIO) in research collaboration. The titer of this seed CVS aliquot was tested (3.60 Log_{10} 50% LD_{50}) and then it was replicated in three mice-brain-passages, increasing the titers to 5.40 Log_{10} 50% LD_{50}.

Samples

A total of 50 mice were inoculated with CVS-31 suspension (0.03ml), through intracerebral route, and observed daily. The encephalic tissues (central nervous system) from the infected mice were used. The brains of all mice that have died after the inoculation, or that have been euthanized when prostrate (terminal clinical signs of rabies), were removed. After disinfection with 70% ethanol, the skin of the head and neck was cut away with forceps and scissors, exposing the skull. The skull was grasped in the orbits with mouse-tooth forceps and the skullcap was detached with curved scissors, thereby exposing the brain[7]. The tissues (0.2-0.4g each) were removed with a curette and transferred to a sterile universal collector. A composite sample was made in which material collected from several individuals was combined and homogenized in a pooled sample, reducing the subject-to-subject variability and thus increasing the power of statistical validity.

Cryoprotectants agents

A selection of some of the most widely used cryprotector agents in microbiology was made. In literature, dimethyl sulphoxide (DMSO) concentrations varies from 1 to 32% (median ~10%), glycerol (GLY) (1,2,3-propanetriol) was applied at concentrations of 2–55% (median 10%), polyethylene glycol (PEG) has been used at concentrations of 5–45% (median 10%) and sucrose, at concentrations of 1–68% (median 10%)[3]. The median and maximum concentrations of the cryoprotectants agents cited on microbiology literature were used. The median of cryoprotectants concentration were chosen because, in certain analysis, it may reflect more accurately the measure of central tendency in the presence of outlier values than is the mean. Cryoprotectants maximum concentrations were employed to verify if in higher concentrations it causes decline of rabies virus viability. The Phosphate Buffered Saline (PBS pH7.2) was used as diluent.

Experimental procedure

The encephalic tissues pooled sample containing rabies virus particles was divided into 10 positive aliquots (0.5g each), in duplicate. Ten aliquots were used to analyze the viability of rabies virus samples immediately after cryoprotectants agents contact (T_0). The other aliquots were taken randomly, submitted to freezing/thawing protocols and analyzed after a 30-days period (T_{30}).

Virus isolation by mouse inoculation test and direct fluorescent antibody test were performed to evaluate rabies virus viability after the exposure to DMSO, GLY, PEG or SUC, before and after the cryopreservation experiments. The cryoprotectants were prepared in double straight concentration using PBS pH 7.2 as diluent. Eight aliquots (0.5g each) of the pooled sample were transferred to vials containing 200µl of cryoprotectant solution at 10% and 32% DMSO, 10% and 55% GLY, 10% and 45% PEG or 10% and 68% SUC – each in duplicate. Two aliquots were used as positive controls, one in pure state (sample control, PC-CVS) and the other in PBS pH7.2

solution (cryoprotectants control, PC-PBS). One duplicate was destined for the viability tests and the other for the cryopreservation treatment. An extra duplicated aliquot of normal mice brain was employed as negative control (NC-NMB). The positive controls were submitted to viral titration before and after the freezing/thawing protocol.

Samples were exposed to the cryoprotectants for 20 min at 20 °C and in PBS pH 7.2. After the exposure period, the specimens were washed to remove the cryoprotectant as follows: suspensions containing the virus were diluted in 2.0 ml PBS, equilibrated for another 5 min at room temperature, and then centrifuged at 150-200G for 5 min. This procedure was performed a second time. The samples were finally suspended in 2.0 ml viral diluents and the viability tests were made immediately.

Freezing and thawing process

For freezing, samples were equilibrated in 10% and 32% DMSO, 10% and 55% GLY, 10% and 45% PEG or 10% and 68% SUC and in PBS pH 7.2 for 20 min at 20 °C, as in the viability test. After the equilibration period, the vials were transferred to a -20 °C freezer and kept at this temperature for a 30-days period.

For thawing, the vials were taken from the freezer, warmed in air at room temperature for 1 min and immersed in a water-bath at 37 °C until it thawed. Thereafter, the cryoprotectants were removed as described above and the samples were submitted to the viability tests as follows.

