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Cryoprotectant toxicity neutralization ☆,☆☆

Gregory M. Fahy *

21st Century Medicine, Inc., 14960 Hilton Drive, Fontana, CA 92336, USA

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ABSTRACT

Cryoprotectant toxicity is a fundamental limiting factor for the successful cryopreservation of living systems by both freezing and vitrification, and the ability to negate it would be attractive. Past attempts to demonstrate "cryoprotectant toxicity neutralization" (CTN) have had many ups and downs. First convincingly introduced by Baxter and Lathe in 1971, the concept that certain amides can block toxic effects of dimethyl sulfoxide (Me_2SO) was contradicted by direct experiments in 1990. But in 1995, the opposite mode of CTN, in which Me_2SO blocked the damaging effects of formamide, was robustly demonstrated. Recent experiments have verified the original 1995 results and extended them to urea and acetamide, but no CTN was detected for *N*-methylamides (*N*-methylformamide, *N*,*N*-dimethylformamide, and *N*-methylacetamide). On the theory that the latter amides and acetamide might serve as low-toxicity structural analogs of formamide, urea, or Me_2SO, competition experiments were carried out between them and formamide or urea, but CTN was not observed for these amide–amide systems. The idea that the *N*-methylamides might have non-specific rather than specific toxicity was supported by the fact that the concentrations of these amides that cause toxicity are similar to the concentrations that denature model proteins. Clear examples of neutralization of the toxicity of glycerol, propylene glycol, ethylene glycol, or Me_2SO are presently lacking, but effects of the latter that depend on sulfhydryl oxidation have been reversed with reducing agents. In summary, CTN is a useful phenomenon with significant theoretical and practical implications.

The importance of cryoprotectant toxicity

Although more subtle mechanisms of action of permeating cryoprotectants have sometimes been suggested (e.g., [36]), the basic role of these agents is to suppress ice formation and thereby the biological consequences of ice formation during slow freezing [42,45,46]. Their effectiveness in this regard depends in part on their solubility in water at all temperatures relevant to freezing and on their lack of intrinsic toxicity. The latter property, lack of toxicity, generally determines which membrane-permeable chemicals are useful cryoprotective agents and which are not. If cryoprotectants had no toxicity and were able to reach and penetrate all cells requiring cryopreservation, they could be used at arbitrarily high concentrations, and in principle all freezing injury could be prevented other than injury related to cooling per se (chilling injury). Therefore, cryoprotectant toxicity can be seen as the single most limiting factor for the success of cryopreservation by both freezing and vitrification and for the scope of cryobiological protocols [18].

Even though permeating cryoprotectants can sometimes be tolerated at remarkably high concentrations and at remarkably high temperatures [32,38], the fact that cryoprotectants can and sometimes do have toxic effects (as distinguished from osmotic effects), particularly at higher temperatures, is well established (e.g., [13,18]) and is generally accepted. This appreciation for the toxicity of cryoprotectants, particularly at higher concentrations and higher temperatures, has arisen at least in part due to the acknowledged difficulty of utilizing vitrifiable concentrations of cryoprotectants to eliminate ice formation altogether. The need to avoid toxic effects of "vitrification solutions" [54] has inspired a great deal of research on the formulation of minimum-toxicity mixtures of cryoprotectants for vitrification (e.g., [1,15,18,21,22,29,39,40,44,54,55]), but although these efforts have met with considerable success, much of this success has been dependent upon discovering methods for using less total permeating cryoprotectant and/or shorter exposure times rather than methods for reducing the intrinsic toxicity of higher concentrations. This approach is helpful for small samples, but not as helpful for larger samples such as tissues and whole organs.

While the significance of cryoprotectant toxicity is well appreciated by those involved in vitrification research, there is probably

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* Fax: +1 909 466 8618.
E-mail addresses: gfahy@21cm.com, gfahy@sbcglobal.net

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less awareness of the relevance of cryoprotectant toxicity on the part of those who use freezing as their method of cryopreservation. However, even the earliest systematic investigations of the mechanism of cryoprotection showed that the efficacy of different agents is not strictly accounted for by their colligative effects, some permeating agents having considerably lower efficacy than expected on a colligative basis, or in some cases even no efficacy at all after freezing to sufficiently low temperatures, even when the cryoprotectant has acceptable toxicity before freezing [43]. In 1977, Fahy [9] and Fahy and Karow [8] pointed out that damage after freezing and thawing in certain cases is actually correlated not with the amount of ice formed but with the concentration of permeating cryoprotectant during freezing and thawing, and that therefore cryoprotectants can exert damaging effects as they are concentrated in the frozen state. Meryman et al. independently reported in the same year that toxic effects of methanol, ethanol, and ammonium acetate in the frozen state are also detectable in thawed erythrocytes [46]. These non-nucleated cells did not show injury attributable to glycerol or Me_2SO in the latter experiments, but evidence continued to emerge in support of putatively toxic effects of cryoprotectants during freezing [2,10,12,14,28], including such effects even in glycerolized erythrocytes [47,50,53], and by 1986 the overall evidence had become quite strong [17].

If cryoprotectants can be toxic in the frozen state, this should be demonstrable by showing that the outcome of freezing and thawing correlates with the toxicity of the cryoprotectant in the absence of freezing. Fig. 1 shows one example of such a demonstration. In this case, rabbit renal cortical slices were frozen to −40 °C in the presence of either dimethyl sulfoxide (abbreviated as D in this figure) or mixtures of D and one of three amides (urea, U; formamide, F; or acetamide, A). In this example, toxicity resulting in only a 16% decrease in the achievable K^+/Na^+ ratio of tissue slices prior to freezing [comparing the D + A (DA) mixture to dimethyl sulfoxide alone (D)] translated into a 40% decrease in K^+/Na^+ ratio after thawing. Compared to freezing with D only, the absolute K^+/Na^+ ratio after thawing with DA was 68% higher. Analogous results were obtained as well by Clark et al. [5] and Hawkins et al. [33] by comparing the freezing of rabbit renal cortex in the presence of dimethyl sulfoxide (Me_2SO) to freezing with either ethylene glycol (EG) or glycerol. In their experiments, Me_2SO was the least toxic agent at 25 °C [4], and it yielded the highest freeze–thaw recoveries under essentially all freezing conditions examined.

