Original Article

Time Limiting Boundaries of Reversible Clinical Death in Rats Subjected to Ultra-Deep Hypothermia

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ABSTRACT

Background: It is well known that body temperature maintenance between 20 and 35 C prevents hypoxic damage. However, data regarding the ideal duration and permissible temperature boundaries for ultra-deep hypothermia below 20 C are rather fragmentary. The aim of the present study was to determine the time limits of reversible clinical death in rats subjected to ultra-deep hypothermia at 1–8 C.

Results: Rat survival rates were directly dependent on the duration of clinical death. If clinical death did not exceed 35 min, animal viability could be restored. Extending the duration of clinical death longer than 45 min led to rat death, and cardiac functioning in these animals was not recovered. The rewarming rate and the lowest temperature of hypothermia experienced did not directly influence survival rates.

Conclusions: In a rat model, reversible ultra-deep hypothermia as low as 1–8 C could be achieved without the application of hypercapnia or pharmacological support. The survival of animals was dependent on the duration of clinical death, which should not exceed 35 min.

Keywords: Cardiac arrest, clinical death, survival rate, suspended animation, ultra-deep hypothermia

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BACKGROUND

Hypothermia is a state in which the body temperature of an organism drops lower than is required to maintain normal metabolic functioning. During hypothermia, metabolic rates decrease, which result in the reduction of oxygen consumption. Different stages of the core temperature drop are classified as mild (33–35°C), moderate (30–32°C), deep (20–30°C), and ultra-deep (<20°C) hypothermia.[1,2] At present, the most studied stages of hypothermia are mild and moderate. Small overcooling to 32–34°C has been widely used for decreasing the risk of undesirable complications of insufficient blood supply or trauma.[3,4] Furthermore, in limited published studies of deep hypothermia, authors have described mainly the negative consequences of cooling warm-blooded animals to temperatures that have ranged from 18 to 28°C.[5-7] It has been noted that such cooling is accompanied by biochemical and visceral disturbances, which may result in the death of the organism.[8-10] Among the primary causes of death associated with deep hypothermia are intracranial pressure rise caused by cerebral edema, disturbance of cardiac performance, and renal damage.[11-14] Medications used to facilitate the entrance to and exit from a deep hypothermia state include L-type Ca-channel blockers, antioxidants, polyethylene oxides, and urea and its analogues.[4,5,9,15]

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The majority of studies investigating ultra-deep hypothermia have largely concentrated on the development of emergency preservation and resuscitation (EPR) technology to extend the golden hour (a period after a traumatic injury, when emergency treatment is most likely to be successful) in life-threatening situations. Controlled ultra-deep hypothermia enables successful resuscitation in cases involving serious injuries of the chest cavity that are accompanied by blood loss with a fatality rates as high as 90%.[16] The EPR protocol provides the time required for surgical care by cooling the body to 10°C.[17,18]

The aim of the present study was to a) investigate the possibility of reaching ultra-deep hypothermia at temperature level below 10°C in rats under conditions of forced external cooling following Shcherbakov's method with modifications (Shcherbakov *et al*., 1989) without the application of hypercapnia and pharmacological drugs to obtain initial data on capacities of an organism on which the hypothermia control and management methods could be based; b) evaluate the duration throughout which ultra-deep hypothermia is tolerated and normal vital functions are restored, and c) define optimal cooling and rewarming procedures.[19]

METHODS

Experiments were conducted using male Wistar rats (200–250 g) that were obtained from the vivarium of ICB RAS. In total, 53 rats were used in three series of experiments. The study protocol was approved by the Institutional Animal Care and Use Committee of ICB RAS.

