

Cryoprotectant solution containing ethylene glycol and dimethyl sulfoxide, used for vitrifying cerebral tissues

Abstract

A method for cryopreserving cerebral tissues by vitrification, which is a method of preventing ice formation. The method includes the saturation of cerebral tissues with a cryoprotective agent mixture and cooling of the saturated tissues to negative (-) 130°C. The vitrification mixture contains ethylene glycol and dimethyl sulfoxide. The mixture can be varied depending on the cooling and warming rates. Ethylene glycol can be varied 28% to 45%. Dimethyl sulfoxide can be varied 16% to 35%. The carrier solution consists of potassium chloride, glucose and tris-HCl buffer.

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Claims

What is claimed is:

1. Cryoprotectant solution containing ethylene glycol (EG) and dimethyl sulfoxide (DMSO) can be used for vitrifying whole brains or cerebral tissues.
2. The cryoprotectant solution of claim 1, wherein the optimal concentrations of the EG and DMSO components for vitrification of cerebral tissues by using rapid cooling and warming rates are from 32% to 28% for EG and from 27% to 16% for DMSO resulting in overall cryoprotectant concentration of 48-55% in total.
3. The cryoprotectant solution of claim 1, wherein the optimal concentrations of the EG and DMSO components for vitrification of whole brains by using rapid cooling and warming rates are from 30% to 45% for EG and from 35% to 20% for DMSO resulting in overall cryoprotectant concentration of 65-70% in total.
4. The cryoprotectant solution of claim 3, wherein simple carrier solution is consisting of only three components: potassium chloride, glucose, and tris - HCl buffer.
5. The cryoprotectant solution of claim 3, wherein saturation of the whole brains through the vascular system with CI-VM-1 solutions is performed in three steps: (1) 10% EG at 0°C, (2) 30% EG at -5°C, and then (3) 70% CI-VM-1 at -15°C. CI-VM-1 consists of 35% EG plus 35% DMSO in order to decrease cryoprotectant toxicity.

Description

BACKGROUND OF THE INVENTION

The field of the invention is cryobiology and cryonics.

Cryobiology started to develop in 1949 when cryoprotective agents (CPAs) were discovered for cryopreservation of cell suspensions. About 200 compounds have been tested as CPAs. However, the number of effective cryoprotectants is limited by twenty derivatives of the three chemical classes, namely polyols (diols, glycerol), amides, and sulfoxides (Y. I. Pichugin, *Problems of Cryobiology* 2: 3-9, 1993). The best CPAs are glycerol, dimethyl sulfoxide, ethylene glycol, 1,2-propanediol, dimethylformamide, 2,3-butanediol, dimethylacetamide, 2-methoxyethanol.

Intracellular crystallization of water is a very harmful factor for biological systems during their cryopreservation. CPAs are used to prevent this intracellular crystallization. There is a great difference between cryopreserving suspensions of cells in comparison to cryopreserving tissues and, especially, cryopreserving whole organs. Cell suspensions can be cryopreserved by freezing.

Freezing is a process of ice crystal formation that can damage cells. Vitrification is a process of cooling biological materials with cryoprotectants to -130°C or below without ice formation.

Cells in suspension can be sequestered into unfrozen liquid channels by growing ice crystals during the slow freezing process. At the same time cryoprotective agents are concentrating in the channels. The freezing process under certain conditions (optimal CPA concentration and cooling and warming rates) can enable successful cryopreservation of cells in suspension but not in tissues because ice crystals cannot sequester the cells into unfrozen channels without damaging the tissues (P. Mazur, *Am. J. Physiol.*: 247, C125-C142, 1984).