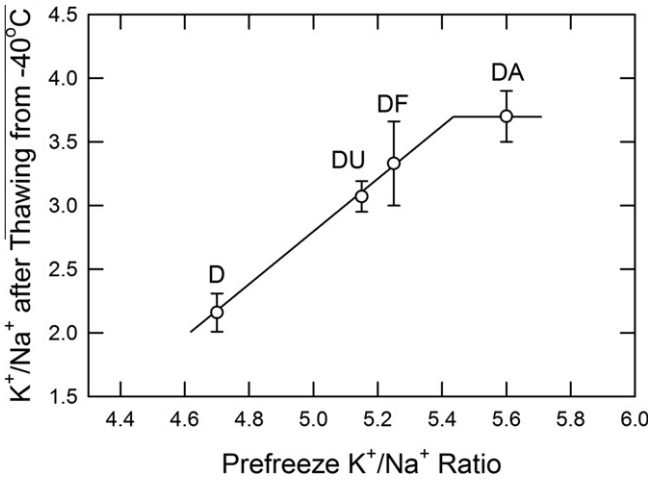

Fig. 1. Correlation between freeze–thaw recovery of rabbit renal cortical slices and the toxicity of the cryoprotectant solution prior to freezing. D, DU, DF, and DA refer to dimethyl sulfoxide (D) and mixtures of D with urea, formamide, and acetamide, respectively (details of which are provided in [12]). The total concentrations before freezing were 30% w/v in all cases; total concentrations in the frozen state were presumably as needed to achieve equilibrium with ice at −40 °C [14] (i.e., at approximately constant water perturbation). K^+/Na^+ is the mean value attained after removal of cryoprotectant and incubation at 25 °C with 100% oxygen for 90 min followed by elution of extracellular cation by brief rinsing with isotonic mannitol as described elsewhere [13]. For further details, see [12]. Drawn from the tabular data of [12].

Cryoprotectant toxicity neutralization

The mixtures of Me_2SO and amides employed in the studies of Fig. 1 were inspired by the biochemical studies of Baxter and Lathe, which indicated that the inclusion of these amides with a given concentration of Me_2SO can block the damaging effect of that concentration of Me_2SO [3,19]. This type of effect was termed "toxicity neutralization" by Fahy and colleagues [12,14–16,18,19], and it became a cornerstone of many efforts to develop low-toxicity vitrification solutions [11,13,15,18,20,22,29,30]. Considerable evidence was accumulated that cryoprotectant toxicity neutralization (CTN) was operative in the experiments shown in Fig. 1 as well as in many toxicity experiments not involving freezing [13,14,18,29]. Particularly indicative was evidence that exposure to higher concentrations of Me_2SO was associated with higher recoveries and a lack of correlation between toxicity and Me_2SO concentration when amides (upper curves of Fig. 2) rather than polyols alone (lower curves of Fig. 2) were present in combination with Me_2SO [14,18]. Such results seemed to imply that the effect of Me_2SO had been "neutralized".

However, a direct test of the idea that amides block the toxicity of Me_2SO yielded negative results: the addition of 3–15% w/v formamide to 40% w/v Me_2SO failed to reduce the toxicity observed with 40% Me_2SO exposure alone, and addition of 5–10% urea actually increased toxicity [19]. This resulted in a paradox: how could it be that amides allow higher concentrations of Me_2SO to be tolerated so much better than do polyols and render toxicity insensitive to the concentration of Me_2SO if the amides do not exert a toxicity neutralizing effect for Me_2SO?

The answer began to emerge in 1995 from studies intended to look at a different aspect of CTN. The original Baxter and Lathe paper had also reported that Me_2SO is able to prevent biochemical damage from very high concentrations of urea [3]. In agreement, Russian investigators had also found that Me_2SO is able to prevent damage to the Na,K-ATPase otherwise induced by urea exposure [48]. With this background, experiments were conducted to investigate the toxic effects of formamide and acetamide, with or without simultaneous exposure to Me_2SO [27].

The key results were obtained with formamide and are illustrated in Fig. 3. Formamide was found to be toxic at concentrations between 10% w/v and 20% w/v, but the toxicity of concentrations up to 20% w/v could be completely prevented in a dose-dependent way by the simultaneous presence of 15–30% w/v Me_2SO, even though this raised the total cryoprotectant concentration to as high as 50% w/v. Experiments with acetamide showed that this agent had little toxicity at concentrations up to at least 35% w/v, the highest concentration tested. Adding Me_2SO to 35% acetamide therefore gave ambiguous results with respect to CTN, but, as in the case with formamide, total solution concentrations of up to about 50% w/v were well tolerated. In contrast to both amides, ethylene diamine was found to be highly toxic even at 5% w/v, and the addition of even low concentrations of Me_2SO only exacerbated toxicity [27], so CTN and compatibility with Me_2SO were not associated with the presence of an $-NH_2$ group per se, but only with the presence of an amide $-NH_2$ group.

The results obtained with formamide and acetamide suggest that the advantage of amides over polyols, at least in the presence of higher concentrations of Me_2SO, arises because amides in combination with Me_2SO are less intrinsically toxic than polyols even