In the first series of experiments, we studied the possibility of reaching reversible ultra-deep hypothermia in rats. We performed fast cooling of animals using an external cold-water flow and achieved temperatures 1–8°C. Animals were subsequently rewarmed to 30°C at a rate of 1.2°C × min^{-1} [Figure 1]. Changes in cardiac function were evaluated by assessing heart rate (HR) dynamics during the cooling–rewarming processes. Forced deep cooling of animals was performed in accordance with Scherbakov *et al*. (1989) with some modifications (the surgical introduction of a pressure sensor into the subclavian artery was excluded to reduce the occurrence of traumatic injury; rewarming rates were increased). Rats were anaesthetized with ether in a transparent glass jar. Then, anaesthetized rats were placed on a surgical table on their backs. To perform artificial pulmonary ventilation (APV), animals were intubated with original tubes (D = 1.8–2.2 mm). The rat ligamentous apparatus reflexively compressed the intubation tube, thus protecting the lungs from water entering airway. To record an electrocardiogram (ECG), needle-shaped electrodes were attached to the paws of each animal. APV was performed with an EPM-2 device (1st MGMU, Russia) using a tidal volume of 1 ml × 100 g^{-1} and a frequency of 70 min^{-1}. Rectal temperature was measured with an electronic thermometer (Actacom-ATT, Taiwan). ECG in the second standard lead was recorded using a veterinary monitor IM-10 (ZooMed, China). Each rat was placed into the plastic chamber with circulating water at 1–4°C at a water level 2

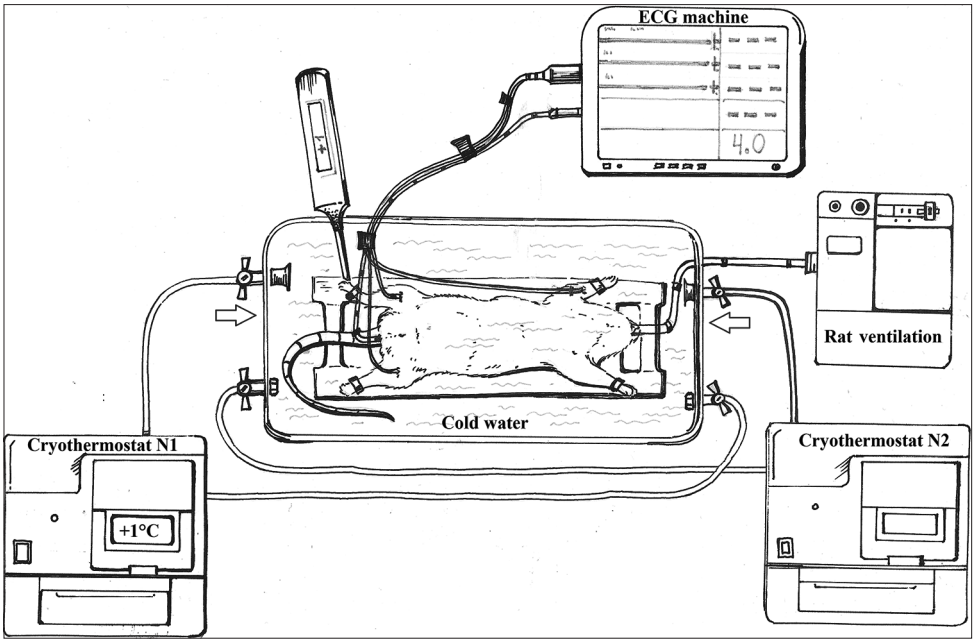

Figure 1: Positioning of the animal in the hypothermic chamber (scheme)

cm higher than the uppermost portion of the body of the animal. Water cooling and circulation were carried out using a Ministat 230 W cryothermostat [Figure 1; Huber, Germany]. Our experiments showed that after reaching a rectal temperature of 10°C, cardiac functioning of rats ceased and only slight electrical activity and some ECG spikes were observed. At 8–9°C, cardiac functioning was completely terminated. Therefore, 10°C was accepted as a starting point for reversible clinical death. Spontaneous recovery of a heartbeat throughout the rewarming period was also observed after reaching a rectal temperature of 10°C. The time period that extended from the point at which the body temperature reached 10°C in the cooling process to 10°C when the animal was rewarmed was designated as the duration of reversible clinical death. APV intensity was gradually reduced to 20 min^{-1} as the rectal temperature decreased. After the cardiac arrest, APV was turned off. The lowest point of hypothermia was regulated by cooling time with indicators of rectal temperature used as a beacon. From 2 to 3.5°C, before the required hypothermic level was reached, the cryothermostat was switched to a heating mode at a given speed to compensate for the inertia that occurred while cooling/rewarming rat body.

At the rewarming stage, each rat was removed from the water on the basis of three indicators that included a rectal temperature of 27–30°C, heart rate above 378 ± 22 beats × min^{-1} and visual signs of motor activity. At this time, the intubation tube, ECG electrodes, and rectal thermometer were removed. Final rewarming was carried out with a dryer by blowing hot air (~50°C) on each animal.