Mouse inoculation test

Virus isolation was conducted by mouse inoculation test (MIT)[7]. The encephalic tissues were triturated in a mortar (pooled samples), diluted to 20% in phosphate buffered saline (PBS) pH 7.2, added 500UI of penicillin and 2 mg of streptomycin per ml of tissue suspension to prevent animal death from possible bacterial contamination of the tissue suspension, and clarified by centrifugation at 900x g for five minutes. The supernatant was collected and 0.03ml was used to inoculate each of 10 to 12 Swiss albino mice, three-four weeks old, through intracerebral route. It was used the BD Ultra-fine II™ U-100 insulin syringes – short needle – 31 gauge, 8mm (5/16") for 30 units or less. The inoculated animals were observed daily up to 21 days. Mice receiving diluents only were used as negative controls. Animals dying within the first four days after inoculation and without signs suggestive of rabies were excluded.

Virus titration test

Controls and stock virus titrations were performed at designated intervals. A 10% suspension of the pooled sample was prepared in PBS pH7.2 solution[7]. Serial 10-fold dilutions (10^{-1} to 10^{-6}) were made of supernatant fluid, and 0.03 ml of each dilution was inoculated by intracerebral route in either of six Swiss albino mice (*Mus musculus*), three weeks old, weighting 10-12g and examined daily. Rabies virus LD_{50} titers were calculated8 and expressed as Log_{10} 50% lethal doses (Log_{10} 50% LD_{50}).

Table 1. Effects of freezing and storage temperature on rabies virus viability tested by mouse intracerebral inoculation and direct fluorescent antibody test after exposure to internal and external cryoprotectants

Samples	MIT[1]					FAT		
	MST[2] (days)		Mortality NAM[3]/NT[4]		%	Results	Degree*	
	T_0	T_{30}	T_0	T_{30}			T_0	T_{30}
NC-NMB	21	21	0/11	0/10	0	Negative	–	–
PC-CVS	6.5	7.8	10/10	10/10	100	Positive	++++	+++
PBS	7.0	7.0	10/10	10/10	100	Positive	+++	+++
DMSO (10%)	7.0	6.9	10/10	10/10	100	Positive	+++	+++
DMSO (32%)	7.0	7.0	10/10	10/10	100	Positive	++	++
GLY (10%)	7.0	7.0	11/11	10/10	100	Positive	+++	+++
GLY (55%)	7.0	6.9	11/11	10/10	100	Positive	+++	+++
PEG (10%)	7.0	6.7	10/10	10/10	100	Positive	+++	+++
PEG (45%)	7.0	7.0	12/12	10/10	100	Positive	+++	+++
SUC (10%)	6.8	7.0	10/10	10/10	100	Positive	++++	++++
SUC (68%)	6.8	7.0	11/11	10/10	100	Positive	++++	++++

[1]Mouse Inoculation Test; [2]Mean Survival Time; [3]Number of Affected Mice; [4]Number Tested;
**Evaluation of positivity degree: –, +, ++, +++, ++++*

