Biophysical and Physiological Responses Promoting Freeze Tolerance in Vertebrates

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Freeze tolerance, an overwintering adaptation of at least 10 species of ectothermic vertebrates, is promoted by integrated biophysical and physiological responses to ice forming within tissues. Application of physiological principles of natural freeze tolerance has accelerated the development of protocols for cryopreserving mammalian organs.

Introduction

Amphibians and reptiles inhabiting temperate areas are confronted with seasonal cold from which they seek refuge within thermally buffered aquatic or terrestrial microenvironments. However, some species, particularly those that overwinter in relatively shallow and poorly insulated hibernacula, are exposed to subzero temperatures. Organisms may survive such episodes by avoiding crystallization (i.e., remaining in the metastable supercooled state), yet a more physiologically interesting alternative strategy is exemplified by those species that tolerate ice forming and propagating within body tissues and organs.

Freeze tolerance has long been known in various invertebrate animals but was only recently described in vertebrates. In his seminal report of this phenomenon, W. D. Schmid (12) established freeze tolerance as an adaptation promoting winter survival by demonstrating that three species of terrestrially hibernating frogs recovered fully after being frozen several days with body temperatures of -6°C. In contrast, species that hibernate under water, where subfreezing temperatures are relatively uncommon, did not survive freezing. Subsequent research has added two frogs, a salamander, a snake, and three species of turtles to the short list of amphibians and reptiles that share this distinction.

Although research concerning the nature of vertebrate freeze tolerance is still in its germinial stages, significant advances have been made by several laboratories working on various fronts, including the biochemical regulation of cryoprotectant synthesis (for review, see Ref. 14); mechanisms of cryoprotection at cellular, tissue, and organ levels of organization (1-4, 13); and various aspects of postthaw recovery (5, 7). The majority of these studies have focused on the wood frog, Rana sylvatica, a North American species that inhabits moist temperate woodlands ranging north to the Arctic Circle.

Problems of freezing and thawing

The physiological stresses associated with freezing and thawing are profoundly influenced by the dynamics of temperature, osmotic pressure, and the physical state of body fluids. It is generally believed that the formation of ice within protoplasm disrupts structural organization and ultimately causes cell death (10, 11). Consequently, the wood frog can survive freezing only if ice is restricted to extracellular spaces.

Osmotic stress. The primary stress associated with freezing and thawing is osmotic disequilibrium across cell membranes (10). Because only water molecules join the growing ice lattice, rejected solute raises the osmotic pressure of the as yet unfrozen fluid, withdrawing water from within adjacent cells. Ultimately, these events lead to additional ice growth that progressively dehydrates and shrinks cells, potentially leading to irreparable membrane damage and hyperosmotic injury. These stresses are reversed upon thawing, since the melting ice creates large pools of dilute fluid in extracellular spaces.

Mechanical stress. The physical isolation of cells embedded in an ice matrix obstructs both intercellular and humoral communication systems. Cells trapped between merging ice fronts may become critically compressed and contorted. Additionally, ice propagating within the vasculature may distend and rupture small vessels, thus precluding tissue perfusion after thawing (6). The potential for such damage is heightened by recrystallization, a time-dependent process involving the progressive growth of existing ice crystals at the expense of others.

Metabolic perturbations. Because breathing and blood flow ultimately cease during freezing, energy production in the frozen state is primarily governed by anaerobic glycolysis (14). Hypoxic tissues may accumulate large quantities of lactate and alanine and, during protracted freezes, may suffer a decline in ATP content and energy charge. The use of glucose as a cryoprotectant requires that the wood frog tolerate extreme hyperglycemia. Although its clearance begins immediately after thawing, elevated glucose levels may persist in some tissues for more than a week.

Physiological limits of vertebrate freeze tolerance

The limit of organismal freeze tolerance is influenced by the proportion of body water that has frozen, which, in turn, is determined by the temperature and osmolality of body fluids (10). Wood frogs survive the freezing of up to 65-70% of their body water, a level attained when tissues have cooled to approximately -2.5°C (2, 6). Interestingly, this maximum survivable body ice content accords with the tolerable limit of cell dehydration for vertebrates (14).
Considered from an ecological perspective, the capacity for freeze tolerance is most appropriately defined in terms of thermal and temporal constraints. The lowest body temperature tolerated by frozen wood frogs is between -3 and -6°C, depending on physiological condition and geographical origin, and is representative of the lower lethal temperatures of other freeze-tolerant vertebrates. Much less is known about the temporal capacity for freeze tolerance, although freezing bouts lasting 2 wk can be survived (14). The available data suggest that these capacities permit the survival of freezing for extended periods under most overwintering conditions.

