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Improved vitrification solutions based on the predictability of vitrification solution toxicity☆,☆☆

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Abstract

Long-term preservation of complex engineered tissues and organs at cryogenic temperatures in the absence of ice has been prevented to date by the difficulty of discovering combinations of cryoprotectants that are both sufficiently non-toxic and sufficiently stable to allow viability to be maintained and ice formation to be avoided during slow cooling to the glass transition temperature and subsequent slow rewarming. A new theory of the origin of non-specific cryoprotectant toxicity was shown to account, in a rabbit renal cortical slice model, for the toxicities of 20 vitrification solutions and to permit the design of new solutions that are dramatically less toxic than previously known solutions for diverse biological systems. Unfertilized mouse ova vitrified with one of the new solutions were successfully fertilized and regained 80% of the absolute control (untreated) rate of development to blastocysts, whereas ova vitrified in VSDP, the best previous solution, developed to blastocysts at a rate only 30% of that of controls. Whole rabbit kidneys perfused at −3 °C with another new solution at a concentration of cryoprotectant (8.4 M) that was previously 100% lethal at this temperature exhibited no damage after transplantation and immediate contralateral nephrectomy. It appears that cryoprotectant solutions that are composed to be at the minimum concentrations needed for vitrification at moderate cooling rates are toxic in direct proportion to the average strength of water hydrogen bonding by the polar groups on the permeating cryoprotectants in the solution. Vitrification solutions that are based on minimal perturbation of intracellular water appear to be superior and provide new hope that the successful vitrification of natural organs as well as tissue engineered or clonally produced organ and tissue replacements can be achieved.

Keywords: Cryoprotective agents; Organ preservation; Engineered tissues; Tissue banking; Dimethyl sulfoxide; Formamide; Ethylene glycol; Ice blockers; Polyvinyl alcohol; Polyglycerol; Polyvinylpyrrolidone; LM5; TransSend; X-1000; VM3; 9v; Z-1000

The medical need for cell, tissue, and organ replacements is immense [30,33] and can only be expected to increase as world populations age. Several biotechnological approaches to alleviating current and projected shortages in the supply of needed replacements are currently being explored [9,10,31,32], but little attention has been focused

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on the critical problem of inventory control of the more complex of these perishable laboratory-produced replacements. Vitreous state or ice-free cryopreservation below the glass transition temperature is becoming increasingly recognized as the most likely solution to the inventory control problem [24,28,43] due to its ability to circumvent mechanical damage from ice formation [12,14,17,20,25,40,44]. However, it is apparent that despite some encouraging progress toward the demonstration of successful organ vitrification using conventional cryoprotectant technology [15,28,43], less toxic vitrification solutions than those reported to date are still required [3,4,29]. Presently, it appears that cryoprotectant toxicity is the single most important barrier to successful vitreous preservation of complex, spatially extended living systems.

The mechanisms of toxicity of vitrifiable solutions have not been elucidated [19]. In part for this reason, it is not presently possible to predict the toxicity of either individual cryoprotective agents or mixtures thereof, and there is a virtually unlimited number of possible mixtures to choose from in composing candidate vitrification solutions. It would therefore be of considerable practical utility to have a simple method for predicting the toxicity of a complex mixture of highly concentrated cryoprotectants from first principles.

In the present contribution we show that a simple new compositional variable (qv^*) can rationally account, in an organized mammalian tissue, for the toxicity of many complex cryoprotectant mixtures composed to be at total concentrations that are just sufficient to permit vitrification at slow cooling rates at both ambient and elevated pressures. This new compositional variable is proposed to reflect the strength of cryoprotectant hydration within the solution. Based on this interpretation, we were able to predict and successfully test several superior new vitrification solutions with low toxicity for mouse ova, kidney slices, whole rabbit kidneys, and other sensitive systems. These results provide substantial new support for the possibility of developing successful methods for the long-term banking of medically needed tissue and organ replacements.

Materials and methods

All vertebrate animal use was conducted with the approval of the institutional animal care and use committees of 21st Century Medicine or of the University of Wales. All procedures performed at 21st Century Medicine were in compliance with current USDA and NIH guidelines.

In all solutions described below, cryoprotectants were present in place of water, so that the molar concentrations of other components were not altered by the presence of the cryoprotectants.

Rabbit renal cortical slices

Slice preparation, cryoprotectant treatment, and determination of K^+/Na^+ ratio were all essentially as described elsewhere [11,16,18]. The protocols selected for cryoprotectant addition and washout are believed not to introduce any injury attributable to osmotic forces [11,18,19], and therefore all injury reported is believed to reflect the intrinsic physicochemical effect of the solutions being tested. The solutions whose effects are described in Figs. 1–4 are described in Table 1. Each of these solutions had a concentration equivalent to the threshold concentration required for vitrification (C_V) of that particular formula at an applied pressure of 1000 atm in the presence of an RPS-2 physiological support solution [16,18]. All toxicity testing involving these solutions was conducted at 1 atm. The solutions retested in Fig. 3 were solution numbers 1–2, 4–5, and 7–13 from Table 1. For all kidney slice figures each point generally represents the mean of two replicate groups of six slices each ($n = 12$ for each point, in total). Normalized K^+/Na^+ equals 100% times the raw K^+/Na^+ ratios of the treated slices divided by the mean K^+/Na^+ ratio of the untreated control slices. For the experiments shown in Figs. 5 and 6, all solutions were vitrifiable at ambient pressure when cooled at $\leqslant 10$ °C/min. The exposure time to each full-strength vitrification solution was 40 min in all slice figures except for Fig. 5, in which the exposure time was 30 min. The physiological support or carrier solution used in Fig. 5 was LM5, which is equivalent to RPS-2 [16,21] in which the glucose concentration has been reduced to 90 mM