In the second series of experiments (paragraph 3.3 in the results section), we studied the influence of the rewarming rate on the recovery of vital functions in rats cooled to 3°C (group 1; 12 animals) and 8°C (group 2; 12 animals). The rewarming speed was 0.6 (6 animals) and 1.2°C × min^{-1} (6 animals) for each of 2 groups.

In the third series of experiments (paragraph 3.4 in the results section), 12 rats were cooled to 8°C and 6 of the cooled rats were kept at the temperature for 10 min, while the other 6 rats were kept at 8°C for 30–75 min. To stabilize the entrance to the hypothermic temperature plateau, a cryothermostat N2 preheated to 8°C was additionally used. All animals were rewarmed at a rate of 1.2°C × min^{-1}.

Fully recovered animals were returned to vivarium and observed for a month. Animals were housed in standard cages and were provided food and water ad libitum for an equal duration (12 h) of the light–dark cycle. Data analysis was performed using Sigma Plot 12.5 software (Systat Software Inc, US) and all data were expressed as mean ± standard deviation (SD).

RESULTS

Survival of rats cooled between 1 and 8°C

The survival of rats cooled between 1 and 8°C varied according to the level and duration of hypothermia achieved. The heart functioning of animals cooled below 3°C did not recover upon rewarming. The heart functioning of rats cooled to temperatures above 3°C was capable of spontaneously recovering as rectal temperatures increased to temperatures above 10°C, except in animals that were exposed to temperatures of 3–8°C longer than 45 min. Overall, in the first series of experiments, 12 of 17 rats fully recovered after being clinically declared dead. Rectal temperature changes during cooling and rewarming steps are shown in Figure 2.

Heart rate dynamics of rats that survived rewarming

Heart rate dynamics during cooling and subsequent rewarming of surviving rats are shown in Figure 3. As expected, throughout the cooling process, HR decreased as rectal temperature declined. As the figure illustrates, at the same body temperature, HRs were higher during the rewarming than cooling. For instance, at 20°C, the average HRs during cooling and rewarming steps were 110 ± 9 and 150 ± 10 beats × min^{-1}, respectively, and at 30°C, the average HRs observed were 295 ± 33 and 378 ± 22 beats × min^{-1}, respectively.

Influence of rewarming rate on the recovery of vital functions

In order to study the influence of rewarming rate on the restoration of vital functioning, we carried out experiments

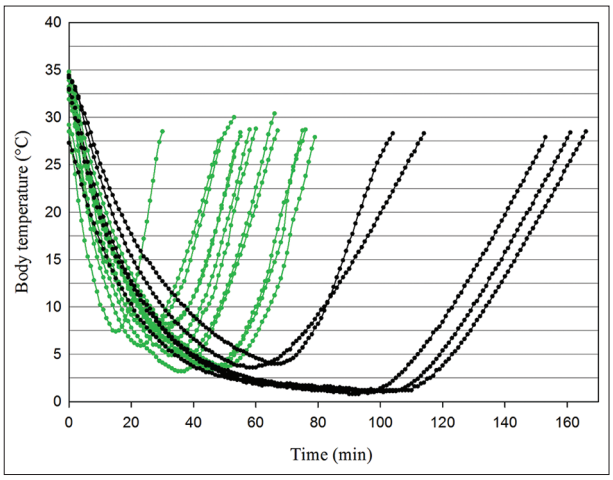

Figure 2: Changes in the rectal temperatures of rats during ultra-deep hypothermia that had been cooled to 1-8°C and subsequent rewarming steps to 30°C. Green lines indicate rats that survived cooling and were successfully rewarmed (n = 12); black lines show rats in which vital functions were unable to be restored (n = 5)

in which rats were cooled to 3°C and 8°C and subsequently rewarmed at different speeds [Figure 4]. In the first case, when cooled to 3°C [Figure 4a], vital activity of animals was recovered after rewarming at a speed of 1.2°C min^{-1}. On the other hand, the cardiac function of rats in the second group, which were rewarmed at 0.6°C min^{-1} was not restored, despite attempts to reanimate the animals. In the second case, when animals were cooled to 8°C, rewarming speed did not affect survival rates of rats in either group, and the vital activities of all animals were restored [Figure 4b].

