SMALL ANIMAL WHOLE BODY CRYOPRESERVATION: PAST AND FUTURE

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PART 1

In this installment of Cooler Minds Prevail, we will talk at length about resuscitation of non-hibernating rodents from circulatory arrest at ultraprofound hypothermic and high subzero temperatures. This will set the stage for developing a model for whole body cryopreservation.

Prior work in hypothermia began in the early 1900s, but because cardiac and respiratory arrest were observed in the animals around 15°C, researchers assumed they were irreversibly dead and made few attempts to resuscitate them from temperatures below this point.

Then came a thermophysiologist named Radoslav K. Andjus working in the Physiology Department at the University of Belgrade after World War II. Since the university's library had been destroyed in an air raid, he was unaware of the "conventional wisdom" that the lethal body temperature of rats was 15°C, and quickly developed a method of reviving animals from temperatures between 0 and 2°C.

His technique, published in 1951, was surprisingly simple. First, he lowered the core body temperatures of rats from physiological (37°C) to around 20°C by enclosing them in glass jars which were then placed in a refrigerator (with the rats re-breathing their own expired air, a method of inducing anesthesia via carbon dioxide inhalation). To further cool the rats, they were packed in crushed ice until colonic temperatures reached about 1°C. They were held at this temperature for 40-50 minutes before resuscitation was attempted.

Andjus first attempted to rewarm the entire body at once in a hot bath, but these animals failed to revive. He quickly determined that the circulation must first be re-established by applying heat locally to the cardiac area before rewarming the whole body. He did so by heating a spatula in the flame of a Bunsen burner and applying it to the chest wall over the heart. Artificial respirations were also given throughout the resuscitation attempt.

While this method was successful, the rate of success (only 20% of rats lived more than 24 hours) left something to be desired. A high proportion of the rats which regained heart beat and respiration died during subsequent warming of the whole body or within a few hours or days of regaining normal body temperature and behavior. When Andjus met Audrey U. Smith, they collaborated in 1955 to determine whether the reanimation failures and high mortality rate after initial success were the result of damage during cooling or due to imperfections of the reanimation protocol. They began by comparing Andjus' initial cardiac heating method to an alternative method employing a high-intensity lamp to project focused light on the chest wall. They found that the easier technique of focusing a powerful beam of light from a projection lamp was even better—76% of these rats survived more than 24 hours, and 68% survived more than 66 days.

In both protocols, rats were cooled according to the previously described method of placing an animal in a jar and then putting the vessel in a refrigerator.

Once rats were cool and lethargic, they were immersed in dishes of melting ice and buried under crushed ice. They remained under ice for exactly 1 hour from the time colonic temperature reached 15°C and for approximately 40 minutes after it had reached 6°C. In general, respiration ceased soon after ice immersion, the heart beat slowed suddenly between 13 and 10°C, and final heart beats were observed at or above 8°C. The rats were removed from ice with body temperatures between 0 and 1.8°C.

When attempting resuscitation using the spatula method, the spatula was warmed in a Bunsen flame and applied as frequently as 20 times per minute. When the first heart beat was observed, artificial respiration began using a small hand-bulb attached to tubing inserted into the nostrils. Local cardiac heating was performed less frequently as heart rhythm became regular, and was discontinued when heart rate increased spontaneously. At 10-11°C the animal's neck was heated under hot tap water and artificial respiration was continued until spontaneous breathing resumed.

When attempting resuscitation using a beam of light, the rats were placed on a platform under a duralite shield with an aperture that allowed for focusing of the light on the praecordium. The intensity of the light/heat could be controlled by changing the variac setting. The neck was warmed under the light beam when colonic temperature reached 10-12°C and artificial respiration was given until spontaneous breathing resumed. Rewarming of the whole

body in both protocols occurred by placing the rats in a 37°C water bath until they could maintain normal posture, at which point they were transferred to an incubator for short-term recovery, then to a warm cupboard for long-term care.

Results obtained by the spatula method were similar to those obtained in Andjus' initial experiments. Out of 25 rats, 4 exhibited irregular heartbeats and then succumbed, 4 exhibited regular heart beats but no spontaneous breathing and were dead within an hour, 6 exhibited spontaneous breathing but no reflexes and died in the 37°C bath, 5 exhibited an apparently complete recovery and died within 24 hours, and only 5 survived for more than 66 days.

Results obtained by the beam of light method were better. Using the initial protocol, which involved a large number of changes in variac settings at lower intensities over the course of warming, 11 of 25 rats survived more than 66 days. Modifications to start at a higher intensity and reduce the number of changes in variac settings resulted in 17 of 25 rats surviving more than 66 days, representing a long-term survival rate of 68%. In addition, far fewer (2) delayed deaths occurred using this protocol.