Table 1
Composition and properties of 15 model vitrification solutions

Solution number	Solution name[a]	Total % w/v	Gram quantities per deciliter of:[b]							Density (grams/ml)	qv^*	K^+/Na^{+c}
			D	F	A	E	P	PVP	PEG			
1	D + PVP	47	41	0	0	0	0	6	0	1.0775	6.02	0.85 ± 0.06
2	$D(2)FP_{10}$ + PVP	49	25.62	7.38	0	0	10	6	0	1.0718	3.28	3.49 ± 0.22
3	$D(1)FP_{10}$ + PVP	50.5	21.88	12.62	0	0	10	6	0	1.0716	2.66	4.13 ± 0.15
4	$D(.8)FP_{10}$ + PVP	51	20.34	14.66	0	0	10	6	0	1.0749	2.49	4.22 ± 0.21
5	$D(1)AP_{10}$ + PEG	46	17.08	0	12.92	0	10	0	6	1.0592	3.39	3.51 ± 0.20
6	$D(.8)AP_{10}$ + PEG	47.5	16.19	0	15.31	0	10	0	6	1.0605	3.07	3.31 ± 0.18
7	$D(3)EP_{10}$ + PEG	49	26.09	0	0	6.91	10	0	6	1.0703	3.67	2.07 ± 0.12
8	$D(1)EP_{10}$ + PEG	49	18.39	0	0	14.61	10	0	6	1.0701	3.10	2.89 ± 0.13
9	$D(.8)EP_{10}$ + PEG	48	16.06	0	0	15.94	10	0	6	1.0698	3.12	3.30 ± 0.12
10	$D^1E^2P^1$ + PVP	48	10.50	0	0	21.00	10.50	6	0	1.0702	2.82	3.41 ± 0.11
11	E + PVP	50	0	0	0	44.00	0	6	0	1.0770	2.11	3.06 ± 0.08
12	P + PVP	42	0	0	0	0	36.00	6	0	1.0503	3.47	2.96 ± 0.13
13	D^1P^1 + PVP	46	20.00	0	0	0	20.00	6	0	1.0640	4.02	2.34 ± 0.13
14	E^1P^1 + PVP	46	0	0	0	20.00	20.00	6	0	1.0619	2.67	2.92 ± 0.08
15	$(D(1)A)^1P^1$ + PVP	45	11.10	0	8.40	0	19.50	6	0	1.0572	3.36	3.05 ± 0.16

Notes. These solutions vitrify upon cooling at about 10 °C/min at 100 MPa [18]. The C_V of solution 6 was interpolated. For solution 3, the tested (estimated) C_V was higher than the actual C_V by 1.5% w/v permeating cryoprotectants.

[a] Nomenclature: A number in parentheses refers to the mole ratio of the cryoprotectant before the parentheses to the cryoprotectant after the parentheses (for example, D(2)F means that there are 2 mol of dimethyl sulfoxide (D) for every mole of formamide (F)). Subscripted numbers refer to absolute concentrations in % w/v units. For example, P_{10} means that there are 10 g/dl of propylene glycol (P). Superscripts refer to weight ratios between cryoprotectants. For example, $(D(1)A)^1P^1$ means that for every gram of D(1)A, there is 1 g of P present in the solution.

[b] Abbreviations: D, dimethyl sulfoxide; F, formamide; P, 1,2-propanediol (propylene glycol); A, acetamide; E, ethylene glycol; PVP, polyvinylpyrrolidone K30 ($M_r \sim 40{,}000$ Da); PEG, poly(ethylene glycol) of mean molecular mass 8000 Da. $qv^* = M_W/M_{PG}$, where M_W is the molarity of water, M_{PG} is the molarity of polar groups on permeating cryoprotectants, and this ratio is obtained when the vitrification solution is at C_V as defined under standardized conditions (e.g., at a constant cooling rate).

[c] For data set shown in Figs. 1 and 2: mean ± 1 SEM.

and 45 mM lactose and 45 mM mannitol have been added (patent pending). For the experiments of Fig. 6, the carrier solutions for panels A, B, and C, were respectively LM5, MHP-2 [21], and RPS-2 [16,21].

Estimation of solution water content

To obtain the data for Fig. 4, solution water content was determined in two independent ways. First, it was calculated by assuming that all solution components occupy the same volume in solution as they occupy in the pure state. By subtracting the sum of all component volumes other than water, including the volume occupied by RPS-2 solutes, from the known total volume of the solution, the volume of water was estimated, and this estimated volume was then equated with the mass of water in the solution assuming 1 ml of water = 1 g of water. Information used for these calculations is provided in Table 2. Second, water content was determined by weighing the complete solution (tabulated densities in Table 1 represent the weight of 100 ml of solution divided by 100) and subtracting the known weights of all components other than water to yield the weight of water in the solution. The consequences of using both

Table 2
Some nominal component volumes for predicting qv^*

Component	Component volume
Dimethyl sulfoxide	71.03 ml/mol
Ethylene glycol	55.77 ml/mol
Propylene glycol	73.17 ml/mol
Formamide	39.72 ml/mol
Acetamide	50.92 ml/mol
PEG 8000	0.827 ml/g
PVP K30	0.782 ml/g
RPS-2 components	2.00 ml/dl of solution

methods of determining water content were then compared (Fig. 4).

Mouse ova experiments

Virgin 6- to 8-week-old CBA/Ca × C57BL/6 female mice were superovulated [38] and euthanized [38], and the oviducts were removed. Oocytes surrounded by the cumulus mass were released from oviducts into 150 IU/ml hyaluronidase (Sigma, UK) contained in phosphate-buffered medium plus 4 mg/ml bovine serum albumin [38] to remove the cumulus cells. Cumulus-free oocytes were rinsed twice in PBF (phosphate-buffered medium plus 5% fetal bovine serum). Oocytes from the same cohort were distributed to control or experimental groups.

VSDP (6 M dimethyl sulfoxide plus 1 mg/ml PEG, M_r 8000), was made up in PBF. VM3 (patent pending) consisted of 16.84% w/v ethylene glycol, 12.86% w/v formamide, 22.3% w/v dimethyl sulfoxide [21], 7% w/v PVP K12 (polyvinylpyrrolidone of $M_r \sim 5000$ Da), and 1% w/v final concentrations of commercially available Superol X-1000 [45] and Supercool Z-1000 [46] ice blockers in PBF at a pH (as estimated with a standard pH electrode) of 8.0. X-1000 was heated briefly to 80 °C to make it visibly less cloudy before use. From VSDP 25 and 65% dilutions and from VM3 9.2, 26, and 90% dilutions were made using PBF. A 1 M sucrose solution for dilution was also made up in PBF.