The vitrification method was primarily discovered by G.M. Fahy in 1981-1984. Since that time many researchers have tried to use vitrification for cryopreservation of various tissues and organs. Researchers have been successful in vitrifying small sizes of tissues but have not been successful in cryopreserving whole organs such as kidneys, livers, and hearts for transplantation. The main problem in attempting to vitrify organs is the requirement to use high concentrations (60-65%) of CPAs to vitrify them at relatively slow cooling rates and to avoid devitrification at slow warming rates. Until recently researchers could not completely overcome the toxicity of these high concentrations for the organs (G. M. Fahy et al., *Cryobiology* 48: 22-35, 2004). Cryoprotectant toxicity in vitrification methods has been the limiting factor for the recovery of biological systems.

Cryonics uses achievements of cryobiology to cryopreserve whole human bodies or heads after legal pronouncement of death. Cryopreservation of the whole human brain by vitrification is a main goal for cryonics. Previously some vitrification mixtures (VMs) were found for renal tissues and blood vessels. G. M. Fahy et al. developed low toxic vitrification mixtures such as VM-3 and M22 for cryopreservation of rabbit kidneys (G. M.

Fahy et al., *Cryobiology* 48: 157-178, 2004). In 2006, an article devoted to the study of some VMs (Vegs and VM-3) for vitrification of adult rat hippocampal slices was published (Y. I. Pichugin et al., *Cryobiology* 52: 228-240, 2006). However, the rapid cooling and warming rates which were employed to obtain very high survival of small pieces of the cerebral tissue cannot be used for cryonics technology.

The cryonics company, Alcor uses M22 (G. M. Fahy et al.) VM for vitrification of cryonics patients' brains. This vitrification mixtures is very complex and very expensive. The goal of the Cryonics Institute (CI) was to find simple and cheap VMs with sufficient vitrification efficiency at feasible slow cooling and warming rates. Besides good vitrification efficiency, perfect VMs must have low toxicity for cerebral tissues, penetrate rapidly into tissues and cells, and have low viscosity at low temperature. Perfect vitrification solutions allow for rapid and uniform perfusion of whole human brains or bodies with few CPA toxic effects on tissues.

SUMMARY OF THE INVENTION

The invention provides a new cryoprotectant solution that was specially designed for vitrifying whole brains or cerebral tissues. This solution was named CI-VM-1 (the Cryonics Institute Vitrification Mixture one). It can be used for vitrification of large whole brains such as the human brain as well as for small pieces of cerebral tissues such as rat hippocampal slices.

CI-VM-1 has high vitrification tendency and enhanced resistance to devitrification even for large volumes (2-4 liters) at very slow cooling and warming rates. CI uses this CPA solution to vitrify CI patients' brains.

CI-VM-1 also has low toxicity for cerebral tissues. Cerebral tissues are very sensitive to various harmful factors. The invention provides a procedure for saturating the cerebral tissues and the whole brains with CI-VM-1 solutions with minimal CPA toxicity. The invention also provides a procedure for cryopreserving cerebral tissue slices which could be banked for neurophysiological research.

An optimal method of perfusion for human brains is very important for cryonics. To decrease time of perfusion, it is necessary to use VMs with low viscosity and rapid permeability in cerebral tissues at low temperature. The patent solution has these properties that are greatly superior to other VMs. CI-VM-1 does not contain high molecular components such as polyvinylpyrrolidone (PVP) and ice blockers that significantly increase viscosity of VMs.

The invention also provides a simple carrier solution that enable CI-VM-1 to be stable for a very long time. This carrier solution does not contain expensive components like most vehicle solutions in cryobiology. The carrier solution has a low viscosity.

CI-VM-1 vitrification solution and CI-VM carrier solution are very simple and cheap. This is one of most important advantages of CI-VM-1 in comparison with other known VMs.

DETAILED DESCRIPTION OF THE INVENTION

Unless otherwise specified, all percentages used herein are by weight. The systematic study of cryopreservation of cerebral tissues was performed. Adult rat hippocampal slices were used in the study as a very widely used model of the cerebral tissue in neuroscience. The K^+/Na^+ ratio assay was selected for study because it is a sensitive functional test to evaluate viability of biological tissues.