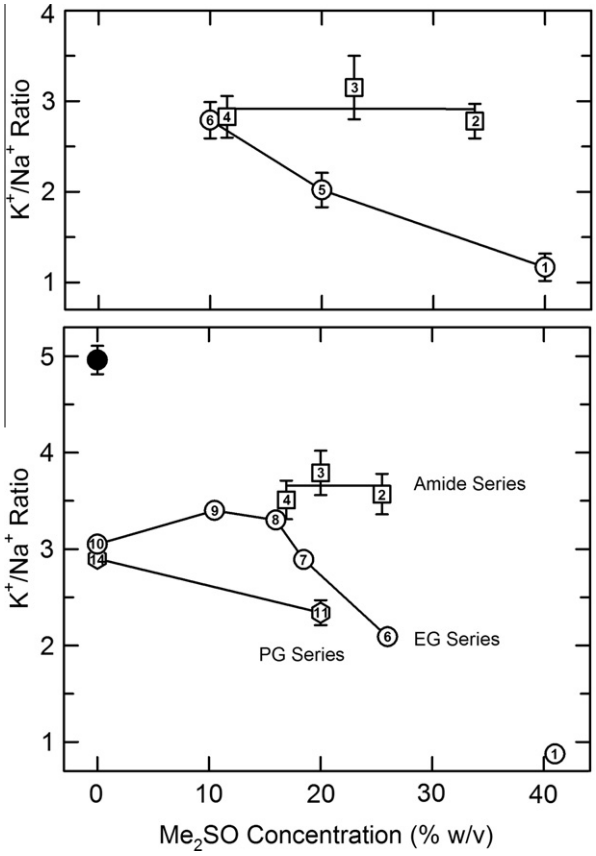

Fig. 2. Ability of amides to uncouple the toxicity of Me_2SO-bearing solutions from their Me_2SO content by a mechanism other than mere dilution of the Me_2SO. (Upper panel) Recovery of rabbit renal cortical tissue ability to accumulate potassium and extrude sodium (at room temperature after cryoprotectant washout [13]) in solutions containing a total of 40% w/v cryoprotectant of various compositions (all cryoprotectant exposure was at 0 °C). Solutions containing no amide show a correlation between total toxicity and the concentration of Me_2SO, but solutions containing amides do not. Point 1 refers to Me_2SO exposure only. Points 2–4 refer, respectively, to 33.55% Me_2SO + 6.45% urea; 22.8% Me_2SO + 17.2% acetamide; and 11.4% Me_2SO + 8.6% acetamide + 20% propylene glycol. Points 5–6 refer, respectively, to 20% Me_2SO + 20% propylene glycol and 10% Me_2SO + 10% ethylene glycol + 20% propylene glycol. Redrawn and renumbered from [14]. Means ± 1 SEM. (Lower panel) Recovery vs. Me_2SO concentration after exposure to vitrification solutions at their threshold concentrations for vitrification at a cooling rate of about 10 °C/min and at 1000 atmospheres of hydrostatic pressure. The number inside each point refers to the number assigned to the solution corresponding to that point in [18] (compositions given in [18] and [19]; unlabeled black point at upper left depicts the untreated control K^+/Na^+ ratio, and solution 1 contains only Me_2SO and 6% w/v PVP K30). Points 11 and 14: propylene glycol (PG)-rich mixtures (no amides). points 6–10: ethylene glycol (EG)-rich mixtures (no amides). Points 2–4: amide-rich mixtures. For both the PG and the EG series, toxicity is correlated with Me_2SO concentrations above 10% w/v, but in the amide series, this is not observed. All vitrifiable mixtures were added and removed at 0 °C in a manner believed to prevent osmotic injury; for details, see [18]. Error bars show ±1 SEM unless the SEM is smaller than the size of the point as drawn. Redrawn from [18].

when the total concentration of amide plus Me_2SO is very high (e.g., 50% w/v). Mixing Me_2SO with a cryoprotectant of lower toxicity should allow total solution toxicity to be lower than it otherwise would be at any particular concentration of Me_2SO, as observed in Fig. 2. In other words, although toxicity is correlated with the concentration of Me_2SO in the lower curves of Fig. 2, the toxicity of the complete solution is not attributable to the Me_2SO concentration per se, but is a property of the solution as a whole. This conclusion is indicated also by the fact that, in Fig. 3, 30% w/v Me_2SO could be added to formamide without contributing any toxicity to the solution. It is implied as well by the observation that in the absence of other solutes, 30% w/v Me_2SO has little (Fig. 1) or no [19] effect on subsequent tissue K^+/Na^+ ratio.

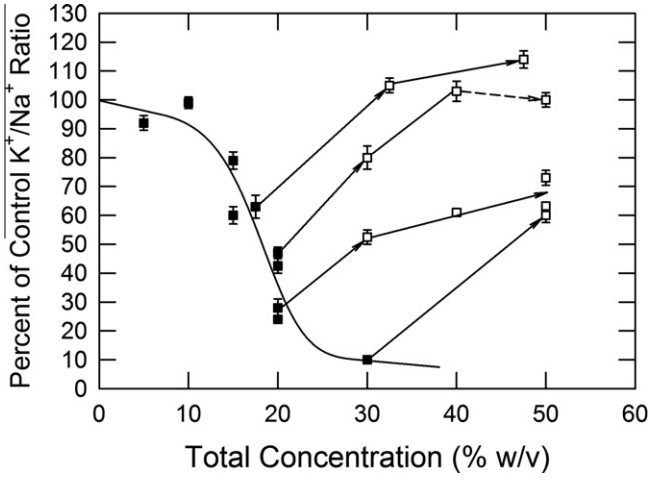

Fig. 3. Neutralization of the toxicity of formamide by the addition of Me_2SO. (Black points) Effect of formamide alone on the viability of rabbit renal cortical slices based on their K^+/Na^+ ratios after cryoprotectant removal and rewarming. (White points) Effect of adding increasing concentrations of Me_2SO to fixed concentrations of formamide. Arrows leading from specific formamide points indicate the concentration of formamide to which Me_2SO was added; white points are plotted at the total solution concentration (consisting of the indicated % w/v formamide plus increasing % w/v Me_2SO) resulting from Me_2SO addition. Dashed line at the end of the upper 20% formamide "titration" indicates that the final point was from a separate experiment. Points represent means ± 1 SEM unless the SEM is smaller than the point as drawn. All cryoprotectant addition and washout was at 0 °C according to the following schedule (where *X* is the highest total concentration of cryoprotectant reached, + refers to the presence of 300 mM mannitol, and each step has a duration of 20 min): 1/8 *X*, 1/4 *X*, 1/2 *X*, 1 *X*, 1/2 *X*+, 3/8 *X*+, 1/4 *X*+, 1/8 *X*+, 1/16 *X*+, 0 *X*+, 0 *X*. Modified from [27]. The smooth line drawn through the formamide-only points is a 4-parameter sigmoid fit.

But why should CTN by Me_2SO result in mixtures that are less toxic than equally vitrifiable Me_2SO–polyol solutions? The apparent answer emerged from studies in which CTN helped to reveal the origin of non-specific cryoprotectant toxicity in general [22]. The non-specific toxicity of a given solution appears to depend on the strength of hydrogen bonding between water and the polar groups on the permeating cryoprotectants in the solution. The evidence for this connection is summarized in Fig. 4, which plots the recovery of K^+/Na^+ from the lower panel of Fig. 2 and from a replication of those experiments against a concentration scale known as *q*. *q* is the molarity of water divided by the total molarity of hydrogen bonding groups in the solution. In this case, the abscissa is designated as qv^* to denote the fact that *q* is determined at the threshold concentration for vitrification (i.e., at approximately constant water perturbation) (indicated by *v*), as determined under standardized conditions (indicated by the asterisk, or "star.") As the number of moles of water per polar group goes down (which means more polar groups are needed to allow the solution to vitrify because the average polar group in the solution is having weaker interactions with water), viability goes up. Based on this interpretation, the hydrogen bond strength decreases in the order propylene glycol > ethylene glycol > amides, and, in combination with Me_2SO, overall solution toxicity can be seen to decrease in the same order [18] (lower panel of Fig. 2). Thus the same effect that explains the superior recovery of the ethylene glycol based solutions of Fig. 2 as compared to the propylene glycol based solutions also explains the further advantage of the amides over ethylene glycol and the particularly advantageous nature of mixtures that combine both amides and ethylene glycol with Me_2SO [20–22]. In Fig. 4, the highest recoveries obtained (square, ×) were obtained with vitrification solutions containing the highest concentrations of formamide.