**Ice nucleation: the initiation of freezing**

The wood frog employs freeze tolerance, rather than enhanced supercooling capacity, as an overwintering strategy because its water permeable skin is a poor barrier to the propagation of environmental ice within body fluids. This species overwinters in shallow depressions in the forest floor covered by damp leaf litter and, when present, snow (6). In its hibernaculum, freezing probably is initiated when a frog has cooled to -0.5°C, the approximate freezing point of body fluids, and is seeded by contact with ice in the environment.

Although such “inoculative” freezing is likely the primary mechanism initiating freezing, crystallization may occur even in relatively dry microenvironments, owing to the bacterium *Pseudomonas putida*, a normal constituent of the gut flora having potent ice nucleating activity at -2 to -3°C. Such reserve system ensures that freezing commences at relatively high temperatures, a critical result that slows the rate of ice crystal growth, minimizes transmembrane osmotic stress, and facilitates the formation of extracellular ice (8).

**Biophysical and physiological responses promoting freeze tolerance**

In the laboratory, ice nucleation in wood frogs occurs when the cooling specimen reaches, or falls below, the freezing point of body fluids. If the frog has supercooled appreciably, the body temperature abruptly rises above the crystallization temperature before stabilizing near the equilibrium melting point of the tissues (Fig. 1A). This “exotherm,” the liberated heat of fusion, is protracted, usually lasting 10–20 h. One obvious benefit of exothermy is that ice accumulates and propagates slowly throughout the tissues. Our research suggests that slow cooling, the norm under natural conditions, is necessary to effect the cryoprotective responses critical to natural freeze tolerance (4).

With the survivable freezing of 65% of its total body water, the wood frog appears solidly frozen; it has a distinctly rigid trunk and limbs, frosted skin, and opaque eyes. Pulmonary breathing and cardiac activities are absent, and there is no blood flow. Although some ice has formed within tissues and organs, dissection of a frozen specimen reveals that ~25% of all ice is sequestered in the coelomical cavity and subcutaneous lymph sacs (8).

In the laboratory, frogs are passively thawed by exposing them to a low temperature (e.g., 4°C) such as is encountered in nature. Depending on the size of the specimen and the amount of ice that has formed, thawing is completed in only a few hours.

Physiological and neurobehavioral functions rapidly are restored during the thawing and recovery sequence (Fig. 2). The earliest sign of viability is the return of cardiac activity, which soon is followed by the resumption of tissue perfusion and pulmonary breathing. The frogs rapidly recover motor faculties. Simple hind-limb reflexes are reestablished before more complex behaviors, such as the righting response, but within 14–24 h of thawing, most frogs exhibit normal neurobehavioral responses. Typically ≥95% of the frogs fully recover (6).

Release of the latent heat of fusion effects a virtually instantaneous increase in metabolism, as evidenced
by a surge in heart pulse rate (Fig. 1B). Cardiac activity persists for many hours during freezing despite adverse changes in tissue chemistry and hemodynamics, including the freeze concentration of harmful ions and solutes within myocardiocytes and the doubling of hematocrit. Coupled with the increased pulse rate, such tenacity not only promotes the timely distribution of cryoprotectant to tissues throughout the body, but also facilitates organ dehydration. The initial responses of the cardiovascular system to freezing thus seem paramount in effecting the cryoprotective mechanisms critical to natural freeze tolerance.

Remarkably, heart function later declines and ultimately is lost, perhaps owing to perturbations such as ion imbalance, hypoxia, and metabolic acidosis; however, cardiac contractions spontaneously resume during thawing, even before all ice has melted. The rapid restoration of cardiovascular function undoubtedly is beneficial in supporting recovery in other systems. How the heart functions under prevailing ischemic hypoxic conditions during freezing and thawing is presently unknown.