All addition steps were performed at room temperature (~20 °C). Plastic insemination straws were prepared with a 4-cm column of 1 M sucrose diluent [38] and a 0.5-cm column of 100% VSDP or 90% VM3, which was placed 0.5 cm from the diluent column, and held until required. Groups of 10–25 oocytes were first pipetted into a 50 μl droplet of 25% VSDP or 9.2% VM3 and held for 3 min. Next, oocytes were rinsed through a 50 μl droplet of 65% VSDP or 26% VM3 in $\leqslant$ 1 min. Finally, oocytes were pipetted into the 0.5-cm column of 100% VSDP or 90% VM3 in the insemination straw. Within 1 min, the straw was sealed with a wet plastic plug and transferred to −140 °C liquid nitrogen vapor, then held in vapor for 3 min before being plunged into liquid nitrogen for storage.

Vitrified oocytes were rewarmed in air for 10 s and then in 20 °C water for 10 s. The contents of the straw were expelled into 1 ml of 1 M sucrose and mixed well by pipetting, then the oocytes were transferred through two 50 μl droplets of 1 M sucrose, again ensuring good mixing. After a total of 2 min of exposure to 1 M sucrose in the initial dilution and initial droplet steps, the oocytes were transferred to the second droplet of sucrose and held for 3 min before two 10-min washes in 50 μl PBF, the first wash at room temperature and the second at 37 °C.

Control (untreated) oocytes were submitted directly to the IVF procedure [38]. All oocytes were assessed for morphological normalcy (based on polar body extrusion, spherical shape, and refractive cytoplasm) and fertilization at 24 h and for development to blastocysts at 5 days postinsemination.

Whole kidney experiments

New Zealand white rabbits were induced with ketamine and xylazine and maintained using isoflurane [47]. The right kidney was flushed free of blood with 100 ml of TransSend-B organ preservation solution (containing 7 mM K_2HPO_4, 18 mM tripotassium citrate, 2 mM sodium acetate, 10 mM glucose, 1.8% w/v decaglycerol, 45 mM α-lactose, 40 mM NaCl, 5 mM reduced glutathione, 1 mM adenine HCl, 8 mM sodium Hepes, 0.1% w/v chondroitin sulfate A, 9 mg/liter chlorpromazine, 16 mg/liter dexamethasone, 1 mM $CaCl_2$, and 1 mM $MgCl_2$, with a total osmolality of 298 mOsm at pH 7.4; patent pending). The kidneys were then weighed and transferred to a specially constructed, computer controlled perfusion apparatus.

Perfusion pressure was maintained at 40 mmHg at all times. Baseline vascular resistance was established during initial perfusion for 10 min with TransSend-B containing 2% w/v hydroxyethyl starch (HES, $M_r \sim 450{,}000$), then VMP solutes (VMP is equal to VM3 minus polyvinylpyrrolidone) were added at ~50 mM/min. The perfusion temperature of 3.5 °C was lowered linearly starting at 2 M VMP solutes and ending at 5 M VMP solutes and −3 °C. After 10 min of perfusion with 5 M VMP at −3 °C, full-strength (8.4 M) VMP was

perfused for 20 min at −3 °C. VMP was prepared in the ice blocker-compatible LM5 carrier solution described above without HES. Washout of VMP began by perfusing half-strength VMP plus 300 mM mannitol and 2% w/v HES for 10 min at −3 °C. The concentrations of both cryoprotectant and mannitol were then reduced gradually (at 30 and 1.5 mM/min, respectively) while proportionally raising perfusion temperature from −3 to 3.5 °C and transitioning from LM5 back to TransSend-B.

Following removal of all cryoprotectant and all but about 20 mM mannitol, the kidneys were transplanted orthotopically back to the site of the left kidney of the donor rabbit using an ultrarapid (~3 min) anastomosis technique [47], and the left kidney was discarded. The animal was closed and allowed to recover over a 2-week postoperative period, with daily charting of serum creatinine levels, other metabolites, and general well-being.

Statistical methods

Linear regressions and associated statistics were calculated using SigmaPlot (SPSS Science, Chicago, IL). Comparison of means of normally distributed groups of similar variance was done using Student's *t* test.

Results

Insight into the nature of cryoprotectant toxicity was initially sought by attempting to account for variations in toxicity between 15 different vitrification solutions on the basis of various global aspects of their compositions. Each vitrification solution was carefully composed to be within 1% w/v of its threshold concentration for vitrification (C_V) as described elsewhere [13,17,18,20] in order to permit meaningful comparisons on an equal physical basis. Fig. 1 presents a re-analysis of previously published K^+/Na^+ ratios [18,19] of rabbit renal cortical slices measured after exposure to and washout of the vitrification solutions described in detail in Table 1. Linear regression analysis revealed a paradoxical but statistically significant weak positive correlation between viability and either the total molarity or the total molality of the solution (top and middle panels, respectively). The results for another traditional measure of concentration (mole fraction) were closely similar (data not shown). Further, a

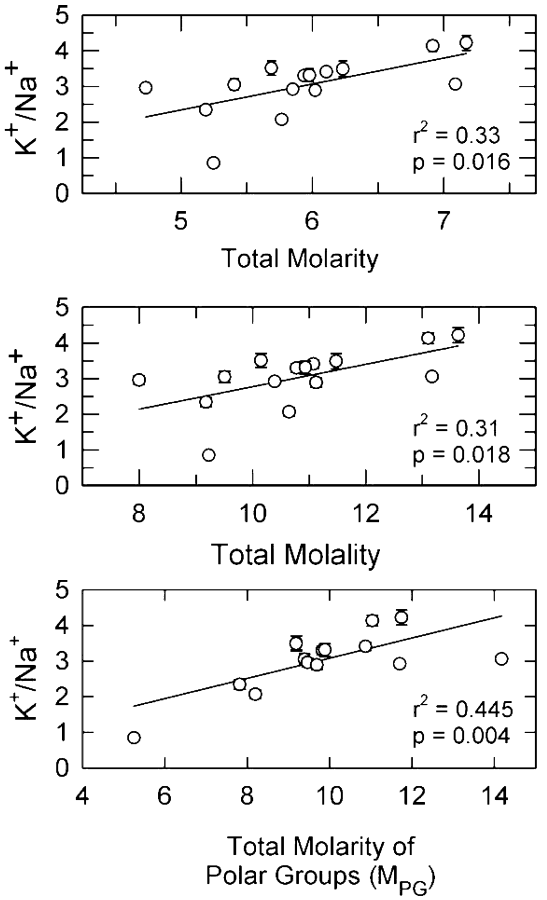

Fig. 1. Relationship between the recovery of electrolyte transport capacity after cryoprotectant washout (measured as the steady state K^+/Na^+ ratio attainable after 90 min of incubation at the optimum temperature of 25 °C) and the total concentrations of 15 vitrification solutions described in Table 1. Top panel: K^+/Na^+ ratio vs. the sum of the molarities of all permeating cryoprotectants in the solution. Middle panel: K^+/Na^+ ratio vs. the sum of the molalities of all permeating cryoprotectants in the solution. Lower panel: K^+/Na^+ ratio vs. the sum of the molarities of all polar groups present on the permeating cryoprotectants in the solution, where the polar groups are defined as OH, S=O, C=O, and NH_2. Cryoprotectant addition and washout procedures were as previously described [18]. Means ± 1 SEM unless 1 SEM is too small to depict. Lines plotted are least squares regression lines.

considerably stronger correlation was obtained using an analogous but non-traditional measure of concentration, i.e., the total molarity of all polar groups on the penetrating cryoprotectants in the solution (M_{PG}, lower panel; p value 4-fold lower than for molarity).