Fig. 5 indicates both the reproducibility of CTN in the case of the formamide–Me_2SO system and the applicability and reproducibility

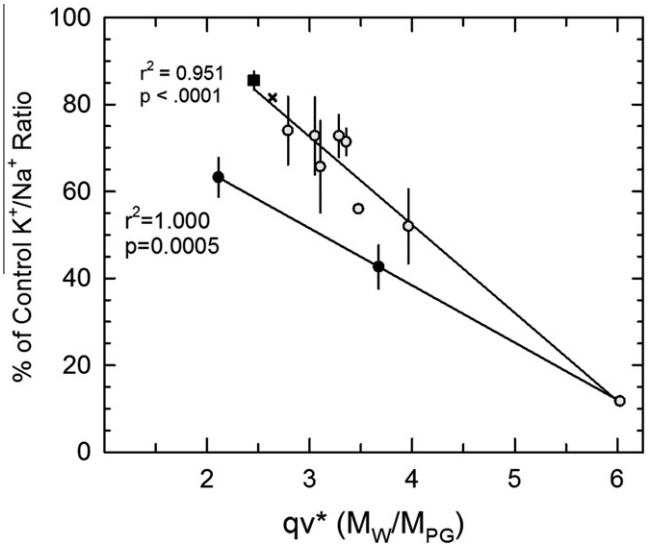

Fig. 4. Relationship between viability of rabbit renal cortical slices (as indicated by K^+/Na^+ ratio) after exposure to different vitrification solutions and qv^*. qv^* is an index of the strength of hydrogen bonding between water and the polar groups on permeating cryoprotectants. Points (except the *X*) represent the means of the means of experiments done in 1987 and 2000; vertical lines represent ± half of the difference between the mean results obtained in those two years. Lines represent regressions through the mean data points shown. (Upper line) Vitrification solutions showing a steeper correlation between viability and qv^*. For this line, the highest point (square) was obtained with the solution containing the highest concentration of formamide [14.7% w/v, which, without CTN, is in the toxic range of formamide concentrations (Fig. 3).] The *X* indicates the result of using a solution containing 12.6% w/v formamide (the second highest concentration used) that was tested in 1987 but was not re-tested in 2000. (Lower line) Outliers having a more shallow relationship between viability and qv^*. For additional details, see [22]; modified from [22]. *X* is for solution 3 of Table 1 of [22].

of CTN in the case of the urea–Me_2SO and acetamide–Me_2SO systems. Original data for formamide and acetamide from 1995 (obtained in Maryland [27]) and for urea from 1999 (obtained in California [20]) are indicated with gray points; black points (for the pure amides) and white points (for amide + Me_2SO) indicate data obtained ca. 2007 [25] to 2009 (obtained in California; not previously published). For all three systems, good reproducibility was demonstrable. Both formamide and urea show robust CTN, while acetamide, with its much lower intrinsic toxicity, shows less but still clear-cut CTN upon Me_2SO addition within a limited concentration range. In the case of acetamide, the benefit of adding Me_2SO may be limited by excessive total solution concentrations (lack of available water). For urea, and most likely for formamide as well, complete toxicity neutralization was possible at lower but not at higher concentrations of amide.

Stoichiometry of amide toxicity and CTN

To better characterize the molecular basis of CTN, Fig. 6 shows the relationship between recovery of viability and the Me_2SO: amide mole ratio for different initial molar concentrations of amide (indicated at the beginning of each line and corresponding to the amide percent w/v values shown in Fig. 5). For the regions of strong toxicity and strong CTN, Me_2SO-to-amide mole ratios of 0.5–1.0 were optimal for both formamide and urea, indicating that "toxicity titration" by Me_2SO acts with respect to the whole amide molecule rather than with respect to the number of $-NH_2$ groups in the molecule. This suggests either that only one $-NH_2$ group in urea can participate in toxicity at any given time or that toxicity is more closely related to the overall polarity of the molecule or of the carbonyl oxygen than it is to biological interactions with

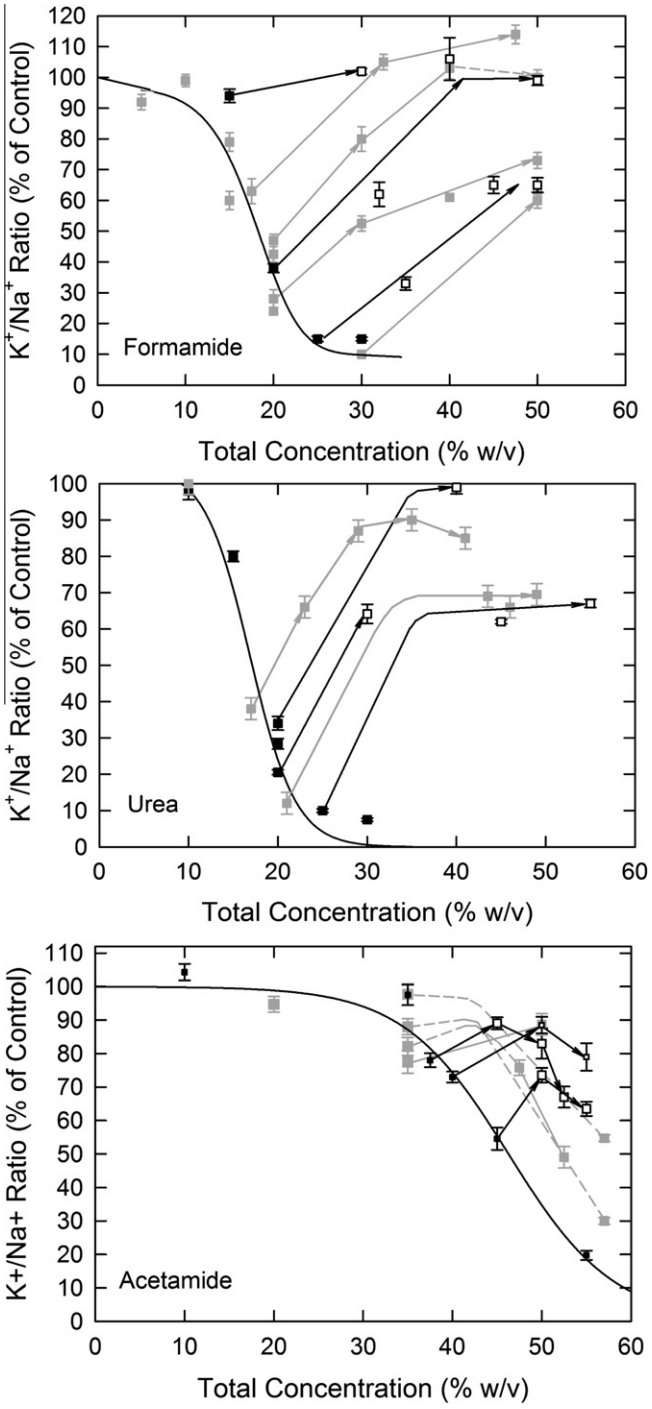

Fig. 5. Demonstration of CTN in formamide, urea, and acetamide systems, and its reproducibility for rabbit renal cortical slices. Gray points represent data obtained several years before reinvestigation of the same systems; black points represent follow-up data for the pure amides, and white points represent follow-up data for amide–Me_2SO mixtures. Modified from [27] and [20]; black and white points are previously unpublished data and were obtained using the same methodology as was used for the data of Fig. 3. In the formamide panel, the black point at 20% formamide is the mean of three separate experiments. In the acetamide panel, three inconsistently low points at 10, 20, and 30% w/v were omitted from the older dataset. In the same panel, dashed gray lines show speculative trajectories between amide-only points and their related amide-plus-Me_2SO points. Smooth curves in middle and lower panels represent 3-parameter sigmoid curve fits carried out by the curve fitting subroutine of SigmaPlot 11.0. In the original datasets for formamide and acetamide, 0.3 was subtracted from the raw K^+/Na^+ value for both experimental and control data before normalizing to account for the fact that dead slices have non-zero K^+/Na^+ ratios. This small correction was neglected for the black points. Points represent means ± 1 SEM.