Vitrification yields much better results (average 80% recovery) than freezing (about 20% recovery) for cryopreservation of biological tissues (contractile tissues, cartilage, and blood vessels (M.J. Taylor et al, in: *Life in the Frozen State*, B.J. Fuller et al, eds., CRC Press, 2004, pp. 603-641).

Various CPAs in various concentrations (15% to 60%), modes of CPA exposure, cooling and warming rates, and final freezing temperatures were tested. However, freezing methods did not give good results. The maximal survival of the cerebral tissues after freezing was only around 20% of the control.

CRYOPROTECTANT TOXICITY

The study of the vitrification method was started by testing the toxicity of the best individual CPAs on rat hippocampal slices. First of all, the best CPAs must have no toxic effect or at least very low toxicity in high concentrations (50% to 70%). However, cryobiological practice has shown that there are no completely non-toxic compounds in concentrations higher than 50-55% for biological tissues. Even such neutral compounds as ethylene glycol and glycerol in concentration 60% have a certain moderate toxic effect on cerebral tissues according to the K^+/Na^+ ratio assay. None of individual CPAs is good for the vitrification method. To decrease CPA toxicity and to increase CPA vitrification efficiency, a mixture of CPAs should be used. Vitrification methods employ a mixture of CPAs, but not individual CPAs. Specific, biochemical toxicity of CPA mixtures may be rather less toxic than toxicity of individual CPAs having the same concentrations as CPA mixtures because the effective concentration of each individual CPA is lower in the mixtures.

Five CPAs were chosen as the best potential CPAs for vitrification mixtures. These were ethylene glycol (EG), glycerol (GL), 1,2-propanediol (PG), dimethylformamide (DMF), and dimethyl sulfoxide (DMSO). The number of relatively low toxic CPAs is limited to these compounds. It was rational to use the least toxic CPA in a higher concentration than the other more toxic CPAs for a vitrification mixture. This CPA was named the primary CPA or the basic component of the vitrification mixture. EG was selected as the primary CPA because it is the least toxic for cerebral tissues. 1,2-propanediol, dimethylformamide, and dimethyl sulfoxide should not be the basic component of VMs because they are too toxic for this role.

Glycerol has almost the same toxic effect on cerebral tissues as EG. It could be considered for use as a primary CPA for this reason. However, glycerol has very high viscosity and very poor vitrification efficiency and so it cannot be used as the primary CPA in

vitrification mixtures. In addition, glycerol penetrates too slowly through the blood brain barrier and too slowly into cerebral tissues and cells. As a result, glycerol causes excessive dehydration of the brains, tissues, and cells. The use of glycerol as the basic component in VMs increases their viscosity by several times. These negative properties of glycerol make perfusion of whole brains and bodies ineffective. 1,4 and 2,3-butanediols are very similar to glycerol in this regard.

Secondary CPAs in VMs may be glycerol, 1,2-propanediol, dimethylformamide, and dimethyl sulfoxide. It was found that the best secondary CPA for VMs is DMSO.

The toxic effect of CPA mixtures significantly depended upon exposure temperature. One of most important laws of cryobiology is that CPA toxicity may be decreased at lower temperatures. So, cerebral tissues should be saturated with VMs at low temperatures. The highest temperature for the beginning of CPA exposure is 0°C. The least toxic cryoprotectants are used in the beginning of the saturation of brain slices with VMs. Experiments with rat hippocampal slices demonstrated that 30-35% EG solutions are not toxic at 0°C. Melting points of EG solutions prepared in CI-VM vehicle solution were -4.3°C for 10%, -9.5°C for 20%, -14°C for 30%, and -23.5°C for 40%. To decrease toxicity of more toxic secondary CPAs, cerebral slices should be exposed with them at temperatures lower than 0°C. Saturation of the slices with 30% EG or 40% EG allowed combining with secondary CPAs at -14°C or -23°C, respectively, decreasing CPA toxic effects. This saturation procedure was a new one in comparison with previous procedures of other researchers, for example Dr. Fahy et al. The best vitrification methods have been described by Dr. Fahy et al (US patent 6,395,467). Cryobiological terms were well defined in that patent, which was chosen as a prototype for improvement of vitrification procedures.