These trends clearly establish that the toxicity of a vitrification solution is not caused by high concentrations of cryoprotectants per se. Instead, it is apparent that some solution property other than the concentrations of added solutes must govern toxicity. The remaining component of vitrification solutions is water. Plotting post-treatment K^+/Na^+ ratio against the molarity of water (M_W) in the solution (Fig. 2, top panel) yields a non-significant ($r^2 = 0.18$, $p > 0.05$) trend toward higher K^+/Na^+ ratios at lower solution water contents. However, factoring in the influence of both M_W and M_{PG} (Fig. 2, bottom panel) yields a highly significant ($p = 0.0001$) correlation whose significance is 40 times stronger than that of the correlation with M_{PG} alone and 140 times stronger than the correlation obtained for molarity. This result suggests that toxicity is related more to a relationship between water and the hydrogen bonding groups of cryoprotectants than to either water or hydrogen bonding group concentrations per se. Also remarkable is the fact that this particular correlation entirely accounts for the toxicity of a solution containing dimethyl sulfoxide as the only penetrating cryoprotectant (point with the lowest K^+/Na^+ ratio), whereas all previous attempts to correlate toxicity with solution composition have shown this solution to be the most deviant outlier [18,19].

To verify reproducibility of these observations, 11 vitrification solutions were selected from the original 15 primarily on the basis of having non-clustered K^+/Na^+ ratios, and the effects of these solutions were retested on rabbit renal cortical slices using the same exposure and washout protocol as in the original experiments [18]. The old and the new data sets were normalized to each group's respective untreated controls to facilitate comparison of the results. As indicated in Fig. 3, the results show that, if anything, the correlation was even stronger in the replication experiments than in the original data set, and stronger still

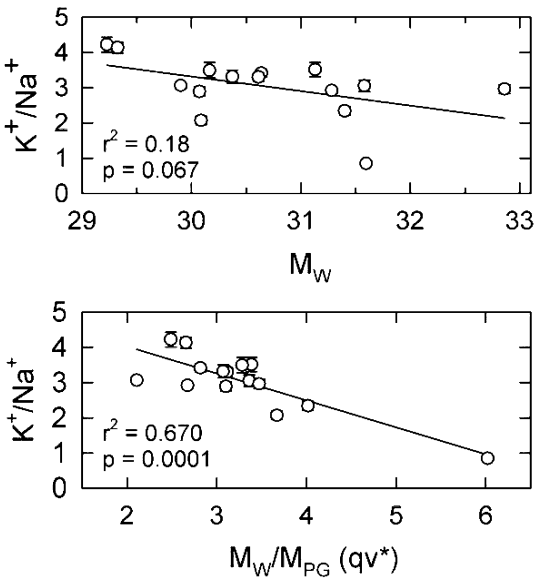

Fig. 2. The role of water content and the water content per water-bonding group in determining the effects of vitrification solutions. Upper panel: ion transport capacity as a function of the molarity of water (M_W) in the tested vitrification solutions. Lower panel: the relationship between ion transport capacity and the ratio of the molarity of water to the molarity of all polar groups on permeating cryoprotectants in the vitrification solution (M_{PG}). This ratio, when determined at C_V when C_V is defined at a standardized, slow to moderate cooling rate (about 10 °C/min in the current case) is defined as qv^*. A similar analysis that includes polar groups on the polymers in the solutions weakens the correlation obtained (data not shown).

when the two sets of data were averaged to minimize random variations. All but two (boxed points) of the 11 averaged results consistently showed deviation from the correlation of under 10%. With the exception of these outliers (whose significance is considered further below), the correlation is able to account for 95% of the variance of the group means of the pooled data set ($r^2 = 0.95$).

Based on the results of Figs. 2 and 3, the compositional variable M_W/M_{PG} appears uniquely able to account for the toxic effects of a variety of complex mixtures of cryoprotectants when this variable is defined under meaningful conditions. For this reason, the new compositional variable as properly defined was given a unique name, qv^* (qv star), wherein q refers to the quotient M_W/M_{PG} by analogy with previous notation [13,20], v refers to

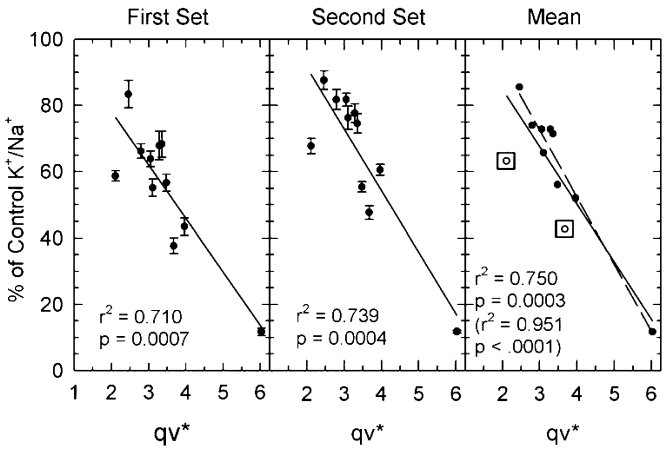

Fig. 3. Reproducibility of the correlation between K^+/Na^+ ratio and qv^* for 11 different vitrification solutions (solutions 1–2, 4–5, and 7–13 of Table 1). The left panel recapitulates K^+/Na^+ data collected originally in Maryland in 1986, while the middle panel shows data collected in California in the year 2000. The right panel shows the average of these two data sets. The boxed points in panel C are results that deviated from the regression line by more than 10% in both data sets. The leftmost boxed point is solution 11 of Table 1, and the second boxed point is solution 7. Except for these two solutions, the linear regression for the pooled results (dashed line) explains 95% of the variance of the data ($r^2 = 0.95$).