Andjus and Smith speculate about the importance of proper cardiac warming for successful reanimation from ultraprofound hypothermia in their discussion of these landmark experiments:

It is likely that the method of reanimation is of great importance. When an animal with a deep body temperature of 0-2°C is transferred to a hot bath at +45°C as in the experiments of Lutz (1950) the skin and superficial tissues must rewarm rapidly and experience anoxia for many minutes before the heart is warm enough to beat and provide an adequate circulation. If, on the other hand, the heart is rewarmed first and a circulation established before the temperature of the bulk of the body rises, the degree and duration of tissue anoxia may be greatly reduced.

They go on to anticipate improvements in their method beyond those already achieved:

It was remarkable that the revival rate in our experiments was increased from 20 to 75% when local heating on the surface of the chest wall was superseded by heating with a beam of light. The amount of heat penetrating to the anterior surface of the heart was undoubtedly increased when the chest wall was irradiated, but the oesophageal thermocouple showed that the temperature of the posterior aspect of the heart lagged behind. These results suggest that a more efficient method for rewarming the heart rapidly should make it possible to revive all rats from body temperatures between 0 and 1°C.

Thus began a steady stream of experiments in hypothermic resuscitation, primarily as a means of determining the best method for resuscitating victims of accidental cooling or freezing and to facilitate the use of hypothermia in cardiac surgery.

Andjus and Smith were delighted that they had managed to modify Andjus' chest-wall heating technique from using a hot metal spatula to using a focused beam of light in order to preferentially warm the heart before warming the whole body. This modification resulted in a substantially larger percentage of rats fully recovering from ultraprofound hypothermic temperatures as well as a significant reduction in the number of delayed deaths after partial recovery. However, some delayed deaths still occurred, and Andjus and Smith speculated that these were likely due to the inevitable burns caused by these techniques. So Andjus collaborated with J.E. Lovelock to further refine the protocol in an attempt to eliminate peripheral tissue damage during heating, as described in their 1955 article in the Journal of Physiology[1].

This was accomplished by using a microwave diathermy apparatus "powered by a 500 W continuous wave magnetron operating at a frequency of 3000 Mc/s feeding into an H01 mode waveguide." An aperture was created in an extension of the waveguide and the animal was placed underneath for preferential heating of the heart. Rise in temperature varied across different parts of the body and was steepest in the left side of the chest.

Cooling was carried out in the same manner described previously, and reanimation procedures began when the animal's colonic temperature was between 0 and 1°C. In a first series of experiments, warming was carried out using microwave diathermy and artificial respiration was given by means of a hand bulb and tubing inserted into the nostrils and discontinued after spontaneous breathing was reestablished. When the animal reached 15°C it was then placed in a 40°C water bath for whole body rewarming to 33°C, whereupon it was placed in an incubator for 3 days before being transferred to long-term care in the animal facility.

This first series of experiments resulted in an 80% full recovery rate—already a 5% improvement on the focused light beam method. However, the strong focus of microwaves through the aperture still resulted in occasional burns to the chest wall. With another slight modification to the protocol (the use of a horn radiator to produce a more even field distribution of microwaves), the recovery rate reached 100% and no burns were observed.

Since they appeared to have perfected the method, they performed an exploratory experiment on one rat, cooling it and reanimating it a total of 10 times (each experiment separated by 2-10 days) using the Series II microwave diathermy technique. This rat also recovered fully each time.

From an initial full recovery rate of 20% using the hot spatula method to a 100% recovery rate using microwave diathermy, Andjus demonstrated his grasp of the issues involved in resuscitating rats from ultraprofound hypothermia and a singular dedication to overcoming obstacles. Because of his primary interest in applying these resuscitation techniques to victims of accidental hypothermia and freezing, the road ahead was clear: now that he had a method for reanimating rats that had reached 0–2°C, he next wanted to know the time limits to resuscitation (how long can a rat be held at these temperatures and still be successfully revived?), the effects of multiple coolings and reanimations, and – the ultimate question – whether a rat could be *frozen* and then resuscitated.

PART 2

Up to this point we have discussed the groundbreaking research in the early 1950s performed by Radoslav Andjus in resuscitating rats from body temperatures between 0 and 2°C. Having determined that preferential heating of the heart improved chances of revival, Andjus perfected the technique a number of times, eventually obtaining a 100% resuscitation

rate by use of microwave diathermy. Having established a technique that ensured a high percentage of recovery, he began to investigate other problems related to resuscitation from hypothermia, including the effects of repeated coolings to zero and the possibility of resuscitating rats cooled to subzero temperatures.

In his 1955 publication in the *Journal of Physiology,* Andjus briefly states that cooling to 0-2°C was performed as described in earlier papers[1]. Cooling to subzero temperatures required immersion of the animal into a bath of propylene glycol between -5 and -20°C. The method of reanimation was microwave diathermy (as described in the first installment of this series).

To study the effect of length of time at ultraprofound hypothermic temperatures, Andjus cooled six groups of ten rats to colonic temperatures of 0-1°C, with each group kept cold for a different length of time before attempting reanimation. The "period of suspended animation" ranged from 60-70 minutes to 110-120 minutes in 10 minute increments. Andjus defined the period of suspended animation as that "spent below 15°C prior to the application of heat."