Toxicities of numerous mixtures of the best CPAs were studied. The mixture of EG and DMSO yielded the best results in cryopreserving cerebral tissue (up to 100% survival using 48-50% CI-VM-1 for fast cooling and warming rates and up to 85% survival using 65% CI-VM-1 for the slower rates). The composition of EG and DMSO in the vitrification mixture must be varied depending on cooling and warming rates.

VITRIFICATION OF LARGE BIOLOGICAL OBJECTS

Cryoprotectant toxicity in vitrification methods will be the limiting factor for survival of cerebral tissues if other parameters of vitrification procedures are perfectly optimized. A final indicator of a perfect vitrification procedure is that viability of tissue before cooling (an indicator of CPA toxicity) is retained without change (within experimental error) after cooling and warming. There are different vitrification parameters for large biological objects such as whole organs or organisms and small ones such as thin slices of biological tissues. The main goal of this invention was a vitrification method especially designed for cryopreservation of post-mortem human brains.

The stability of vitrification of large volumes of CI-VM-1 was studied. The volume of an average human brain is 1.4 liters. Slow cooling and warming rates 0.1-0.05°C/min can be

used for 65-70% CI-VM-1 to get stable vitrification and to avoid devitrification. Even two liters of 65% VMs can be cooled with these rates to -130°C without ice crystallization. The two liters can then be warmed at the slow rate of $0.1-0.05^{\circ}\text{C}/\text{min}$ without devitrification.

One of the important tasks of cryonics is transportation of patients to the CI facility (Clinton Township, Michigan) within 24-48 hours. The possibility of the long term storage of human brains saturated with 65% CI-VM-1 was studied. Experiments demonstrated that cerebral tissues completely saturated with 65% CI-VM-1 can be stored in dry ice for at least 48 hours without decreasing their viability. As a result, a new method or criterion of determination of vitrification stability was developed.

It is very important for perfusion of whole human brains to take into account viscosity of VMs and permeation of CPAs through the blood brain barrier, as well as into cerebral tissues and cells. CI-VM-1 containing only EG and DMSO has low viscosity and relatively good permeability at low temperatures. However, known carrier solutions increase the viscosity of VMs and can make them unstable for storage at low temperature. A new type of carrier solution was designed for CI-VM-1. This solution is named CI-VM carrier solution.

A "carrier" or "vehicle" solution is the portion of VM other than the CPAs. Carrier solutions are usually used in the absence of CPAs to support the viability of organs, tissues, or cells outside of the body. They have also been employed as cold storage solutions for various biological objects. High molecular mass agents such as proteins, dextrans, hydroxyethyl starch (HES), polyvinylpyrrolidone, and others can be present in carrier solutions in 3-5% to support oncotic pressure (ie, pressure in the blood vessels). All of these agents significantly increase the viscosity of VMs. CI-VM carrier solution contains none of these agents or other similar high molecular mass compounds.

As a rule, carrier solutions have Ca^{+2} , Mg^{+2} , phosphate ions, and inorganic buffers. These agents can make 50-70% VMs unstable at low temperature because similar inorganic salts can precipitate in the VMs at low temperature. These precipitations can block small blood vessels during VM perfusion of organs. RPS-2 or LM5 carrier solutions form precipitations in 70% CI-VM-1 at 0°C . For this reason, CI-VM carrier solution is formulated so as not to contain any agents that can form precipitation. It is not a suitable cold storage solution because it was specially designed for 60-70% VMs like CI-VM-1 to make them stable.

CI-VM carrier solution has a very simple composition, namely 28 mM/L potassium chloride, 230 mM/L glucose, and the 10 mM/L organic TRIS – HCl buffer.