the fact that the variable pertains to solutions at their C_Vs, and the asterisk refers to the fact that C_Vs are to be determined at a standard slow cooling rate (in this case, ~10 °C/min) [13,20], which is both more physically meaningful and more pertinent for organ-sized systems than the ultrarapid rates that are usually employed for vitrification of small samples. As demonstrated in Fig. 4, qv^* can be estimated from known C_V values with sufficient accuracy by using handbook data for component densities to calculate water content and thus M_W (see Table 2 for typical reference data). In other words, for all practical purposes, solute volumes in solution can be assumed to be the same as they are in the pure state, any errors being insignificant for purposes of calculating qv^*. This provides a convenient alternative to determining water content by weighing the solution and subtracting the masses of all other components, particularly for retrospective analysis of previously known solutions.

qv^* is a measure of the glass-forming efficacy of the cryoprotectants that compose the vitrification solution. If the value of qv^* is low, only a relatively small number of water molecules can be restrained from crystallization per hydrogen bonding group, which means that the average glass-forming tendency of the polar groups in the solution is weak. Based on the evidence from Figs. 1–3, it appears that weak glass formers favor higher viability. On this basis, new vitrification solutions were devised that were predicted to have lower toxicities than previously known solutions.

To provide a point of reference, a new test solution was prepared by analogy to the standard vitrification solution referred to variously as VS41A [16,36] or VS55 [7,8,24,42]. VS41A was modified by substituting an equal mass of the weak glass former, ethylene glycol, for the strong glass-former, propylene glycol, normally present in VS41A. As shown in Table 3, the resulting solution (referred to as V_{EG}) was both more concentrated than and less toxic than VS41A, in accordance with prediction. Next, the components of V_{EG} were proportionally increased in concentration by a total of 2% w/v to attain C_V, and the proportion of ethylene glycol to other components was varied to enable toxicity to be plotted as a function of qv^*. When renal cortical slices were exposed to these new solutions, their recoveries were found once again to be directly related to qv^* (Fig. 5; $r^2 = 0.873$; $p = 0.013$), and a substantial advantage over VS41A persisted despite the considerably higher concentrations of these solutions.

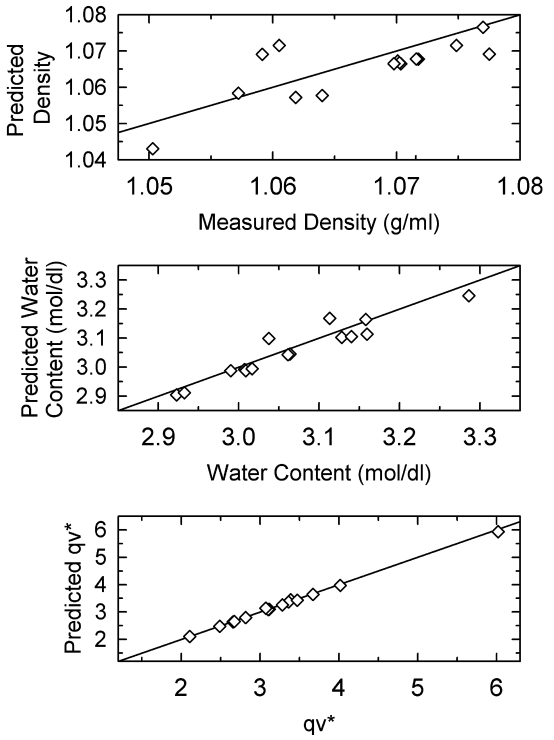

Fig. 4. Predictability of qv^* from handbook data. Accurate determination of qv^* requires a knowledge of the water content of the solution. This can be determined by measuring the density of the solution, multiplying by the volume of the solution, and subtracting the masses of all components in the solution other than water to yield the mass of water in the solution. Alternatively, it can be assumed that the volumes occupied by all solution constituents are the same in the solution as they are in the pure state, from which the expected density of the solution, and therefore the water content, can be calculated assuming 1 ml of water equals 1 g. The volume displacement of the RPS-2 vehicle solution constituents was determined directly by measuring solution volume before and after adding these constituents and was found to be 2 ml/dl of solution. The error in density introduced by using handbook data for penetrating and non-penetrating cryoprotectants (Table 2) is shown in the upper panel for the 15 solutions of Table 1. The maximum error in density above or below expectation (vertical distance from the line indicating identical values for measured and predicted densities) was 1%. Translating the predicted density into a predicted water content yielded a maximum error of about 2% (middle panel). However, this error is visually imperceptible over the scale of qv^* values studied here (lower panel), and thus will generally be negligible.

To complete the development of more general solutions for vitrification, we combined V_{EG}-like solutions with non-permeating polymers (Fig. 6).

Table 3
Effect of replacing propylene glycol with ethylene glycol in a standard vitrification solution

Feature	VS41A	V_{EG}
Dimethyl sulfoxide	3.10 M	3.10 M
Formamide	3.10 M	3.10 M
Propylene glycol	2.21 M	0.00 M
Ethylene glycol	0.00 M	2.71 M
Total molarity	8.41 M	8.91 M
K^+/Na^+ [a]	4.93 ± 0.15	7.17 ± 0.15
% of control K^+/Na^+	59.9 ± 1.8	86.9 ± 1.8*

[a] The time of exposure to the vitrification solutions was 40 min at 0 °C.
* $p < 0.001$ vs. VS41A.

