PPA: Method of Cryopreservation of Whole Brains

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Provisional Patent Application

Title:
Method of Cryopreservation of Whole Brains

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Reference Documents:

US PATENT DOCUMENTS
5,592,168 Sep., 1999 Wowk, et al
6,395,467 May, 2002 Fahy, et al.

FIELD OF THE INVENTION

The field of the invention is cryobiology and cryonics.

BACKGROUND OF THE INVENTION

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. Tissues cannot be successfully 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 -130ºC or below without ice formation.

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 cryopreservating 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 preservation of renal tissues and blood vessels. G. M. Fahy et al. developed vitrification mixtures having low toxicity 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 rat thin 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.

One of main problems of cryopreservation of whole brains is their severe dehydration during VM perfusion. All CPAs have much slower penetration rates through the intact brain blood barrier than water has. Water leaves the brain much more rapidly than CPAs enter into the brain during CPA perfusion. As a result, severe dehydration of the brain occurs during perfusion. Severe dehydration is a very harmful factor because it dramatically increases CPA toxicity. CPAs penetrate into thin cerebral slices by simple diffusion and so the slices do not experience severe dehydration.

BRIEF SUMMARY OF THE INVENTION

The invention provides a method that was specially designed for vitrifying whole brains. All the known freezing or vitrification methods result in very poor survival of cerebral cells of whole brains after their cryopreservation. This new method can significantly increase survival of cerebral cells. This method will be referred to as the CI (Cryonics Institute) cryopreservation method.

DETAILED DESCRIPTION OF THE INVENTION

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). Freezing methods have not been effective for cryopreserving cerebral tissues. 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

Study of the vitrification method was begun by testing the toxicity of the best individual CPAs on rat hippocampal slices. 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.

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 0ºC. 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 0ºC. Melting points of EG solutions prepared in CI-VM vehicle solution were -

4.3ºC for 10%, -9.5ºC for 20%, -14ºC for 30%, and -23.5ºC for 40%. To decrease toxicity of more toxic secondary CPAs, cerebral slices should be exposed with them at temperatures lower than 0ºC. Saturation of the slices with 30% EG or 40% EG allowed combining with secondary CPAs at -14ºC or -23ºC, 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. This solution was named CI-VM-1 (the Cryonics Institute Vitrification Mixture one). CI-VM-1 can cryopreserve cerebral slices with up to 100% survival using 48-50% CI-VM-1 for fast cooling and warming rates and with 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.

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.

It is very important for perfusion of whole human brains to take into account viscosity of VMs and permeation of CPAs though 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. 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.

A most significant problem of cryopreservation of whole brains is their severe dehydration during VM perfusion. All CPAs have much slower penetration rates through the intact brain blood barrier (BBB) than water has. Water leaves the brain much more rapidly than CPAs enters into the brain during CPA perfusion. As a result, severe dehydration of the brain occurred during perfusion. Severe dehydration is a very harmful factor because it dramatically increases CPA toxicity. CPAs penetrate into thin cerebral slices by simple diffusion and so the slices have no severe dehydration.

THE STUDY OF DETERGENTS AS MODIFIERS OF THE BLOOD BRAIN BARRIER

There is great benefit to be gained by the use of BBB modifiers. For example, 30% ethylene glycol (EG) is not toxic for cerebral tissues under certain conditions. Rat

hippocampal slices exposed to 30% EG at 0ºC had 100% survival according to the K/Na ratio assay. Rat brains that were perfused with 30% EG at 0ºC without BBB modifiers had only around 40% survival. The brains had about 35% dehydration. When modifiers of the rat BBB were used, the brains retained their normal volumes during perfusion. As a result, the rat brains that were perfused with 30% EG at 0ºC with proper concentration of BBB modifiers had 100% survival.

The main task was to modify the BBB so that the brain retains its normal volume during VM perfusion. We studied twelve compounds of the detergent chemical class. Four of twelve compounds showed desirable activity. We describe them as modifiers of the blood brain barrier or BBB modifiers.

There is no good theory of the effect of detergents on biological membranes, cells or the BBB. Researchers use empirical methods for selection of the best detergents for their purposes.

Rats were used to test all the detergents. However, the better BBB modifiers were also tested on sheep brains. The sheep brains retained volume during VM perfusion as well as the rat brains did. The best one of the four good modifiers was selected for this new CI cryopreservation method.

All the known cryopreservation methods result in very poor survival of cerebral cells of whole brains after their cryopreservation. This new CI method significantly increases

survival of cerebral cells. The proper use of the method may preserve about 80% of cells after cryopreservation of whole brains.

EXAMPLE 1

Toxicity of individual cryoprotective agents for rat cerebral tissue were studied.

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-mehtoxyethanol	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 0ºC.

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 were studied.

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 detail because Fahy and Wowk have already studied this system (The US patent 6,395,467).

EXAMPLE 3

Ethylene glycol – dimethyl sulfoxide vitrification mixtures were studied.

TABLE 3

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 4

The influence of various detergents on rat brain volume was studied.