It has also been found that an optimal procedure for saturation of whole post-mortem brains and heads through the vascular system with CI-VM-1 solutions should be performed in three steps: (1) 10% EG at 0°C, (2) 30% EG at -5°C, and then (3) 70% CI-VM-1 consisting of 35% EG plus 35% DMSO at -15°C in order to decrease perfusion time and CPA toxicity. It is preferable to use 70% CI-VM-1 instead of 65% in order to accelerate saturation of cerebral tissues with VM-1 and to achieve 60-65% concentration in the tissues in a shorter time period.

VITRIFICATION OF THIN SLICES OF CEREBRAL TISSUES

Thin rat hippocampal slices allow for the use of faster cooling and warming rates to achieve vitrification and avoid devitrification. The faster the rates that can be used, the lower the VM concentrations which are required to get vitrification and to avoid devitrification during warming. 48-55% VMs require cooling rates of about 30°C/min to resist ice crystallization and to achieve a vitreous state. Warming rates faster than 80°C/min must be used to avoid devitrification of 48-55% VMs. These rates can be reached for small rat cerebral slices, but not for rat heads or larger biological objects.

Proper composition of CI-VM-1, exposure time and temperature, as well as cooling and warming rates were found for vitrification of rat hippocampal slices to give survival of up to 100% the survival seen for controls.

These compositions of CI-VM-1 and these procedures can be used for low temperature banking of cerebral slices with recovery of their functional activity. There are currently no banks of cerebral slices.

The scientific literature contains only two articles describing the use of EG plus DMSO solutions. The first article (A. M. Saeed et al, *Theriogenology*, vol. 54, 2000, pp. 1359-1371) was devoted to vitrification of cumulus cells using 3.58M (22%) EG plus 2.82M (18%) DMSO. The second article (N. Oberstein et al, *Theriogenology*, vol. 55, 2001, pp. 607-613) reported about the use of 17.5% EG plus 17.5% DMSO for vitrification of equine embryos. However, such low concentration VMs and carrier solutions were designed specifically for those cells and are therefore ineffective for vitrification of whole brains or cerebral tissues.

EXAMPLE 1

Toxicity of individual cryoprotective agents for rat cerebral tissue

TABLE 1.

Toxicity of most known and usable CPAs for rat cerebral tissue.

CPA	% survival
ethylene glycol	66.3±10.4
glycerol	60.0±4.8

1,2 propanediol	31.5±14.8
dimethylformamide	25.9±4.0
dimethyl sulfoxide	17.9±2.6
dimethylacetamide	8.2±1.6
2-methoxyethanol	6.3±1.8
methylformamide	5.1±2.9
formamide	2.1±0.5

Experiments were performed using the same experimental conditions (15 minute exposure of the rat hippocampal slices with 60% VMs at -10°C). There was no attempt to find optimal experiment conditions for every CPA.

Methylacetamide was tested as well but its aqueous solutions in comparison with dimethylacetamide were not stable and formed precipitates during overnight storage at 0°C.

The procedure of the experiments with rat hippocampal slices have previously been described in details previously (Y. I. Pichugin et al., *Cryobiology* 52: 228-240, 2006). Survival of the hippocampal slices after treatment was expressed as a percentage of the survival of the untreated controls. This comparison standard was used for all the experiments described below.

It is clear that the less survival, the more toxic the CPA effect. Most amides and 2-methoxyethanol were very toxic for cerebral tissues.

EXAMPLE 2

Toxicity of some binary vitrification mixtures for rat cerebral tissue

TABLE 2

Toxicity of the four binary vitrification mixtures for rat cerebral tissue

VM composition	VM %	survival, %
1,2 propanediol – dimethylformamide	40 – 25	42.7±4.6
1,2 propanediol -- dimethyl sulfoxide	40 – 25	35.1±3.4
dimethylformamide -- dimethyl sulfoxide	40 – 25	28.8±4.5
ethylene glycol – glycerol	40 – 25	52.6±4.0

The experiments were performed using the same experimental conditions (25 minute exposure of the rat hippocampal slices with 65% VMs at -20°C).