**Dynamics of water and ice**

Crystallization results in an initial burst of ice formation, the magnitude of which is proportional to the degree of supercooling. Subsequently, ice propagates through the vasculature and forms within extracellular spaces throughout the body. The period of ice accumulation may last from hours to days, depending on the specimen’s body size, ambient temperature, efficacy of external insulation, and other factors governing the rate of heat loss from the body (Fig. 1C). It is critical to the survival of the organism that ice forms gradually. An equilibrium ice content is attained when the body temperature has declined to the lowest point permitted by the cooling potential of the environment.

**Cryoprotective mechanisms**

Freezing survival of the wood frog is attributed to two principal adaptations that mitigate the stresses associated with freezing and thawing. Unlike the many invertebrates that initiate cryoprotective responses in autumn, well in advance of the actual freezing episode, in the wood frog defenses against cryoinjury are invoked only after freezing has begun.

**Chemical cryoprotection.** Ice nucleation in the wood frog triggers a rapid mobilization of glucose, from hepatic glycogen reserves, which is distributed via the blood to other tissues (Fig. 1C). This response apparently is mediated by a β-adrenergic adenosine 3',5'-cyclic monophosphate-dependent mechanism and regulated by adjustments in the activity and quantity of key regulatory enzymes. Wood frog hepatocytes synthesize glucose at extraordinarily high rates (e.g., 35 μmol·g⁻¹·h⁻¹), even at the low body temperatures associated with overwintering. Blood and liver concentrations increase severalfold within the first few minutes of freezing and may ultimately exceed 300 μmol/g. Because the quantity of glucose produced is constrained by the size of the hepatic glycogen reserve, the capacity for freeze tolerance shows considerable individual variability.

Investigators initially surmised the cryoprotective role of glucose from the pattern of its rapid synthesis and accumulation. Experiments using glucose supplements to reduce cryoinjury to cells and tissues in vitro and in vivo have largely supported this hypothesis. However, direct evidence of glucose’s efficacy as a cryoprotectant at the organismal level was obtained only recently by demonstrating that wood frogs collected in late winter (which are relatively glycogen poor and thus synthesize only modest quantities of glucose) were supplemented with saline (control) or saline containing glucose before being frozen to -5°C. Shown are treatment effects in promoting organismal survival (A), reducing body ice content (B), and reducing cell damage, as represented by hemoglobin leakage from critically damaged erythrocytes (C). Values are means ± SE of 9 or 10 frogs per group. Means identified by different letters were statistically distinguishable (P ≤ 0.05). (Adapted from Costanzo et al.)

**Freeze-tolerant frogs** uses it as a cryoprotectant. On the other hand, the benefits of using glucose include its rapid turnover in the liver and distribution to other organs, as well as its role in providing energy for frozen anoxic tissues. To minimize hyperglycemic stress, frogs begin converting free glucose to liver glycogen immediately after they thaw. Nevertheless, several days may elapse before glucose homeostasis is fully restored.

The energetic implications of this system have received only cursory study, although available data suggest it is costly and potentially limiting. For example, some of the liver glycogen mobilized during a freezing
episode (~25% of the total reserve) is expended, presumably in supporting the metabolism of frozen tissues and postthaw recovery processes. Also, glucose copiously appears in the urine of recently thawed frogs. Although some excreted glucose might be recovered via absorption through the skin, glucosuria represents a potentially critical avenue for energy loss (8).

**Tissue dehydration.** During freezing, much of the ice that forms becomes sequestered within the coelom and lymph sacs. The water translocated to these compartments likely originates from adjacent organs which, consequently, may lose more than one-half of their original water. These tissues rapidly rehydrate after thawing (Fig. 1E).

The dynamics of dehydration during freezing and rehydration during thawing are organ specific (9). For example, whereas the liver and intestine lose ~50% of their total water during freezing, skeletal muscle loses only 20–30%. The heart, which ultimately loses >50% of its initial water, functions for many hours during freezing (3, 5) and resumes function soon after thawing begins, even as ≥30% of the body water remains frozen.

Reversible organ dehydration benefits wood frogs by limiting the mechanical damage caused by ice propagating extensively in the vasculature (8, 9), a chief cause of cryoinjury to isolated organs (11). However, such dehydration (together with that caused by the freeze concentration of extracellular solutes; see Ref. 13) also promotes cell shrinkage, which potentially leads to irreversible membrane damage. Organ dehydration maximizes glucose’s effect in mitigating these stresses by concentrating the cryoprotectant in a reduced solvent volume (8).