10 out of 10 animals recovered completely from the group that spent 60-70 minutes in hypothermic circulatory arrest, but the longer the period of suspended animation beyond that, the lower the recovery rate, with 6 animals recovering from 70-80 minutes, 4 from 80-90 minutes, 3 from 90-100 minutes, 1 from 100-110 minutes, and none from 110-120 minutes. Several delayed deaths also occurred in animals revived after 70-100 minutes of "suspended animation."

Another series of rats was cooled to 0-0.5°C for 60-70 minutes and resuscitated repeatedly. The results of this experiment were extremely interesting. Initially, a rat was cooled and resuscitated every other day for a total of 8 coolings over 16 days. The rat lost a lot of weight, was unable to regulate its body temperature, and died 18 days after the last cooling. The next rat was cooled repeatedly but had longer intervals between coolings and was allowed to regain its initial weight after the first cooling.

Interestingly, this rat was able to tolerate a longer periods of suspended animation (80 minutes) after several coolings, and recovered fully from a total of 10 coolings over 43 days.

Other rats were then repeatedly cooled to zero and allowed to regain their initial weight before each cooling. Andjus noticed that the time to regain weight also decreased with successive coolings, noting that "one rat needed 11 days to regain its initial weight after the first cooling, 6 days after the second, and 1-3 days after the third to eighth cooling. The means taken from the results obtained with a group of seventeen animals show the same tendency."

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This trend in improved recoverability after repeated coolings appeared to hold true across the board:

Further improvements in the recovery after reanimation were noted in repeatedly cooled rats. For a few hours after the first reanimation and artificial rewarming to 37°C the rat is not able to maintain its normal body temperature in a cold environment. When left in a refrigerator at 0 to +3°C the reanimated rat steadily cools down. By contrast, a number of rats reanimated for the sixth to eighth time were perfectly capable of maintaining their normal body temperature in the refrigerator.

It was also noted that rats reanimated for the first time, and having just resumed their heart beat and respiration, with a body temperature of 15°C (see Andjus & Smith, 1955) were not capable of spontaneous rewarming to 37°C when left at room temperature (21-23°C), and died after a few hours if the rewarming

was not completed artificially. By contrast, a number of rats reanimated for the fifth to eighth time spontaneously regained their normal body temperature when left on the bench with colonic temperatures of 15°C.

In addition, one rat cooled for the third time and another for the sixth time tolerated 120 minutes of suspended animation with full subsequent recovery. None of the ten control rats even recovered spontaneous respiration after cooling the first time to 0°C for 120 minutes.

Finally, Andjus investigated the recovery of rats from subzero temperatures. Some rats were "supercooled," while others were allowed to undergo ice crystallization. In supercooled rats, both subcutaneous and colonic temperatures dropped steadily to temperatures as low as -5.7°C subcutaneous and -3.3°C colonic and for as long as 40 minutes in the subzero range. Andjus reported that "all rats supercooled without crystallization were reanimated, recovered completely, and resumed growth."

Animals that underwent crystallization did not fare so well. Eight of nine recovered heart beat and spontaneous breathing, but all died during rewarming or within 24 hours post-reanimation.

These experiments mark the beginning of investigation into the effects of hypothermic temperatures on mammalian physiology in a number of laboratories. And while Andjus had determined a method for achieving excellent (75-100%) recovery rates in rats cooled to 0-2°C by local cardiac heating prior to warming the whole body during resuscitation, in simultaneous experiments, Audrey Smith quickly found that hamsters appeared to require whole body warming for resuscitation from ultraprofound hypothermic temperatures. In fact, no hamsters were revived using the local cardiac heating technique, which prompted Smith to question whether it was really necessary for resuscitating rats and other non-hibernating mammals. As usual, the answer to this question was to be determined through experimentation, this time in collaboration with S.A. Goldzveig in 1955 (results were published in the Journal of Physiology in January 1956)[2].

Goldzveig and Smith first replicated

previous experiments by cooling 104 rats (150-190 g) to between 0.1 and 1.2°C and attempting resuscitation using local cardiac heating with microwave diathermy. Of these rats, 78 were long-term survivors.

These experiments proved that local cardiac heating is not necessary for complete recovery of adults rats and mice from ultraprofound hypothermic temperature, and, almost as importantly, that a simple bench lamp was as effective as a microwave magnetron in recovering animals from this state of "suspended animation."

Whole body warming was initially carried out on 12 rats using three 100 W bench lamps. The rat was placed supine on a wire grid and two lamps were placed as close as possible to the ventral surface and a third beneath the dorsal surface of the body. Three rats resumed breathing but died soon afterward. The authors reported that "the appearance of the skin suggested that the animals had been overheated."