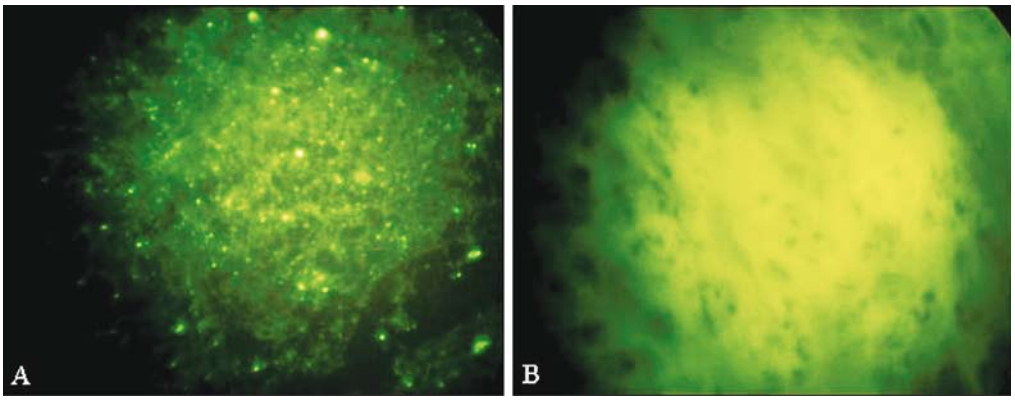

Figure 1. Photomicrograph with immunofluorescent staining of the samples impression smear shows bright apple-green viral nucleocapsid antigen targeted by virus-specific fluorescent-labeled antibodies and seen using fluorescein isothiocyanate filter in standard incident light ultraviolet microscope (Carl Zeiss, Germany), with *x400* magnification.
A: antigen very abundant in every field and amount per field was "not possible to count" (++++): Positive Control – Challenge Virus Standard (PC-CVS).
B: no antigen throughout whole impression (-): Negative Control – Normal Mice Brain (NC-NMB)

Fluorescent antibody test

The viral antigens in infected samples were detected by the direct immunofluorescent antibody staining technique (FAT)[9]. The impression smears were made on multi-well immunofluorescence glass slides and fixed for 30 min in cold acetone at a -20 °C environment, and air-dried. The samples were then incubated at 37 °C with fluorescein isothiocyanate-labeled rabbit polyclonal antinucleocapsid antibody absorbed with 10% normal mouse brain (BIO-RAD Laboratories-France, Catalog Number 357-2112) for 60 minutes at 37 °C in a humid chamber. Slides were washed with PBS pH 7.2 in two successive 5-minute wash periods. After rinsing with distilled water, the smears were air-dried and examined using a standard incident light ultraviolet microscope (Carl Zeiss, Germany), with x40 objective and x10 eyepiece lenses (total magnification *x400*).

Each smear was examined and graded by two readers independently and where disagreement occurred the respective cases were re-examined and a consensus reached. All the smears were graded from (–) to (++++) according to the amount of fluorescent antigen, as follows: (–), no antigen throughout whole smear; (+), antigen very scanty, one or more particles in fewer than 50% of microscope fields; (++), antigen scanty, one or more particles in fewer than 100% of fields, but more than 50% of fields; (+++), antigen abundant, one or more particles in each field, but the amount per field was 'possible to count'; (++++), antigen very abundant in every field and amount per field was 'not possible to count'. For the slides read of rabies samples smears, scores 3 and 4 were considered 'easy', while specimens with scores 1 and 2 were considered 'difficult' to diagnose[10].

RESULTS

There was a decrease of viral titers when CVS strain samples were exposed to freezing/thawing procedure. In a 30-day period (T_{30}), the titre of the pooled sample was reduced from 5.40 to 4.69 Log_{10} 50% LD_{50} compared to fresh sample (T_0).

The results of viability tests in rabies virus samples, after exposure to internal and external cryoprotectants, are set out in Table 1. Before the freezing/thawing procedure, differences were observed in the immunofluorescent staining degree between the CVS strain positive control (PC-CVS), positive control exposed to PBS pH 7.2 (PC-PBS) and cryoprotectants treatments. While the PC-CVS (T_0) sample demonstrated the maximum fluorescence classification, there was a decrease on fluorescent degree from PC-PBS, as identified in samples exposed to DMSO (10%), GLY (10% and 55%) and PEG (10% and 45%). Decline in fluorescence profile was more severe in DMSO (32%) treatment. However, the outcome of sucrose cryoprotectant treatment (using either 10% or 68%) was equivalent to that observed in the PC-CVS (control not treated) Fig. 1-A.

Table 1 either shows the effects of freezing and storage temperature on rabies virus viability tested by mouse inoculation and direct fluorescent antibody test after a 30 days period. No difference was observed in the immunofluorescent staining degree subsequently to cryopreservation, irrespective of the cryoprotectants treatments or the concentration used. Otherwise, the

fluorescence intensity found in PC-CVS (T_{30}) following the cryopreservation was lower than in fresh samples and the mean survival time (MST) in the mouse inoculation test increased one day. Only when the pooled samples were cryopreserved without cryoprotectant (CP-CVS) did the fluorescence degree differ in the viability tests. Assays on negative control of normal mice brain (NC-NMB) were uniformly negative Fig.1-B.