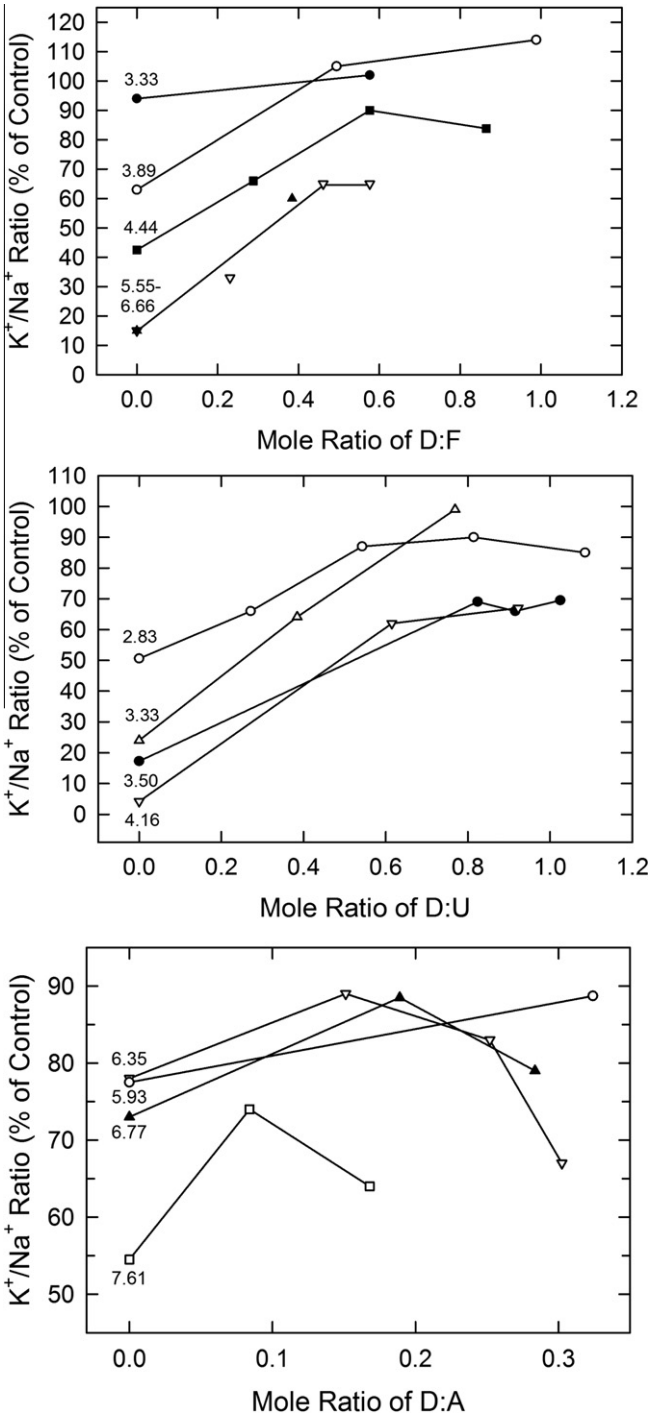

Fig. 6. Relationship between toxicity and the Me_2SO:amide mole ratio based on the data of Fig. 5. D, Me_2SO; F, formamide; U, urea; and A, acetamide. The numbers given at the onset of each line represent the molar concentration of amide to which Me_2SO was added. For the top panel, data presented for solutions containing 20% w/v formamide are the averages obtained at compositions used in more than one experiment; open triangles are for 25% w/v formamide (5.55 M) and black triangles are for 30% w/v formamide (6.66 M). For the urea panel, the percent recoveries plotted at a D:U mole ratio of zero are the values derived from the master curve fit shown in Fig. 5, which was obtained by fitting both the new and the old urea results as a single data set.

the $-NH_2$ groups per se. It is interesting that the slopes of the dose–response curves for CTN in Fig. 6 are essentially the same for formamide and urea, and that the magnitude of the rescue attained starting from a given level of baseline damage is also similar between the two amides. Acetamide, of course, falls into a different category, and the optimal Me_2SO:amide mole ratio for CTN in its case was only 0.1–0.3, apparently due to toxicity exacerbation at higher ratios.

In the absence of Me_2SO, the concentration of urea that "irreversibly" inactivates about 50% of active sodium and potassium transport is about 2.87 M, versus about 4.09 M for formamide. Thus, urea, with twice as many moles of nitrogen per molecule as formamide, is only 1.4 times more potent with respect to toxicity per mole than is formamide, and only 0.7 times as potent per mole of nitrogen. Acetamide is much less toxic per mole than formamide. Possible reasons for this could be reduced amide nitrogen polarity for hydrogen bonding, steric hindrance of access to sites of toxicity of formamide due to the bulk of the acetamide methyl group, or less chemical reactivity of acetamide.

It should be noted that the optimum Me_2SO:amide mole ratios for Me_2SO neutralization of amide toxicity are quite different from the ratios prescribed by Baxter and Lathe for amide neutralization of Me_2SO toxicity (4:1 for urea, 2:1 for formamide, and 1:1 for acetamide [3]). To use such high ratios for neutralization of amide toxicity would require lower concentrations of amide, but in that case, there would be no amide toxicity to block and therefore no advantage to be obtained from CTN. (For attempted neutralization of Me_2SO toxicity by formamide, Me_2SO:amide mole ratios ranging from 1.5 to 3.0 and above were ineffective [19]).

N-Methylamide toxicity and toxicity neutralization

To enable more insight into the mechanisms and structure–activity relationships of amide toxicity and of CTN, the same kind of "toxicity titration" experiments were carried out for *N*-methylformamide (NMF), *N*,*N*-dimethylformamide (DMF), and *N*-methylacetamide (NMA), and the results are presented in Fig. 7. Unlike the non-*N*-methylated amides, no sign of CTN was demonstrable for any *N*-methylated amide. Addition of a small amount of Me_2SO to NMF was possible without exacerbating toxicity, but as a total of 50% solute was reached, viability decreased. The damaging effect of Me_2SO addition was even more dramatic in the case of DMF and NMA: in these cases, adding Me_2SO was about as damaging as adding extra amide, gram for gram. In summary, *N*-methylation not only abolishes CTN for amides, but it also renders the presence of relatively low concentrations of Me_2SO actively detrimental. Both of these effects are qualitatively different from the behavior of the non-*N*-methylated amide systems. A possible explanation for both effects is suggested below.

Interestingly, addition of either one or two methyl groups to the amide nitrogen of formamide resulted in lower toxicity at any given concentration than was seen with formamide itself, suggesting a role for the amide nitrogen of formamide in its mechanism of toxicity. On the other hand, *N*-methylation results in greater toxicity when the parent molecule is acetamide, for reasons to be suggested below.