So they tried again. This time they placed the two 100 W ventral bench lamps further away, leaving an air gap 5-8 cm from the body. The third lamp was reduced to 60 W and also placed 5-8 cm from the dorsal surface of the body. All twelve of these animals recovered completely, suggesting that the position of the lamps was paramount to recovery. Thinking that intensity of illumination might also be of importance, they played with reducing illumination further: 4 of 6 rats rewarmed using one 40 W and two 100 W lamps recovered completely, but further reductions in illumination did not produce better results. In general, rats resuscitated by local cardiac heating began breathing spontaneously much earlier (within 11-15

minutes) than rats resuscitated by heating the whole body (14-23 minutes), but no other differences in rats recovered by the two methods were observed.

Mice (22-38.5 g) of two different strains (one of which was susceptible to bacterial hepatitis) were used for further cooling experiments. They were cooled in the same manner as rats, but due to having much less body mass they cooled much more rapidly. They ceased breathing between 4.5 and 5°C, and the heart stopped beating between 2.5 and 3.8°C. Mice were left in ice for 55-60 minutes after respiration and heartbeat had stopped.

Two methods of resuscitation of ice-cold mice were attempted. Nine of eleven mice were long-time survivors of local cardiac heating by microwave diathermy (intensity of the microwave was reduced to account for smaller body mass). The first attempt at recovering mice by whole-body illumination under a 60 W bench lamp resulted in a 100% recovery rate, but was followed by 10 delayed deaths at 3 weeks, which upon necropsy were found to be due to fulminating hepatitis. The experiment was repeated using another strain of mice, of which all recovered fully and 19 of the 21 were long-term survivors.

These experiments proved that local cardiac heating is not necessary for complete recovery of adults rats and mice from ultraprofound hypothermic temperature, and, almost as importantly, that a simple bench lamp was as effective as a microwave magnetron in recovering animals from this state of "suspended animation." Goldzveig and Smith admitted that even these results could probably be improved upon by further manipulation of variables, but stressed that "…from a theoretical point of view, these results are of great importance and suggest that the danger of tissue anoxia has been exaggerated."

PART 3

After successfully reanimating rats from deep body temperatures of 0–2°C and subsequent respiratory and cardiac arrest, Radoslav Andjus allowed the survivors to live for many months afterward in order to observe any long-term effects of hypothermia. What he noticed, beyond

temporary weight loss and a couple of rats with impaired temperature regulation, was that animals that had been cooled did not appear to suffer any gross or debilitating effects. Although food intake and sexual behavior were initially diminished, the rats regained healthy appetites within a few days and went on to produce normal offspring within 3 months of cooling.

Andjus, having pioneered a method of resuscitation of rats from ultraprofound hypothermia, also had occasion to take the first look at the effects of hypothermia on learning and memory. In his brief 1955 *Nature* publication, "Effects of Hypothermia on Behaviour,"[1] Andjus first compared the ability of cooled (0–1°C) vs. untreated (control) rats to learn a serial problem-solving task. Next, he compared two groups of rats cooled to different temperature ranges (0–1°C and 13.4–18.5°C) to controls in a classical maze-learning paradigm. Rats were trained on the maze, cooled, tested for retention, and finally trained on a serial problem-solving task.

The results showed a significant impairment in problem-solving ability in rats cooled to 0-1°C compared to controls, but not in rats cooled to 13.4–18.5°C. However, the effect was only temporary, as demonstrated by the fact that impairment decreased as the interval between cooling and testing increased. And though memory retention was also affected by hypothermia, Andjus stated that "the differences among experimental and control groups were very small, and in no instance were they statistically significant," indicating that even severe hypothermia does not produce permanent long-term physical or behavioral changes.

These initial results were supported in another experiment by N. Mrosovsky in 1963[2], who reported that severe hypothermia did not affect the response of rats to a conditioned avoidance task when cooling was begun only 15 minutes after animals were trained to criterion. In this task, rats were placed in an apparatus with electrified wire flooring such that either side of the cage floor was capable of shocking the animal. To facilitate one-session avoidance learning, the rats were first taught that they could escape from shock by undergoing

20 shock trials at varied time intervals (30, 60, 90, and 120 seconds) in random order. Then they were conditioned to avoid the shock (conditioned response) by responding immediately to a light (conditioned stimulus) that came on inside the dark experimental room 8 seconds before the shock. The light stayed on until the rat crossed the dividing line between the two sides of the apparatus. When they reached the criterion of six successive avoidance responses, experimental animals were returned to their home cages for 15 minutes before cooling was initiated and rewarming was carried out under a bench lamp. Control animals remained in their home cages until retesting.

Both experimental (cooled) and control (untreated) groups were retrained in the avoidance task 13 days after hypothermia. On Day 14, after three successive avoidance responses, training was continued, but the shock came on in the opposite side of the box at the same time as the light (both were on for 8 seconds). The rat was successful in this "reversal procedure" if it stayed on its side of the apparatus while the light was on six consecutive times.