These VMs yielded worse results than the best VM containing EG and DMSO.

Vitrification systems such as amide – DMSO – EG – PG were not explored in details because Fahy and Wowk have already studied this system (The US patent 6,395,467).

EXAMPLE 3

A new saturation and washout protocol

Fahy and et al used the method of gradual increases and then decreases of all components of vitrification mixtures as X, 1/2X, 1/4X, 1/8X, 1/16X, where X was full strength of VM (full concentration of all components of VM). In detail, steps for introducing and removing vitrification solutions were: 1/16th of full strength; 1/8th of full strength; 1/4th of full strength; one-half of full strength; full strength (X) vitrification solution; 1/2X+300 mM mannitol; 3/8X +300 mM mannitol; 1/4X+300 mM mannitol; 1/8X+300 mM mannitol; 1/16X+300 mM mannitol; 0X+300 mM mannitol; ordinary vehicle.

TABLE 3

 Protocol 1 of exposure of rat hippocampal slices with CI-VM-1

Steps of saturation of hippocampal slices with CPA solutions

% CPA	1/16X→1/8X→1/4X→1/2X→X→				
step time	10	10	10	10	20
temperature	10	0	0	-7	-20

Steps of washout of hippocampal slices from CPAs

	→1/2X→+1/4X→+1/8X→+1/16X→+				
step time	10	10	10	10	10
temperature	-20	-7	0	10	10

 Where: X was 65% CI-VM-1 (35% EG plus 35% DMSO). The other solutions were diluted CI-VM-1 solutions. + means the use of 0.3 mole/liter Mannitol to avoid edema of the cerebral slices. Mannitol was usually included in the washout media to avoid osmotic shock. "Step time" means duration of steps in minutes. Each step was 10 min except for X step, which was 20 min. Temperature was in °C.

TABLE 4

 Protocol 2 of exposure of rat hippocampal slices with CI-VM-1

Steps of saturation of hippocampal slices with CPA solutions

% CPA	EG (4.1%→8.2%→16.3%→32.5%)→VM→				
step time	10	10	10	10	20

temperature	10	0	0	-7	-20
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Steps of washout of hippocampal slices from CPAs

% CPA	→+EG(32.5%→16.3%→8.2%→4.1%)→+				
step time	10	10	10	10	10
temperature	-20	-7	0	10	10

Where: VM was 65% CI-VM-1 (35% EG plus 35% DMSO). Other solutions were EG solutions. + means the use of 0.3 mole/liter Mannitol to avoid edema of the cerebral slices. "Step time" means duration of steps in minutes. Each step was 10 min except for X step, which was 20 min. Temperature was in °C.

TABLE 5

Survival of rat hippocampal slices after the use of the Protocols 1, 2

	Survival, %
Protocol 1	65±5
Protocol 2	81±4

EXAMPLE 4

Ethylene glycol – dimehtyl sulfoxide vitrification mixtures

The saturation and washout protocol 2 was used in all the experiments.

TABLE 6

Survival of rat hippocampal slices before and after cooling

VM composition	survival before cooling, %	survival after cooling, %
25% EG, 40% DMSO	65±7	63±6
27% EG, 38% DMSO	73±6	69±5
32.5% EG, 32.5% DMSO	80±8	81±4

45% EG, 20% DMSO	87±2	73±3
50% EG, 15% DMSO	82±2	56±8

 Survival before cooling reflected CPA toxicity. Survival after cooling reflected CPA cryoprotection activity, which was the final result of the entire cryopreservation.

The EG – DMSO VM (50% -- 20%) demonstrated less stable vitrification than the EG – DMSO VM (45% -- 15%) did. A higher percentage of EG in VMs increased their viscosity. Higher percentage of DMSO in VMs decreased their viscosity but increased their toxicity.