Amide–amide competition trials

Given that NMF and DMF are comparatively low-toxicity structural analogues of formamide, it was informative to determine whether they can competitively inhibit formamide toxicity. Similarly, acetamide and NMA are relatively low-toxicity structural analogues not only of urea but also of Me_2SO, so it was important to know whether these agents can competitively inhibit the toxicity of either urea or formamide. The results of many such competition trials are presented in Fig. 8. The added amides either had no convincing effect on the toxicity of formamide and urea or actually exacerbated injury. Acetamide, which most resembles Me_2SO

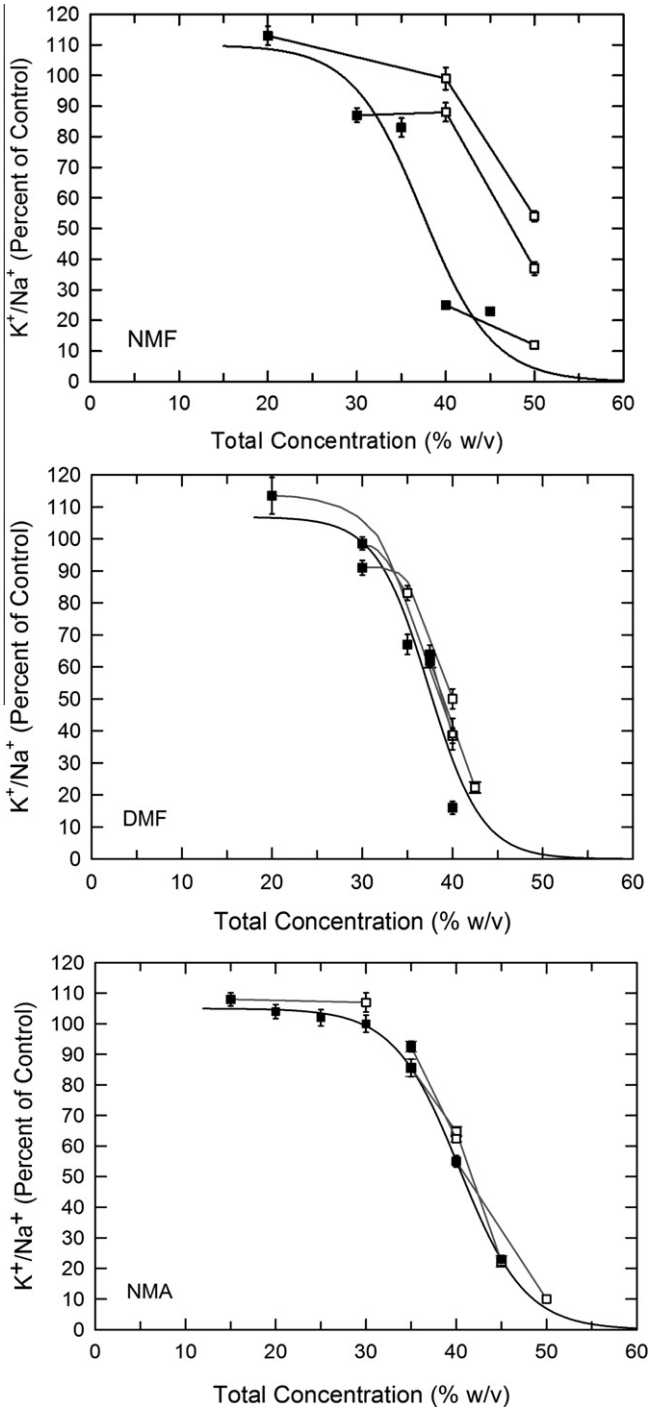

Fig. 7. Lack of CTN in rabbit renal cortical slices exposed to methylamide solutions. The method for adding and removing cryoprotectants was identical to that used to generate the data in Figs. 3, 5 and 6, and the meaning of the depiction is the same as in Figs. 3 and 5. NMF designates *N*-methylformamide; DMF designates *N*, *N*-dimethylformamide; NMA designates *N*-methylacetamide. Points represent means ± 1 SEM unless the SEM is smaller than the point as drawn.

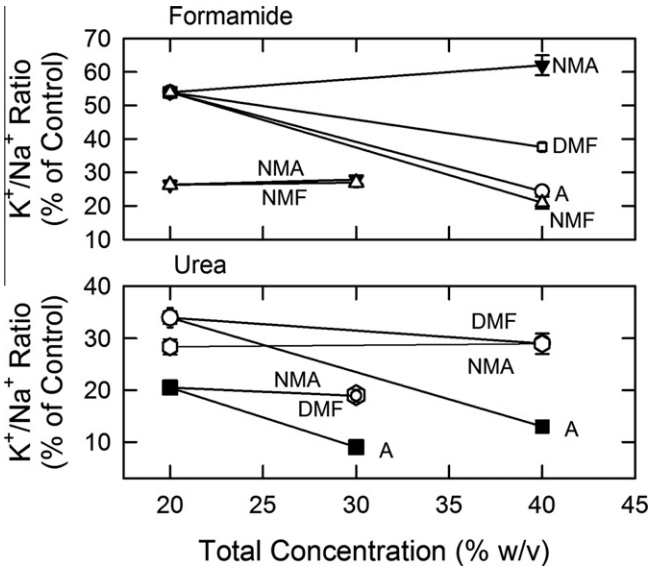

Fig. 8. Lack of neutralization of the toxicity of 20% w/v formamide (upper panel) and 20% w/v urea (lower panel) by acetamide (A) and *N*-methylamides (abbreviations as in Fig. 7). Different results at 20% amide represent different individual experiments. Means ± 1 SEM unless the SEM is smaller than the point as drawn.

structurally, was particularly toxic when added to either formamide or urea. NMF, which most resembles formamide, was particularly detrimental when added to formamide at concentrations above 10% w/v, and more toxic than DMF even though NMF is less toxic than DMF in the absence of other solutes. Similarly, acetamide, which more closely resembles urea than does NMA, was toxic when added to urea even at only 10% w/v, whereas NMA contributed no net toxicity even when added at 20% w/v even though NMA is more toxic than acetamide in the absence of other solutes.

The failure to observe CTN in any of these test systems provides additional clues about the steric, electrostatic, and chemical requirements for CTN on the one hand and for cooperative toxicity on the other. Assuming that the measured changes in ion transport capacity arise as the result of specific molecular interactions between the amides and cellular constituents that may persist in tissue homogenates and fractions thereof, the interactions shown in Fig. 8 in combination with the interactions shown in Fig. 5 might be helpful for the isolation and identification of the biochemical targets of formamide and urea that underlie the toxic effects of these agents.

Cryoprotectant toxicity and protein denaturation

Fig. 9A compares the toxic effects of different amides on a molar concentration scale. Based on the sigmoid curve of best fit for each agent, the amide concentration producing a 50% "irreversible" inactivation of renal cortical tissue potassium and sodium transport capacity was determined and compared, in Fig. 9B, to the concentration of the same agent that produces 50% denaturation of three model proteins at 25 °C. Although the comparison is between 0 °C exposure in the case of the tissue slices and 25 °C exposure in the case of isolated proteins in vitro, the data indicate that the concentrations of DMF and NMF that denature proteins are the same as the concentrations that inactivate ion transport (as indicated by the diagonal line). The toxic concentration of acetamide also borders on the concentration that denatures proteins, but toxicity in the case of urea (U), formamide (F), and Me_2SO (D) is observed at concentrations that are less than half those required to denature proteins, which suggests that mechanisms of toxicity more specific than protein denaturation are likely to be involved for the latter agents.