It is known that toxicity depends much more strongly on permeating cryoprotectants than on extracellular agents [19,20]. Many current vitrification solutions therefore include extracellular polymers, whose contribution to vitrification tendency at least roughly matches that of intracellular proteins [20]. As shown in Fig. 6A, we found it was possible to make V_{EG} vitrifiable by adding extracellular polymer to it in substantial concentrations

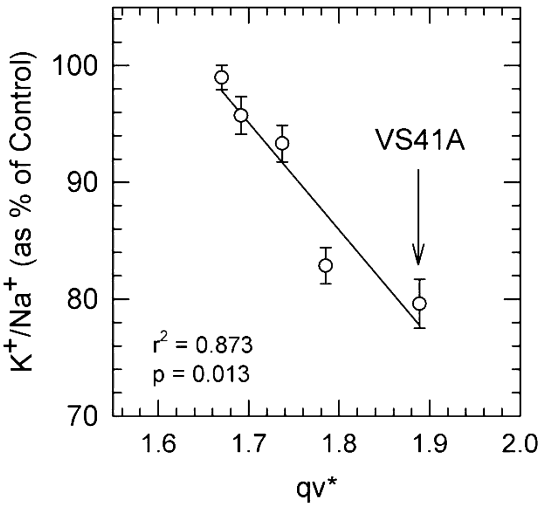

Fig. 5. Variation of electrolyte transport capacity with qv^* for vitrifiable variants of V_{EG}. The variants contained ethylene glycol, dimethyl sulfoxide, and formamide, where the mole ratio of the latter two cryoprotectants was fixed at 1:1. The total concentration was 57% w/v in all cases, and the concentrations of ethylene glycol were, respectively, 5, 10, 15, and 17.449% w/v for the progressively decreasing qv^* values shown.

without reducing viability as reflected by the K^+/Na^+ ratio (upper two bars). By adding even more polymer, the concentrations of dimethyl sulfoxide and formamide in V_{EG} could even be reduced without losing stability against ice formation (Fig. 6A, lowest bar). As shown in Fig. 6B, solutions prepared by reducing permeating cryoprotectant in V_{EG} and adding polymer are substantially less toxic than both VS41A and three previously described vitrification solutions that, like the new solutions, contain both ethylene glycol and extracellular polymers [26,35,41]. Fig. 6C further verifies the superiority of the new solutions over VS41A and also illustrates the utility of including an "ice blocker." Polyvinyl alcohol [45] and polyglycerol [46] have specific and complementary [46] antinucleating properties and therefore are able to disproportionately enhance stability against ice formation. Fig. 6C (bottom bar) shows that V_{EG} rendered vitrifiable by the inclusion of 1% polyvinyl alcohol is no more toxic than the same solution lacking the antinucleator.

A new general-purpose vitrification solution known as VM3 was obtained by combining the final solution of Fig. 6A with 1% w/v polyvinyl alcohol and 1% w/v polyglycerol. VM3 has the same molar concentration as VS41A, but, unlike VS41A, results in essentially no toxicity when exposed to renal cortical slices at 0 °C for 30 min (data not shown). Furthermore, the critical warming rate (defined as the warming rate at which 0.2% of the sample mass will crystallize during rewarming after previous vitrification) is much lower for VM3 (~2.8 °C/min) than for VS41A (~55 °C/min), which makes it possible to safely warm larger objects by simple conduction.

The generality of applicability of VM3 was demonstrated by using it to successfully vitrify a variety of different living systems. Positive results after vitrification of different rat tissues (de Graaf et al., in preparation; Pichugin et al., in preparation) and rabbit renal cortical slices with VM3 will be presented in detail elsewhere. Here we illustrate the potential scope of the present observations by reporting vitrification results for mouse ova and toxicity data for whole rabbit kidneys.

Fig. 7 compares the rates of morphological normalcy, fertilization capacity, and development

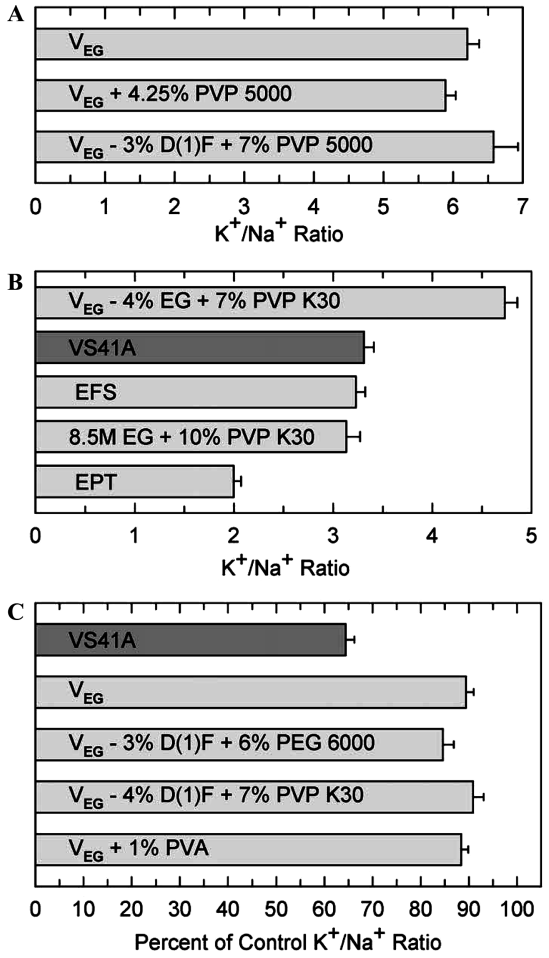

Fig. 6. Superiority of new vitrification solutions based on V_{EG} but containing polymers. (A) Addition of 4.25–7% w/v polyvinylpyrrolidone of $M_r \sim 5000$ Da (PVP K12) was successful with and without the subtraction of 1.1% w/v formamide and 1.9% w/v dimethyl sulfoxide [3% w/v D(1)F, where the (1) indicates that dimethyl sulfoxide and formamide have the same molarity]. Both solutions shown are fully vitrifiable and essentially non-toxic. (B) Direct comparison between a V_{EG}-type solution (PVP K30 has $M_r \sim 40{,}000$), VS41A, and three ethylene glycol solutions advocated in the literature (the EFS solution of Kasai et al. [26], consisting of 40% v/v ethylene glycol, 18% w/v Ficoll, and 0.3 M sucrose; the Drosophila vitrification solution of Mazur et al. [35], consisting of 8.5 M ethylene glycol plus 10% w/v polyvinylpyrrolidone of $M_r \sim 40{,}000$ Da [PVP K30]; and a variant of EFS [41] containing 40% v/v ethylene glycol, 20% w/v PVP K30, and 11.3% w/v trehalose [EPT]). (C) Further comparisons of the toxic effects of V_{EG} and its variants (PEG 6000 refers to polyethylene glycol, $M_r \sim 6000$) to VS41A and demonstration of the lack of toxicity of the "ice blocker" polyvinyl alcohol (PVA) [45] when the latter is present at a concentration sufficient to allow vitrification.