EXAMPLE 5

A new criterion of determination of vitrification stability

Experiments with slow cooling (to -130°C) and warming of 20 ml glass vials with 55%, 60% or 65% CI-VM-1 were performed. The recorded cooling rates (3°C/min to 0.1°C/min) did not play a role in vitrifying these solutions. The recorded warming rates (8°C/min to 0.1°C/min) were important to avoid devitrification for just 55% VM-1 but not for 60% and 65% CI-VM-1. The critical warming rates were 0.5-0.1°C/min. This means that devitrification of 55% CI-VM-1 was observed at warming rates of 0.5-0.1°C/min. The beginning of devitrification for 55% VM-1 was approximately at -115°C. It is very important that there was no devitrification for vitrified 60% or 65% VM-1 even at a warming rate 0.1°C/min.

Biological tissues contain natural nucleators. Simulation of cerebral tissues was performed. Experiments with dry ice (-78.5°C) storage of 20 ml of 65% CI-VM-1 with homogenized rat brain tissues containing natural nucleators were conducted. There were no visible ice crystals observed in the solutions during the 14 day observation period.

45%, 55%, 60%, and 65% CI-VM-1 solutions were tested. 45% solution showed massive ice crystallization in 5 minutes after cooling to -78.5°C. 55% solution showed ice crystallization in 3 hours after cooling to -78.5°C. The ice crystals very slowly grew from nucleators. There were no ice crystals observed in 60% CI-VM-1 solutions during a 7 day observation period. Visible ice crystals in 60% solutions were observed only after 21 days of storage. There were 7-10% visible ice crystals in 60% VM-1 solutions. There were no visible ice crystals in 65% solutions after 21 days of storage.

The stability of vitrification of large volumes of CI-VM-1 was determined. Experiments with storage of unfiltered 2 liters of 65% CI-VM-1 at -78.5°C for 6 days (144 hours) were performed. There were no visible ice crystals in 2 liters of 65% VM-1.

These criteria were verified using slow cooling of 2 liters of 65% CI-VM-1 to -130°C and slow warming from -130°C to -30°C. There was no devitrification of this vitrified solution even with the very slow warming rate of 0.05°C/min. 65% CI-VM-1 in large volume demonstrated very stable vitrification.

Ice crystallization starts with formation of crystals that are too small to be visible. These tiny crystals damage cells if they form inside the cells. In vitrification solutions, the invisible crystals may grow very slow and so they can remain invisible for a long time. Thus, a final verification of the criterion is testing VMs on living tissues because they are very sensitive even to invisible ice crystals.

Experiments with vitrification of rat hippocampal slices in comparison with dry ice storage were performed (Table 7).

TABLE 7

Comparison of vitrification of rat cerebral tissues with their dry ice storage

	survival, %
Vitrification	79.0±7.0
48 hour dry ice storage	81.0±4.3
14 day dry ice storage	44.3±3.1

The decrease in slice survival started only after their 48 hour dry ice storage.

EXAMPLE 6

The vitrification method with rapid cooling and warming rates

The rat hippocampal slices were exposed with various modifications of VM-1 and quickly cooled to -130°C.

TABLE 8

Survival of rat hippocampal slices after using the vitrification method

VM composition	VM %	survival, %
27.5% EG, 27.5% DMSO	55	87.3±7.8
25% EG, 25% DMSO	50	92.7±4.2
32% EG, 16% DMSO	48	97.6±3.8
40% EG, 8% DMSO	48	48.2±5.2

The procedure of the experiments with rat hippocampal slices has previously been described in detail (Y. I. Pichugin et al., Cryobiology 52: 228-240, 2006).

The disclosure of the foregoing article is incorporated herein by reference. It did not make sense to study 48% VMs with DMSO content more than 28% because they are rather more toxic than 25% EG plus 25% DMSO VM.