Fig. 9B may explain why adding Me_2SO to *N*-methylamides results in additional damage rather than CTN: Me_2SO presumably cannot prevent protein denaturation, but can only exacerbate it. Evidently, *N*-methylation both reduces formamide toxicity and eliminates CTN by changing the mechanism of that toxicity from specific to non-specific interactions. Finally, *N*-methylation

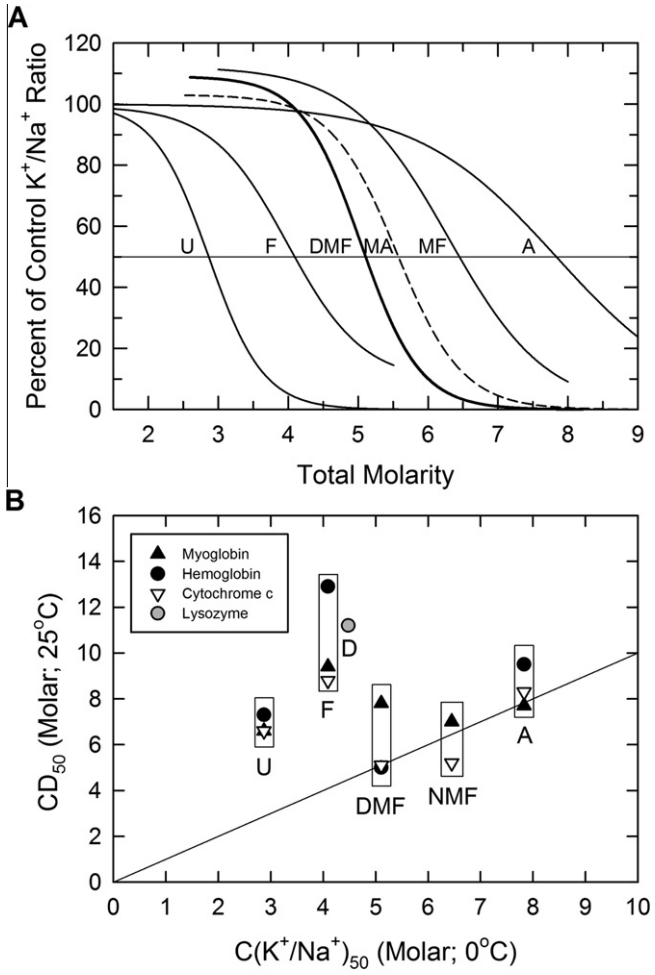

Fig. 9. Association of methylamide toxicity with protein denaturation. (A) Determination of the amide concentrations needed to reduce tissue K^+/Na^+ ratio, after cryoprotectant washout and restoration of active metabolism, by 50%. U, F, DMF, MA, MF, and A refer to urea, formamide, dimethylformamide, *N*-methylacetamide, *N*-methylformamide, and acetamide, respectively (labels located to the left of each curve). Each curve is the best-fit sigmoid from Figs. 5 and 7. (B) Comparison of the concentrations for 50% inactivation of the capacity to generate a normal K^+/Na^+ ratio as identified from panel (A) above (abscissa) to the concentrations needed to induce 50% denaturation of model proteins (whale myoglobin, human hemoglobin, and horse heart cytochrome *c* for the amides; lysozyme for Me_2SO (D)) (ordinate). Boxes designate points for the cryoprotectant whose abbreviation appears below the box (abbreviations the same as in A except for *N*-methylformamide, designated as NMF). The diagonal line is where points would fall if the concentrations producing 50% inactivation of K^+/Na^+ ratio and 50% denaturation were identical. Denaturation data from Table 5 of [19] and from [31]; Me_2SO concentration for 50% inactivation of K^+/Na^+ estimated from, for example, [19].

presumably increases the toxicity of acetamide by converting a molecule with borderline denaturation tendency into one with stronger denaturation tendency.

Is toxicity neutralization possible for Me_2SO?

Me_2SO is perhaps the single most used permeating cryoprotectant, and means of either preventing or, if necessary, reversing its toxic effects would be of great interest. Although Me_2SO is known to have many effects on living cells, one of the most interesting effects from the point of view of possible intervention is the ability of Me_2SO to chemically react with tissue sulfhydryl groups at normal body temperature [27,57,62] and, albeit slowly, with free cysteine even at 2 °C [27]. Five hours of 0 °C storage of rabbit renal cortical slices normally causes reversible depletion of tissue –SH content, but when a vitrification solution containing Me_2SO (VS41A, also known as VS55) was added and removed over the same period at 0 °C, reversal of –SH depletion was prevented [27]. Sulfhydryl oxidation is also likely to underlie [27,49] Baxter and Lathe's classic observation of activation of fructose bisphosphatase by Me_2SO [3].

Sulfhydryl oxidation produces a number of adverse effects, including the activation of extracellular proteinases [27]. My laboratory found that urokinase-type plasminogen activator (uPA), which may be able to induce renal hemorrhage if sufficiently activated [61], is intensely and selectively shed into the urine for at least 30 days after perfusing rabbit kidneys with VS41A or a more dilute variant (V52) at −22 °C [27] (Fig. 10); in fact, the activation seems to increase with time postoperatively. However, uPA activity could be strongly suppressed by either amiloride or 2-mercaptoethanol [27] (Fig. 10). Although this is not the same as neutralizing a toxic effect of Me_2SO when it is present, it at least provides an indication that a toxic effect can be blocked or reversed after Me_2SO is removed, which could have similar benefits.

In unpublished studies in collaboration with Dr. David Clough of the Uniformed Services University of the Health Sciences in Bethesda, Maryland, it was found that exposure of rabbit renal cortical slices to VS41A resulted in a 30% reduction in activity of the crucial SH-dependent membrane protein, Na,K-ATPase, as determined after VS41A removal, and a 48% reduction in the active tissue K^+/Na^+ ratio. Incubation with dithiothreitol completely restored Na,K-ATPase activity. However, it brought active tissue K^+/Na^+ ratio up only to 57% of control values, implying that Na,K-ATPase inactivation is not the primary cause of the depressed K^+/Na^+ ratio.

Besides the Na,K-ATPase, the generation of transmembrane ion gradients requires intact membrane semipermeability and intact mitochondrial ATP generation. Separate studies showed that increased plasma membrane permeability to Na^+ and K^+ was not observed after exposure of renal tissue to an ancestral version of VS41A [18]. Therefore, it is possible that mitochondrial damage (lack of ATP) may be the main explanation of the reduced K^+/Na^+ ratio after exposure to VS41A. This possibility is consistent with the fact that sulfhydryl reagents are known to be mitochondrial poisons [7,34]. It is also consistent with correlations between Me_2SO concentrations during freezing, frank mitochondrial damage, and failure of rat hearts to survive freezing and thawing in Karow's laboratory (see Fig. 3 of [26]). Whether this is a major mechanism of Me_2SO toxicity and whether this mechanism can be prevented or reversed remain questions for future investigation.