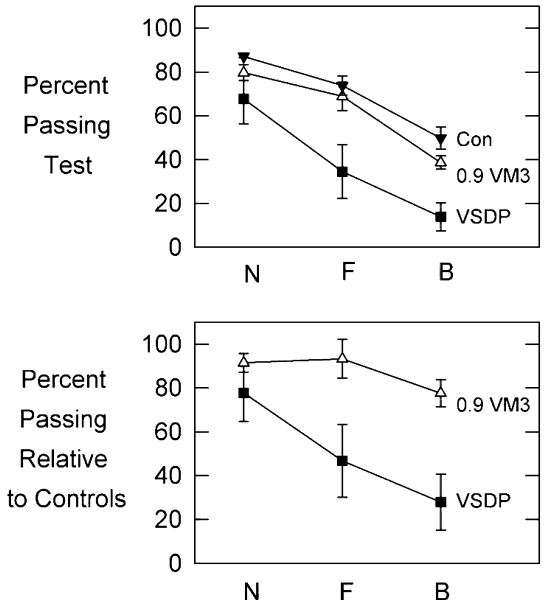

Fig. 7. Comparison of a new vitrification solution, VM3, and a previous solution, VSDP [38,39], for the preservation of mouse ova. All ova were vitrified using either 90% of full-strength VM3 (0.9 VM3, a 7.6 M solution) or VSDP (a 6 M solution) and evaluated for their immediate post-exposure morphological normalcy (N), their ability to undergo fertilization by added sperm (F), and their ability to develop to the blastocyst stage of development once fertilized (B). Results shown represent the cumulative percent of ova and derived embryos passing each test (N, F, and B). Upper panel: absolute results. Lower panel: results normalized to the performance of untreated control ova. For specific methods, see Methods and [38,39].

to the blastocyst stage after vitrification of mouse ova in either a diluted version of VM3 (90% [7.6 M] VM3, which is sufficient for vitrification using rapid cooling and warming rates) or a solution that was previously shown to be particularly effective for vitrification of mouse ova [38,39] (VSDP, consisting of 6 M dimethyl sulfoxide plus 0.1% w/v polyethylene glycol, $M_r = 8000$). The rate of development to blastocysts was 80% of the rate of untreated control ova after vitrification in 90% VM3, but less than half of this rate after vitrification in VSDP.

It is understandably more difficult to achieve comparable results in large organs due in part to the need to distribute the vitrification solution slowly by vascular perfusion and to remove the cryoprotectants in similar fashion. In previous experiments involving perfusion with VS41A, no kidneys survived perfusion at −3 °C [29]. Perfusion of this 8.4 M solution at −25 °C allowed about half of the perfused kidneys to survive, but only when iloprost, heparin, and aspirin were used to compensate for severe damage to the microcirculation [4,28,29]. Even with this pharmacological support, VS41A-perfused kidneys exhibited hematuria, cortical hemorrhages, and chronically elevated serum creatinine after transplantation [4]. Perfusing kidneys with a more dilute solution (VS4, equivalent to 89% of full strength VS41A, or 7.49 M) at −3 °C yielded 100% survival and a lower peak serum creatinine (9.6 ± 1.2 mg/dl for VS4, vs. 14.3 ± 1.1 mg/dl for VS41A −25 °C survivors) but only when iloprost, heparin, and aspirin were used to mitigate still-severe vascular congestion [28].

In contrast, Fig. 8 shows a peak serum creatinine of 2.24 ± 0.24 mg/dl after transplanting rabbit kidneys perfused at −3 °C with the 8.4 M permeating cryoprotectants of VM3 without the benefit of iloprost, aspirin, or heparin. This peak creatinine level is actually lower than that reported for sham-operated (untreated) controls in past experiments [27]. All of these kidneys (7/7) survived perfusion with the new solution, and none showed hemorrhaging or hematuria.

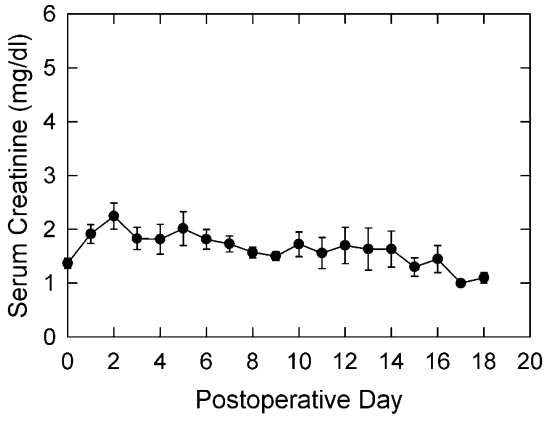

Fig. 8. Mean postoperative creatinine levels of transplanted rabbit kidneys perfused with an 8.4 M solution known as VMP, which consists of VM3 minus the PVP to ensure equilibration. After slow introduction (see Methods), VMP was perfused at −3 °C for 20 min. Serum creatinine levels peaked on day 2 at 2.24 ± 0.24 mg/dl (mean ± 1 SEM; $n = 7$). Further details are given in the Methods section.

Discussion

Past attempts to relate the toxicity of a cryoprotectant solution to its physical properties have not been able to provide useful predictions of the response of nucleated cells or organized tissues to vitrification solutions [1,2,5,18,19,37]. The present report describes new concepts related to the discovery of superior vitrification solutions for complex living systems, and provides initial evidence that the application of these concepts will be of practical significance for the cryopreservation of sensitive single cells, organized tissues, and even systems as complex as whole organs. Our reasoning pertaining to the basic mechanisms underlying the new results is as follows.

In order for a solution to vitrify at normally attainable cooling rates, it is necessary to inhibit water–water interactions. In a practical sense, all solutions at their threshold concentrations for vitrification are equivalent in the sense that water–water interactions in these solutions are inhibited just enough to prevent crystallization regardless of the composition and absolute concentrations of the solutions. However, despite the approximate equivalence of water crystallization tendency between various solutions at C_V, it is apparent that large differences exist in the toxicities of these vitrification solutions. The nature of the relationship between toxicity and qv^* suggests a possible way of understanding this dichotomy.

qv^* is believed to be an indirect measure of the average energy of hydrogen bonding between water and hydrogen bonding groups in the solution. A solution with a high qv^* vitrifies with a higher number of water molecules per water-bonding polar group than a solution with a low qv^*, implying that each polar group in the former solution must interact with and restrict a larger number of water molecules, presumably because of stronger hydrogen bonding with water. This conclusion is strongly supported by direct measurements of the effects of different cryoprotectants on the proton chemical shift of water, which showed that glass-forming tendency, as measured by C_V, depression of the homogeneous nucleation temperature, and elevation of the glass transition temperature, is directly related to the basicity (water-bonding ability) of the cryoprotectants [22,34]. Given that solutions with a high qv^* are more toxic than those with a low qv^*, it appears that the toxicity of vitrification solutions increases as the strength of water-cryoprotectant hydrogen bonding increases.