**Evolutionary development of freeze tolerance**

Freeze tolerance in the wood frog is promoted by several integrated responses to ice forming within body tissues that may represent fundamental stress responses that were modified by selective pressures for their present cryoprotective roles. We investigated the evolution of anuran freeze tolerance by comparing the freezing responses of the closely related, but freeze-intolerant, leopard frog (*Rana pipiens*) with those of the wood frog (3). Physiological responses critical to the wood frogs’ freeze tolerance, such as the synthesis and distribution of the cryoprotectant glucose, protective organ dehydration, and deferred cardiac failure, were present, but markedly less pronounced, in *R. pipiens*.

The use of glucose as a cryoprotectant requires that the wood frog tolerate extreme hyperglycemia. Because *R. pipiens* also survives hyperglycemia, such tolerance may represent an important preadaptation to freeze tolerance. Interestingly, glucose supplements did not enhance the freezing viability of *R. pipiens*, although in vitro tests of cryoprotectant efficacy revealed that glucose provided comparable protection to erythrocytes of both species. Apparently, the evolution of anuran freeze tolerance involves not only the development of effective cryoprotective mechanisms but also substantial improvements in the tolerance of tissues and organ systems to freezing and thawing stresses.

**Applications to cryomedicine**

Recent advances in surgical science and immunosuppression have allowed the routine transplantation of some tissues and organs. This result has necessitated improved methods for preserving donor material before transplantation. Current transplantation procedures rely on the cold, but unfrozen, storage of organs. The development of organ banks, similar to blood and sperm banks, would not only allow additional transplants but also significantly improve the quality of tissue matching between donor and recipient (11).

During the past four decades, cryopreservation research has relied almost exclusively on mammalian models for experimentation, even though the tissues and organs of endotherms do not naturally experience the low temperatures resulting in the freezing of tissues. On the other hand, freeze-tolerant vertebrates have “solved” not only the problem of successfully freezing individual organs but that of simultaneously freezing multiple organ systems as well. A major rationale of our research is that the study of these animals, which share many physiological characteristics with higher vertebrates, will provide important clues to the development of protocols for cryopreserving and banking human organs.

Indeed, studies of vertebrate freeze tolerance suggest several fundamental approaches that may overcome some of the current difficulties. First, organ and tissue explants should be frozen slowly and stored at high subzero temperatures, rather than at the extremely rapid cooling rates and liquid nitrogen temperatures conventionally used in cryopreservation procedures. Second, unlike isolated cells in a suspension, the cells and tissues comprising organs have a complex structure that is susceptible to mechanical disruption by ice crystal growth. Thus a preparatory, partial dehydration of explants may be beneficial in reducing cryoinjury to the vasculature. Finally, cryoprotectant additives may substantially improve the capacity for frozen tissue storage.

Significant progress has already been made by applying all three of these principles to improve the viability of cryopreserved rat hearts (15). For example, cryoprotectant-perfused hearts stored frozen (−3.4°C) for 4–6 h showed moderate to high recovery of various functional parameters, including cardiac output, systolic and diastolic pressures, and aortic and coronary flows.

Investigations of the mechanisms of vertebrate freeze tolerance may also provide novel insight into other significant biomedical problems. Freezing in wood frogs is intimately linked to a rapid elevation of blood glucose which in other vertebrates would represent a severely diabetic state. Also, because freezing ultimately results in tissue dehydration, ischemia, and hypoxia, freeze-tolerant vertebrates are useful models in the study of water and electrolyte balance, reperfusion injury, and hypometabolism.

**References**


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**Calcium: A Trigger for Long-Term Depression and Potentiation in the Hippocampus**

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**Introduction**

Excitatory synaptic transmission between hippocampal pyramidal cells is subject to a number of regulatory processes that can change either the amount of transmitter released when an action potential invades the axon terminal or the sensitivity of the postsynaptic mem-

brane to presynaptically released glutamate. Long-term potentiation (LTP) of the strength of synaptic transmission has been intensively studied because it represents a likely cellular substrate for learning and memory. Much like forgetting a previous memory, however, it is to be expected that some other process must counteract or reverse LTP by decreasing the strength of those synapses, i.e., long-term depression (LTD). As a result of years of investigation, there is considerable agreement about the re-

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