He reported no significant differences in initial learning, citing a median number of trials to criterion of 9.5 for cooled animals and 11.0 for controls on Day 13 retesting. The median number of shocks received was also similar (3 vs. 2) in both groups. There were also no significant differences in reaching criterion on Day 14 re-testing, nor in the reversal procedure.

Mrosovsky wisely points out in his interpretation of these results that

> It must not however be assumed from the lack of evidence that hypothermia readily disrupts retention that behavior is unaltered. In the work of Andjus et al. (1956) and that of Sudak and Essman (1961), while retention was not changed, the ability on problem solving and habit reversal were decreased, even several weeks after the cooling.

He goes on to mention that the "motivating conditions" of those experiments are different from his own, which may explain differences in results, but also says that it may be possible that initial learning is more likely to be altered than retention after hypothermia. According to this hypothesis, he classes hypothermia along with anesthetics in the category of agents having mild retroactive effects on learning and memory (i.e., those affecting memories consolidated immediately before the interfering event).

After spending a few years perfecting Andjus' technique for resuscitating rodents (rats and hamsters) from ultraprofound hypothermic and high subzero temperatures, Audrey Smith upped the ante and attempted the same feat in larger mammals. In her 1957 publication, "Problems in the resuscitation of mammals from body temperatures below 0°C[3]," she detailed the results of such experiments performed on Dutch rabbits and small primates of the species Galago crassicaudatus agisymbanus.

Andjus, having pioneered a method of resuscitation of rats from ultraprofound hypothermia, also had occasion to take the first look at the effects of hypothermia on learning and memory.

Smith used a modified version of the closed vessel technique to anesthetize and initiate cooling in the rabbits, then placed them in ice water for further cooling. Respiration ceased between 13 and 21°C and the heart stopped beating a couple of minutes later when temperatures were 3 or 4 degrees lower, at which point the rabbits were immersed in -5°C baths. Due to larger body mass, it took much longer for deep body temperature in rabbits to drop from 15 to 10°C than it had in hamsters, though the rabbits' extremities froze quickly. Smith wished to avoid this discrepancy, so she attempted to speed cooling by injecting a cold, creamy saline and serum mixture into the stomach and rectum. Cooling

was certainly faster, but unfortunately the gastric mucous membrane was damaged and sometimes the stomach ruptured, forcing her to abandon this method. Further investigation finally led her to determine that thoroughly wetting the undercoat to remove all insulating air from the fur and vigorously stirring the -5 degree bath led to a fall in deep body temperature to the freezing point of plasma within 20 to 40 minutes of extremities freezing. Galagos were cooled similarly. Their extremities had been freezing for around 40 minutes by the time internal organs began to freeze.

James Lovelock built a larger microwave diathermy apparatus for Smith's rabbit and primate experiments on the assumption that larger body masses simply needed more magnetronic power. Initial warming attempts resulted in severe superficial burns before the rest of the animal had been thawed. Compensating for this effect resulted in the next few animals' visceras being cooked. Finally, a technique was determined for warming from -0.6° to 10 or 15°C within a minute.

Fifteen rabbits and two galagos underwent this treatment and resumed heartbeat and pink mucous membrane coloring when temperature reached around 15°C. Between 20 and 30°C they began breathing and diathermy and artificial respiration was stopped while gentle warming was continued under a heat lamp or in an incubator, while a few rabbits were left at room temperature. Smith describes the results:

> Muscle tone improved and the animals made spontanous movements. Some of them, including the two galagos, sat up and moved around. Within about an hour, however, the reanimated rabbits and galagos all collapsed and died. At post mortem the only obvious lesion was a severe haemorrhage in the upper part of the stomach. This is the part of the stomach which secretes hydrochloric acid.

Smith had noticed similar lesions in the stomachs of hamsters she had frozen which had died shortly after resuscitation, also from the acid-secreting portion of the stomach. She theorized that lowering

body temperature disabled the function of mucous-secreting cells (which protect the stomach from acid) by increasing their permeability to hydrochloric acid and causing the acid in the stomach to diffuse and injure blood vessels. Smith tested this theory by neutralizing stomach acid with sodium bicarbonate during cooling but before freezing. This time, after resuscitation, there was no sign of gastric hemorrhage. Sadly, the rabbits undergoing this treatment still did not live more than 4 hours, and two galagos which seemed to make an excellent recovery died within 24 hours. Though their stomachs were normal, these animals were found to suffer from pulmonary edema and one had bloody fluid in the duodenum and jejunum.

Other topics investigated and reported within her manuscript were the effects of freezing on the hamster placenta and studies on the isolated heart. Observations made on the placentas of hamsters frozen on the 9th, 10th, and 11th days after fertilization of the egg (when the hamster placenta undergoes rapid growth and freezing disrupts fetal development) indicated that bleeding may also be induced by circulatory disorders. Smith speculated that it may be due to derangement of cardiac muscle tissue itself.