We propose that non-specific toxicity of vitrification solutions is governed by the statistical availability of water molecules for hydrating biomolecules. Strong water association with added polar groups may result in lower water availability for hydrating biomolecules even though the absolute water content of solutions bearing strongly hydrated cryoprotectants is higher. In other words, competition between cellular constituents and cryoprotectants for hydration may govern the partitioning of water to cellular constituents.

Because qv^* is a property of the solution as a whole, excellent vitrification solutions could be formed that include balanced [16] concentrations of the strong glass-former, dimethyl sulfoxide [18], and weak glass-formers such as formamide [13], acetamide [12,13,20], and ethylene glycol [18]. This implies that toxicity relates to the statistical average of water distributions around biomolecules rather than to momentary local encounters between biomolecules and strong glass formers. Stated differently, it appears that qv^* is linked to toxicity when toxicity is non-specific in nature. By the same reasoning, injury associated with specific binding, strong denaturants, or perhaps with individual cryoprotectants used in high enough concentrations to produce specific toxic effects would not be expected to correlate with qv^*. At least one outlier of Fig. 3C, in which ethylene glycol was the only penetrating cryoprotectant (solution 11 of Table 1), may be an example in which specific toxic effects were present.

The basic phenomena described here can be reconciled with traditional experience by reference to the schematic diagram of Fig. 9. Although it is the case that the toxicity of a given cryoprotectant solution tends to rise with concentration, there tends to be a threshold concentration specific to each solution below which little toxicity is observed [20]. We suggest that as C_V (especially when expressed in units such as M_{PG} or M_{PG}/M_W) rises,

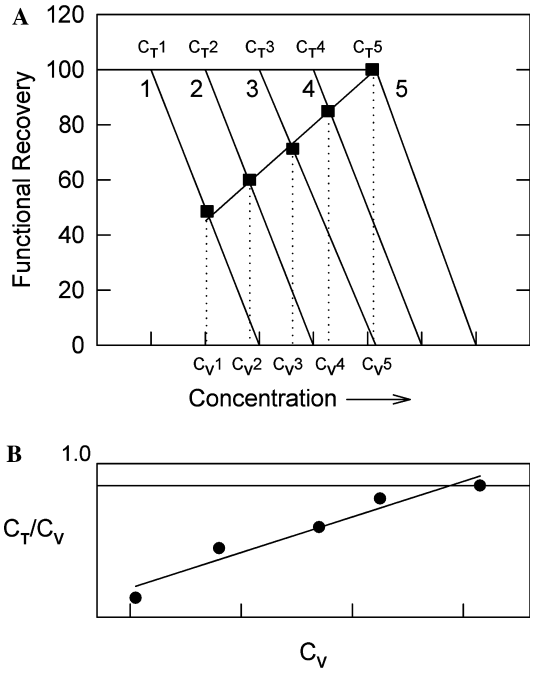

Fig. 9. Illustration of how toxicity can be lower in solutions of high C_V despite the fact that increasing the concentration of any solution tends to elevate its toxicity. (A) Concentration dependence of the toxicities and glass-forming tendencies of five hypothetical solutions 1–5 of progressively decreasing toxicity and decreasing glass-forming tendencies. Solid lines indicate that for every solution, increasing concentration is innocuous until a threshold concentration for toxicity (C_T1 through C_T5) is reached, beyond which functional recovery falls rapidly with further increases in solution concentration [20]. However, viability does not fall to zero before the concentration threshold for vitrification, C_V1–C_V5, is reached, as indicated by the black boxes, which mark the intersection between the toxicity curves and the vertical lines representing C_V (dotted drop lines). If C_V rises less rapidly with decreasing glass-forming tendency than does C_T, then the intersections between C_V and the toxicity curve will occur at higher and higher levels of functional recovery as C_V rises even though the intersections also occur at higher and higher absolute concentrations. This phenomenon is summarized in (B), which plots the ratio between C_T and C_V as a function of C_V, showing that as C_V rises, C_T can rise until it equals or even exceeds C_V. Such a solution can be used for vitrification without inducing significant biological injury. An implication of the close linearity of these plots, as suggested by Figs. 1–3 and 5, is that the rise in C_T is likely to be causally related to the rise in C_V.

the threshold concentration required for toxicity (C_T) rises as well (panel A) and, in fact, that C_T tends to rise faster than C_V (panel B) so that, for solutions with the highest C_Vs, C_T can approach, reach, and even surpass C_V. An implication of the linearity of the plots of Figs. 1–3 and 5 is that the rise in C_T is causally related to the rise in C_V, that is, that the same features that weaken glass-forming tendency weaken toxicity even more strongly.

The mechanism by which reduced hydration of biomolecules caused by the presence of cryoprotectants leads to impaired cellular function remains to be elucidated. Protein stability in the presence of cosolvents has been related to the ability of the protein to remain preferentially hydrated in the presence of the cosolvent [6,23]. This is usually ascribed to a more favorable free energy of interaction between water and the protein in comparison to the interaction between the cosolvent and the protein. This interpretation may underemphasize the possibility that water availability for hydration can be limiting either because of insufficient water content or because of stronger interaction between water and cosolvent than between water and protein. Dimethyl sulfoxide may be a good example of an agent that has a higher affinity for water than for protein since its interaction with formamide in aqueous solution is thermochemically repulsive [19], whereas formamide–formamide chains in solution bear a resemblance to the α helix, the site of most protein–cosolvent interactions [6]. It will be interesting to see whether the behavior of model proteins after transient exposure to vitrifiable cryoprotectant mixtures parallels the qv^* dependence of the functional integrity of organized tissue.

The phenomena described here have allowed an extremely delicate mammalian organ to be exposed to unprecedentedly high concentrations of cryoprotective solutes at relatively high temperatures with subsequent excellent life support function. This observation substantially increases the likelihood that the successful vitrification and recovery of a variety of complex and spatially extended tissue and organ replacements will be feasible. In addition, the successful application of the new solution technologies to systems as diverse as mouse ova and rabbit kidneys suggests the generality of the principles involved in the prediction and control of vitrification solution toxicity.

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