TABLE 4 The results of the experiments with various detergents

	I optimal concentrations in			
	I m-RPS-2	VM-1 solutions	Uniformity	Reproduction
Anionic				
1. SDS	0.01%	---	even	normal
2. SDBS	0.006%	0.002-0.004%	even	medium
3. SC	0.5%	0.3-1.2%	even	normal

4. SDC	0.06%	0.05-0.2%	even	normal

Cationic				
5. CPC	0.01%	---	uneven	medium
6. CTAB	0.01%	0.005-0.02%	uneven	bad

Non-ionic				
7. Triton X100	no	---	even	normal
8. Tweens	no	---	even	normal
9. Pluronic	no	---	even	normal
Amphoteric				
10. CHAPS	0.2%	0.01-0.04%	uneven	bad

Notes: 1. Optimal concentrations of detergents in m-RPS-2 solution represent the first type of experiments. The most powerful detergent is SDBS because it can protect rat brains against dehydration at the lowest concentration. The least powerful detergent is SC because it requires the highest concentration to protect rat brains against dehydration.

2. Optimal concentrations of detergents in VM-1 solutions solution represent the second type experiments.

3. “Uniformity” means the uniformity of saturation of rat brains with VM-1. “Even” means even, uniform saturation of the rat brains with VM-1. “Uneven” means uneven, non-uniform saturation of the rat brains with VM-1. It is a very important indicator. Detergents with uneven VM perfusion are not good detergents for this application.

4. “Reproduction” means the reproduction of the results of the experiments. It also is an important indicator.

The best detergents for our purposes are SDC and SDBS. SC is a good detergent, but it is much less powerful detergent than SDC detergent.

SDS and CPC do not have sufficient solubility in m-RPS-2 or in VM-1 solutions at 2-4ºC. CTAB and CHAPS gave uneven VM saturation of the brains.

EXAMPLE 5

The influence of SDBS detergent on cerebral cell survival was studied.

TABLE 5 Survival of hippocampal slices prepared from rat brains perfused with 65% VM-1 solutions with various concentrations and amounts of SDBS detergent at 2-4ºC

Concentration, %	m	Survival before cooling, %	Survival after warming, %
0.007	1.00	79.1±4.5	21.6±3.9
0.007	1.25	46.9±4.9	43.5±5.9
0.007	1.50	31.7±2.7	34.5±1.8
0.010	1.00	80.0±5.7	
0.012	1.00	36.7	
0.0035	1.00	36.9±5.4	

Where: Concentration, % is concentration of SDBS in 15% EG;

m is ratio of weight of 65% VM-1 to weight of the rat upper part;

Survival before cooling, % reflects Toxicity;

Survival after warming, % reflects Cell Survival after full steps of cryopreservation of the rat brain;

From the Table 5, one can conclude that:

1. The optimal ratio of the weight of 65% VM-1 to the weight of the rat upper part was 1.25 to obtain maximal cell survival after cryopreservation. VM-1 concentration in the rat brains was probably 57% to 60%. VM-1 concentration in the rat brains with ratio 1.50 was probably 60% to 65%. VM-1 concentration in the rat brains with ratio 1.00 was probably less than 55%-57% because vitrification was not stable.

2. Optimal concentrations of SDBS are 0.007% to 0.010%. Concentrations were only compared for ratio 1.00.

3. Decreasing 0.007% concentration of SDBS two times decreased cell survival about two times.

The influence of SDC detergent on cerebral cell survival was studied.

TABLE 6 Survival of hippocampal slices prepared from rat brains perfused with 65% VM-1 solutions with various concentrations and amounts of SDC detergent at 2-4ºC

Concentration, %	m	Survival before cooling, %
0.10	1.00	67.7±11.0
0.15	1.00	24.5±3.2
0.05	1.00	28.9±2.1

Where: Concentration, % is concentration of SDBS in 15% EG;

m is ratio of weight of 65% VM-1 to weight of the rat upper part;

Survival before cooling, % reflects Toxicity;

From Table 6, one concludes that optimal concentrations of SDC are 0.1% to 0.13%. Decreasing 0.1% concentration of SDC two times decreased cell survival about two times.

It was not necessary to perform full cryopreservation steps for SDC because it is enough to have toxicity data to compare SDC with SDBS.

CLAIMS

We claim:

1. Anionic detergents such as sodium dodecylbenzenesulfonate (SDBS), sodium deoxycholate (SDC), sodium dodecylsulfonate (SDS), sodium oxycholate (SO), and others can be used to modify the brain blood barrier for elimination of severe brain dehydration during cryoprotectant perfusion.

2. Anionic detergents of claim 1, wherein the preferable detergents are SDBS and SDC with the optimal concentrations 0.007% to 0.010% for SDBS and 0.08% to 0.12% for SDC.

3. Anionic detergents of claim 2, wherein SDBS or SDC can be used at the perfusion step with 15% of a cryoprotective agent at 0ºC to avoid severe edema of non-cerebral tissues.

4. Anionic detergents of claim 2, wherein the preferable vitrification mixture consists of ethylene glycol and dimethyl sulfoxide.

ABSTRACT

A method for cryopreserving whole brains by vitrification, which is a method of preventing severe brain dehydration and ice formation. The method includes the use of the modifiers of the brain blood barrier during the saturation of cerebral tissues with cryoprotective agents and cooling of the saturated tissues to negative (-) 120ºC. The CI cryopreservation method is effective for all vitrification mixtures, but the preferred vitrification mixture contains ethylene glycol and dimethyl sulfoxide. To modify the brain blood barrier for elimination of severe brain dehydration during perfusion, some detergents were used. The preferable modifiers of the brain blood barrier are sodium dodecylbenzenesulfonate and sodium deoxycholate. Concentration of sodium dodecylbenzenesulfonate can be varied from 0.007% to 0.010%. Concentration of sodium deoxycholate can be varied from 0.08% to 0.12%.