This compelled her to experiment on isolated hamster hearts. Interestingly, although whole hamsters did not survive freezing for 3 hours, isolated hamster hearts resumed beating for several hours when perfused in vitro after freezing for 3 hours. She also found that the isolated rat heart recovered completely after freezing at -2°C for 1 hour, but failed to recover from temperatures below -5°C. Further investigations involving perfusion of hamster hearts with glycerol led to resumed beating of hearts after lowering to -20°C, many of which established a regular beat. These results indicated that the heart may not have been the limiting factor in resuscitating whole animals from subzero temperatures, and that improved methods of cryoprotection might be developed for resuscitation of whole animals from subzero temperatures.

In 1982, P.D. Rogers and G.P. Webb published some of their observations (based on previous papers and a Ph.D. thesis) after carrying out a classroom demonstration of suspended animation in which they cooled rats and then resuscitated them after 30 minutes at 0°C[4]. The demonstration was performed as a means to stimulate discussion among students regarding the characteristics and diagnosis of death, the effects of hypoxia during cooling, and the limitations of ECG measurements.

Because the "Giaja method" of cooling employed by Andjus and Smith induced hypoxia and hypercapnia, the authors were interested in comparing resuscitation rates in hypoxic vs. non-hypoxic animals. They did so by anesthetizing rats and immersing them (except for limbs, tail, and head) in crushed ice and water to induce ultra-profound hypothermia, as measured by rectal temperatures. During the cooling process some animals were artificially ventilated until cardiac arrest (respired rats) while others were not (unrespired rats). After 30 minutes of cardiac arrest at temperatures near 0°C, all rats were ventilated during rewarming in a 40°C water bath until heartbeat returned and reached 60 beats/min, at which point they were removed from the bath and warming was continued under a 100 W lamp. ECG was recorded throughout.

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Rogers found that approximately 90% of respired rats began breathing spontaneously during rewarming and 100% regained heartbeat. On the other hand, less than 10% of unrespired rats recovered spontaneous respiration during rewarming, and when the heart did restart (it often did not), heartbeats were erratic and did not circulate blood due to severe vasodilation assumed to be caused by the combination of hypoxia and hypothermia. Rogers found that he was able to resuscitate 70-90% of unrespired rats by means of abdominal compression (i.e., "abdominal pumping"), but even this method was only successful when the heart restarted.

Though it is easy to assume that hypoxia is the cause of more difficult and less successful resuscitation of unrespired vs. respired rats, Rogers and Webb point out that respired rats may simply be benefiting from the protective effects of hypocapnia on pH changes during hypothermia. They discuss at length the question of "what is the optimal pH in the hypothermic animal," which remains unanswered.

An interesting phenomenon known as "heart block" was also demonstrated by these experiments. ECG recordings obtained from unrespired rats often showed a QRS complex during rewarming, which most people would assume to indicate that the heart had restarted. However, because ECG is simply a record of electrical activity, this is not always the case:

The observation that a QRS complex occurs in the absence of cardiac output illustrates the limitations of ECG measurements. The ECG is a record of electrical activity within the heart, and any conclusions about mechanical events are extrapolation, though usually with sound theoretical and empirical foundation. In fact, when the chest cavity is opened in unrespired animals with temporarily restarted hearts, it is possible to record QRS complexes in the absence of any apparent heartbeat, i.e., dissociation between excitation and contraction.

Suggestions for further hypothermia experiments in rats include measuring blood pressure during cooling and rewarming, removing blood samples for pH and gas analysis during the experiment, and monitoring electroencephalogram (EEG). Having discovered in previous experiments that unrespired rats suffered from a collapse

in blood pressure during cooling prior to cardiac arrest, while cardiac arrest and blood pressure collapse occurred simultaneously in respired rats, Rogers also wonders whether this pre-arrest collapse can be prevented with vasoactive medications and whether this would improve resuscitation rates in unrespired rats. Answering questions such as these would have far-reaching implications in the treatment of accidental hypothermia in humans.

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The method that was used by Rogers et al. to resuscitate rats from ultra-profound hypothermia appears superior in terms of animal welfare and equipments needs. Because hypothermia is not induced by methods that induce hypoxia (as in the experiments of Andjus and Smith), the need for specific warming protocols are greatly lessened. The use of anesthetics and ventilation during cooling allows the researcher to exclusively focus on the mechanisms of cold circulatory arrest and investigate methods (such as administrations of medications or complete blood substitution) to prolong the period rats can tolerate ultra-profound circulatory arrest and even subzero temperatures.

PART 4
INTRODUCTION
In the previous three installments of this series we reviewed a variety of challenges that researchers had to go through to achieve a protocol that allowed for reproducible resuscitation of small animals (rats and hamsters in particular) from ultra-profound

hypothermic temperatures and even high subzero temperatures. Perhaps the most crucial finding in these experiments has been the importance of providing adequate metabolic support during the cooldown and warming phase for good functional recovery.