Other possible cases of CTN?

Dextrose was reported by Clark et al. to prevent irreversible binding of Me_2SO to proteins, and this effect was associated with reduced toxicity [4]. This may be the strongest evidence for CTN to date other than the evidence discussed above. However, it has yet to be independently confirmed.

Occasionally, reports have appeared in which injury in the presence of Me_2SO has been lessened by the addition of agents other than amides or dextrose (e.g., [41,60]). While such reports could theoretically provide additional examples of CTN, it is more likely that other mechanisms are at work in most or all of these cases. For example, Ulrich et al. found that sugar cane cells exposed to a mixture of Me_2SO (10% w/v) and glucose (8% w/v) at 0 °C had reduced triphenyltetrazolium chloride (TTC) staining, but adding 10% w/v polyethylene glycol 6000 (PEG) to this mixture substantially improved staining [60]. However, it is possible the protective effect of adding a non-penetrating agent was caused not by preventing intrinsic toxicity but simply by osmotically reducing cell volume and, therefore, in this case, uptake of Me_2SO and sugar so that there

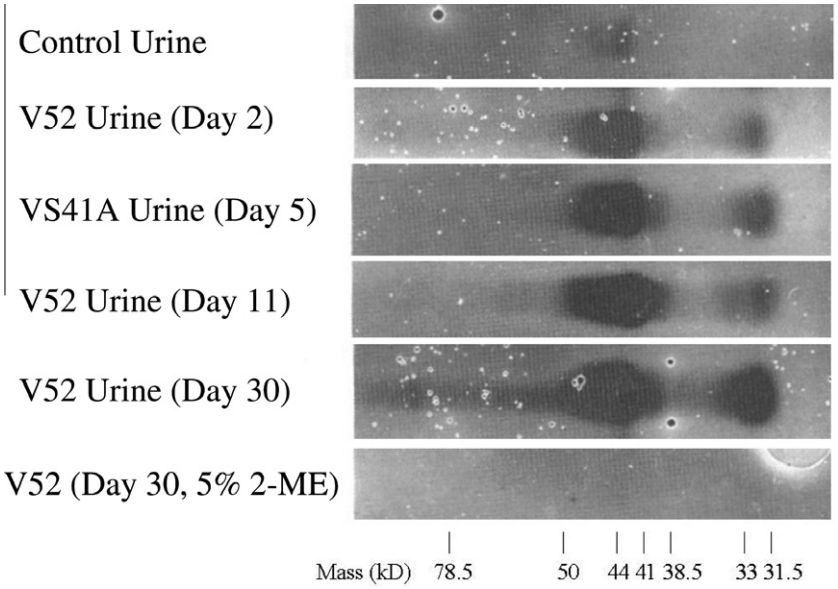

Fig. 10. Zymograms of urine collected from rabbits at various times (2, 5, 11, and 30 days) after receiving transplanted kidneys previously perfused with V52 or VS41A (also known as VS55 [58]) as described in [27]. The gray areas represent an overlay containing Carnation instant non-fat milk; the dark areas are holes in the milk layer caused primarily by amiloride-inhibitable enzymatic activity in the underlying urine sample gel (urokinase-type plasminogen activator activity). The lowest lane contained 5% 2-mercaptoethanol (2-ME) to reduce S–S crosslinks. All lanes contained an equal amount of urine (3.75 μL). For detailed methodology and the formulas for V52 and VS41A, see [27].

would be less osmotic shock upon abrupt (one-step) cryoprotectant washout. In addition, Leibo and Mazur reported that freezing cells with a combination of Me_2SO and PVP gave dramatically better recovery than freezing with either agent alone [41], but such results most likely reflect such effects as mutual dilution of cryoprotectants to sub-toxic levels during freezing [51], altered phase diagram behavior, and summation of differing mechanisms of cryoprotection rather than CTN in the frozen state. Nevertheless, it is certainly possible that bona fide examples of CTN beyond those cited here reside in the literature, and awareness of the existence and possibility of CTN may inspire the identification of such examples.

Conclusions

The ability to reduce the toxicity of a penetrating cryoprotectant by adding more penetrating cryoprotectant is a rather amazing one, and it has been very important for the development of minimum-toxicity solutions [15,21,22,27]. Thus far, the phenomenon has only been clearly documented in amide–Me_2SO systems using amides that, while fully recognized to be cryoprotectants in Karow's classic compendium [37], are not nearly as popular as Me_2SO, ethylene glycol, or propylene glycol. Nevertheless, they provide an example of CTN that can be further studied to gain deeper insights into mechanisms of toxicity and counter-toxicity of significant relevance to cryopreservation.

Whereas pharmacologists have drug antagonists, and biochemists have enzyme inhibitors, cryobiologists have lacked antidotes to cryoprotectant toxicity for the most part. Any sign that such antidotes might be possible suggests that tools more available in other fields might yet become available to the cryobiologist.

The dose–response curves for amide toxicity can all be fit by sigmoid curves that strongly resemble (though on an absolute concentration scale rather than a log-dose scale) the dose–response curves that are typical of pharmacological agents. Similar curves have also been seen for the inhibition of physiological functionality by cryoprotectants at higher temperatures [35]. These dose–response curves presumably arise for specific molecular reasons in the case of at least formamide and urea, and the ability to interfere with and even abolish these curves or to avoid these curves by using closely related alternative molecules provides a unique opportunity for future studies to elucidate the underlying molecular mechanisms involved. Whether low temperature examples analogous to the high-temperature use of atropine to block the physiological effects of acetylcholinesterase inhibition by Me_2SO [56] can be found remains a question for future research to answer. In the case of Me_2SO, a unique toxic mechanism involving sulfhydryl oxidation might one day enable a reduction in or a reversal of the toxicity of this agent.

A major caveat of the effects described here is that they have been observed so far only in rabbit renal cortical and rabbit liver (unpublished results) slices. Although solutions based on the combined use of amides and Me_2SO have proven efficacious for the vitrification or cryoprotection of many biological systems (including mouse embryos [54] and oocytes [22], rabbit veins [58], rat brain [52] and kidney (cortical and medullary) [6] slices, whole rabbit kidneys [21–24], rabbit renal cortical slices [12], and a variety of other diverse systems ([59] and unpublished results of 21st Century Medicine)), it has yet to be established that the CTN effects described here are applicable to any non-renal and non-hepatic cell or tissue, even though such effects might be expected for many cells [48].

Clearly, the study of CTN remains in its infancy. Further investigations are bound to yield results that are interesting on both a theoretical and a practical level.

Conflict of interest

The author is the Vice President and Chief Scientific Officer of 21st Century Medicine, which sells cryopreservation solutions and techniques.

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