DISCUSSION

The measurement of virus titres provides information about the viral viability after the freezing/thawing protocol. In a 30-day period, the pooled sample titre decreases when compared to fresh sample. The data shown indicates that the rabies virus samples had the viability compromised by freezing/thawing protocols in the absence of cryoprotectant agents.

The rabies virion capsid is surrounded by the host cell-derived membrane[11], what can be interpreted as a susceptibility feature of viral particle to mechanical damage caused by ice crystals formation, similar to those inflicted on host cells. According to Howell and Miller[6], enveloped viruses demonstrate greater lability during storage than those lacking envelopes.

Since viruses acquire their envelopes by budding through cellular membranes, the dynamics associated with deleterious changes which occur during freezing and thawing may affect both viruses and cells in a similar manner. The mechanisms of membrane and viral envelope damage appear to be caused by the formation of external and internal ice, excessive changes in pH and solute concentration, and ice recrystallization, during freezing and thawing. In fact, decreases in viral titer correlate with demonstrable changes in areas of the viral envelope presumed to be associated with viral infectivity.

In addition, it was also shown that cryopreserved samples are still viable after 30 days at low temperature (-20 °C), but a decrease of titer occur. The present study showed that almost all treatments demonstrated a reduced quality of virus particles when compared to the control in the fluorescent antibody test. This reduction is presumable due to virus losses caused by the washings to remove the cryoprotectants. This is confirmed by the fact that in the treatment where the samples were not exposed to cryoprotectants (PC-PBS/T_0), but were submitted to washings and centrifugations, the samples slide fluorescence degree was lower than in the fresh control (PC-CVS/T_0). However, when rabies virus samples were frozen without cryoprotectant (PC-CVS/T_{30}), the slides degree examined after thawing was lower than the fresh control (PC-CVS/T_0). In this case, the reduced of slides degree may be due cells rupture caused by the freezing and thawing procedures. Moreover, this inconsistency can be explained by the labile nature of RNA and the high ribonuclease content of brain tissues, and most importantly, degradation of RNA[12] in the pooled stores samples, promoting viral degradation and loss of viability.

The increase of the mean survival time (MST) in the mouse inoculation test (MIT) might be explained by lower viral virulence. This finding is similar to that observed by Lopes et al.[13] studying long-term stored samples, frozen under natural conditions without any preservation substance, that encountered a higher MST (12-17 days). Considering that the average of mouse incubation period is five days and death occurs between one and three days after clinical signs initial manifestation, the mean survival time of mice inoculated with samples from naturally infected animals is generally seven to ten days and five to seven days for Challenge Virus Standard (CVS strain)[7,13].

In the present study, the use of sucrose at 10% and 68% concentrations induced positive effects on the viral particles viability after washing/centrifugation protocols (T_0) to remove cryoprotectant agents and for a 30-days period of cryopreservation (T_{30}). Non-permeable or external cryoprotectants protective action is very complex and attributable to a number of properties, the most important of which is the beginning of the dehydration process. In particular, sucrose does not enter the cell, but exerts its beneficial effects by causing cellular dehydration through changes in osmotic pressure[14]. It was suggested that the increase in the sucrose concentration generates an osmotic gradient across the cell membrane, which draws water out of the cell, causing the cell to dehydrate sufficiently before and during the freezing procedure[15], also exerting a protective effect to the viral particles.

The results either demonstrate that after 30 days of freezing the viability of cryopreserved samples (using DMSO, GLY or PEG) was lower than the viability of fresh samples, but was similar to frozen controls. The viability of rabies virus after cryopreservation without a cryoprotectant was significantly lower than that observed in the other treatments with almost all samples

damaged following this procedure. As shown in this work, titration is an essential step in the evaluation of cryopreserved virus samples.

In conclusion, this study shows that cryopreservation of rabies samples in sucrose allows the maintenance of a better rabies virus viability after short-term cryopreservation. Such results could stimulate further researches in this area. More experiments are needed to clarify the basic aspects of rabies virus cryopreservation, mainly to obtain knowledge of fundamental cryobiological properties, which would minimize the injuries associated with the cryopreservation procedures. Other studies on the features of these cryoprotectant agents would provide new strategies to minimize such injuries and consequently elevate the viability of rabies virus stored samples.

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