While Andjus and Smith were successful in recovering some small animals from high subzero temperatures, Smith recognized that recovering whole animals from temperatures lower than -5 degrees Celsius would necessitate the use of replacing the blood with a cryoprotectant. She writes, "If the artificial circulation was to be used to hasten the process of freezing, the fluid circulated would have to contain a substance to prevent it from freezing at temperatures below zero...So far no technique has been evolved for perfusing individual organs or the whole mammal with glycerol and removing it without damage. If this could be done it might be possible to cool the intact mammal to and resuscitate it from temperatures as low as -70 degrees Celsius. Long-term storage of frozen mammals might then be considered. It must be emphasized that there is no prospect of accomplishing this in the near future."(1). Smith clearly recognized that small animal whole body resuscitation from cryopreservation required a combination of technological advances and specialized skills that were (almost) unavailable to her at the time.

BIOLOGICAL AND TECHNICAL REQUIREMENTS
Here we will outline a number of biological and technological issues that need to be addressed to make a credible attempt to resuscitate small animals from temperatures lower than Smith ever attempted.

1. **Cryoprotection.** While Smith et al had mixed results with resuscitating small animals from high subzero temperatures (between 0 degrees Celsius and 5 degrees Celsius) it is generally recognized that recovery from lower temperatures necessitates the use of a cryoprotectant such as glycerol or DMSO. If the animal were

cooled to the temperature of liquid nitrogen (or the glass transition point of the cryoprotectant) a simple mono-agent like this may not suffice and a contemporary low-toxicity vitrification agent that allows cryopreservation without freezing (such as M22) would be required.

2. **Extracorporeal Circulation.** A corollary of the requirement to use a cryoprotectant is the need for an extracorporeal perfusion circuit to deliver and remove the agent. This will allow the blood of the animal to be replaced with a cryoprotectant to permit lowering the temperature without freezing. Such a perfusion circuit will also permit the researcher to control flow rate, pressure and temperature. To allow the cryoprotectant to be perfused in a controlled manner without causing excessive osmotic injury the researcher would either need to introduce the solution in a series of steps with successively higher concentrations, or build a device that allows instantaneous mixing of the so called "carrier solution" and the cryoprotectant to create a smooth (linear) ramp.

3. **Surgical Access.** To replace the blood of the animal using extracorporeal perfusion microsurgical skills are required to place cannulae in the animal. A number of options are available for the researcher, including cannulation of the tail, femoral, heart and neck vessels. It is important to ensure that these vessels and nearby organs are not injured to a degree that would exclude resuscitation of the animal. For example, the transcardial perfusion protocols that are routinely used for small animal fixation cannot be used because they irreversibly damage the heart and the ability of the animal to breathe on its own.

4. **Temperature Control.** Rigorous control of temperature is important

in all parts of the procedure. During the initial cooldown cooling and metabolic support (respiration) need to be synchronized. Introduction of the cryoprotectant should be conducted at the lowest possible temperature to mitigate cryoprotectant toxicity. Uniform and rapid cooling below zero degrees Celsius is necessary to prevent ice formation and fracturing. Conversely, a suitable warming technology is required to prevent ice formation (or "de-vitrification") and ischemia upon warming.

5. *Viability tests.* In a sense, testing for viability after small animal whole body cryopreservation is straightforward. If the animal recovers (long-term) cardio-respiratory and brain function the experiment can be deemed successful. In reality, however, the researcher may not immediately be successful and more modest viability tests can be used to measure whether progress is being made. Examples of such tests include tests of isolated organs (such as the heart) or electrophysiology measurements of brain slices of animals that underwent the whole body cryopreservation protocol.

CHALLENGES

In the remainder of this article we will discuss a number of specific challenges that need to be resolved to implement this model.

It is of crucial importance to first successfully establish a reproducible hypothermic resuscitation model. Without being routinely able to recover small animals from temperatures close to 0 degrees Celsius as a baseline it is going to be difficult to identify the unique challenges associated with cryopreservation. Along similar lines, it should also be recognized that unless one is able to load and unload the cryoprotectant at 0 degrees Celsius with recovery of the animal, it will not be useful to move to the next step where the temperature is lowered below zero

degrees Celsius. In other words, the next step after hypothermic resuscitation should not be going straight to cryopreservation but replacing the blood of the animal with the cryoprotectant of choice and re-introducing the blood again without loss of integrated functional activity. If this can be done successfully, lower temperatures can be introduced.

Smith clearly recognized that small animal whole body resuscitation from cryopreservation required a combination of technological advances and specialized skills that were (almost) unavailable to her at the time.

Choice of cryoprotectant and concentration is also a non-trivial challenge. This is not just a matter of adapting a human cryopreservation protocol for small animals. After all, the objective of small animal cryopreservation resuscitation research is to *recover* the animal. We do not know exactly when a person is rendered unrecoverable *by contemporary criteria* in modern cryonics but it is a reasonable assumption that the concentrations and protocols currently used still cause too much cryoprotectant toxicity. The most logical step therefore would not be to load the animal with a concentration necessary to vitrify (CNV) but to use a concentration that permits ice-free exposure to high subzero-temperatures first (say -5 degrees Celsius). It is not clear at this point whether this favors using a lower concentration of the least toxic vitrification agent known today or simply a low concentration of the more well-known mono-agents such as glycerol or ethylene glycol, or a combination thereof. When it is possible to load and unload such a cryoprotectant, cool below zero degrees Celsius, unload the cryoprotectant and recover whole body function, then the

concentration can be slightly increased and the same protocol can be used for progressively lower temperatures.

There can be no successful recovery of a whole mammal without avoiding injury to the vessels and major organs of the animal. Audrey Smith already hypothesized that during deep hypothermia the selective permeability of the inner lining of the gastric mucosa is lost, which needs to be addressed in any successful resuscitation attempt. It has also been firmly established that exposure of the brain to a cryoprotectant will produce shrinkage, which can become quite severe with higher concentrations. How much shrinking of the brain is tolerated without loss of viability is an important question for whole body resuscitation because if brain function is not recovered, whole body resuscitation is not possible. As emphasized before, this issue further reinforces the need to first successfully load and unload a cryoprotectant in a whole body without subjecting it to cooling. Last but not least, how vulnerable are organs such as the lungs to cryoprotectants, osmotic injury, and cryopreservation?

Without being routinely able to recover small animals from temperatures close to 0 degrees Celsius as a baseline it is going to be difficult to identify the unique challenges associated with cryopreservation.

The cryopreservation procedures employed by Alcor exploit the perfusion methods originally developed for use in cardiopulmonary bypass. Research in cryonics has long depended upon the use of animal models to develop improved perfusates and related technologies. Previous research at Alcor was largely carried out using dogs. An initial experiment was performed in 1977 to duplicate Alcor's first human cryopreservation, which was carried out in the previous year, and to

hone the skills of the suspension team (2). Afterward, a series of seven total body washout experiments were performed, of which five were successful (i.e., the animals recovered fully) (3). While such pioneering research efforts were instrumental to the development of current cryonics techniques and technologies, progress made toward miniaturization of the extracorporeal circuit renders the use of dogs as experimental animals less clearly necessary and creates an ethical imperative to explore a small animal model like the rat as a substitute.

The rat is a species in which the effects of cerebral ischemia on histology and neurological outcome have been studied and well-characterized. In addition, the cardiovascular system in the rat is similar to that found in humans (4). The ascending aorta, aortic arch, and descending aorta are completely analogous. The only major difference is that the rat has three vena cavae (two superior and one inferior), whereas the human has two.

The extracorporeal circulation circuit (ECC) itself should consist of specialized tubing to contain perfusate, a pump to circulate the perfusate, a membrane oxygenator to oxygenate the perfusate when necessary, a venous reservoir to serve as a buffer for fluctuations in venous drainage, and a heat exchanger for temperature control. Importantly, the priming volume (i.e., the total volume of perfusate within the circuit) should be as small as possible. In the past, rodent CPB circuits have utilized relatively oversized (10-25 times) clinical pediatric devices. Even using the smallest neonatal oxygenators on the market for CPB in the rat results in a disproportionately large prime volume, and therefore excessive hemodilution, as well as an imbalance in the membrane surface area to body mass and priming volume to blood volume ratios. Reducing circuit volume also helps reduce heat loss and confers greater control over perfusate temperature. The past 10 years have seen the design and manufacture of miniaturized extracorporeal circuit components for use in research which can be modified to use in a small animal whole body cryopreservation model.

Surgery and associated procedures required for extracorporeal circulation in a rodent model are the same as those required by human cryonics cases, the most important of which is cannulation of specific vessels for introduction of perfusate to and return of perfusate from the body. The experimental preparation includes anesthesia, orotracheal intubation, ventilation of the lungs, and cannulation of the vessels. The small size of the animal does increase the level of difficulty of (micro)surgery, but most such difficulties are overcome with sufficient practice and expertise. For many years, adequate venous return was a major concern for researchers attempting full bypass using the rat model. But rapid improvements in cannulation techniques have resulted in consistent achievement of optimal flow rates (5). Several different vascular approaches for extracorporeal circulation have been attempted in the rat with varying degrees of success, but the literature now seems to strongly favor cannulation of the tail artery for arterial inflow and the jugular vein for venous return.

How much shrinking of the brain is tolerated without loss of viability is an important question for whole body resuscitation because if brain function is not recovered, whole body resuscitation is not possible.

As this review makes clear, it is now possible to create a physical infrastructure to continue the whole body small animal resuscitation experiments pioneered by researchers such as Andjus and Smith at even lower temperatures. Aside from contributing to the ultimate goal of developing full body suspended animation, a small animal cryopreservation model can also help address specific issues in human cryopreservation such as cryoprotectant toxicity in vivo, and the effects of cerebral dehydration on viability. ∎

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