Influence of the duration of hypothermia on animal survival

The analysis of rat survival in the third series of experiments showed that the duration of clinical death determines whether restoration of the vital activity is possible. Six of six rats survived in the subgroup that was cooled and maintained at 8°C for 10 min, which produced a total period of clinical death that did not exceed 30 min. However, 0 out of 6 rats that were maintained same hypothermic temperature for 30–60 min survived. In this case, the duration of clinical death exceeded 45 min [Figure 5].

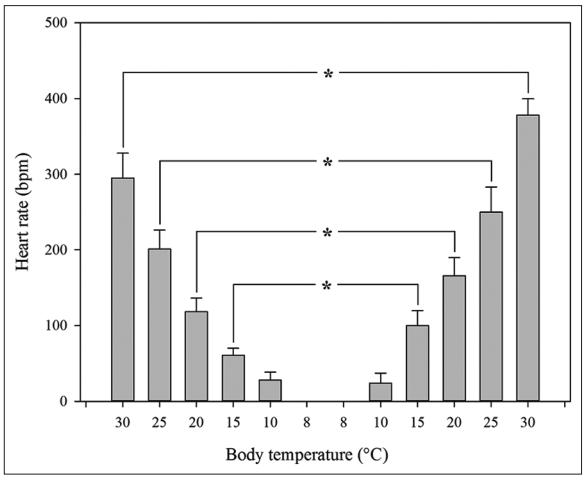

Figure 3: Dynamics of temperature dependent heart rate changes during the cooling and subsequent rewarming of rats (n = 17). The average HR of rats prior to the experiment was 425 ± 30 beats × min-1

DISCUSSION

Results regarding hypothermic rat survival obtained in this study can be explained in terms of the duration of clinical death. The duration of the silent heart, in our view, was a critical factor, which defined whether the animal could be recovered after being exposed to ultra-deep hypothermia at a temperatures 1–8°C. The rewarming rate and the lowest temperature of hypothermia did not affect revitalization. Animal viability could be restored as long as the period of clinical death did not exceed 30–35 min. A period of clinical death that exceeded 45 min led to the death of experimental animals, and cardiac functioning in these animals was unable to be recovered.

Assuming that there is a critical duration of clinical death that determines the capacity of animals to be rewarmed successfully explains observed failures to cool rats to temperatures of 1°C, since it takes about 90 min to reach this temperature. The time required significantly exceeds the duration allowed, and leads inevitably to animal death. Similar results were obtained in experiments with spinal cord neurons, which showed that the duration of hypothermia was the main factor affecting nerve cell survival.[20]

The importance of the duration of clinical death to successful recovery of the vital functions of cooled rats has also been assessed by Andzhus and Hozich (1965). They found that greatest duration of clinical death that facilitated the recovery of the vital functions of rats was

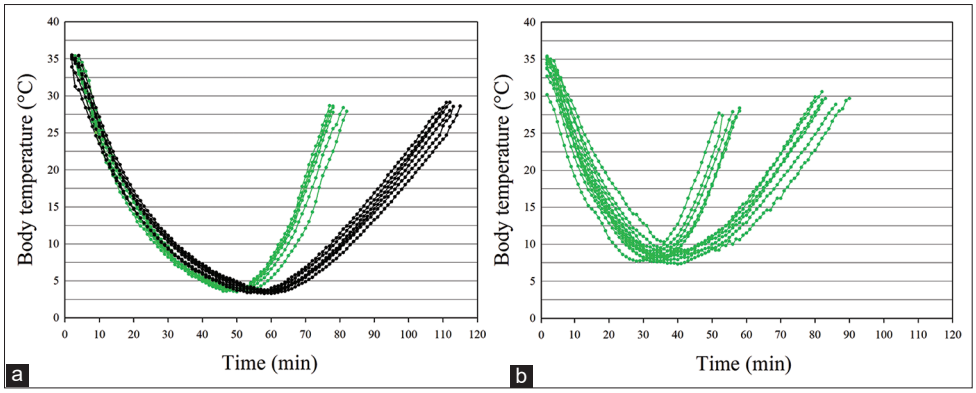

Figure 4: Rectal temperature changes in rats cooled to 3°C (a) and +8°C (b) and rewarmed. (a) green lines indicate rates that survived the hypothermic conditions (*n* = 6) (rewarming was done at a rate of 1.2°C min-1); black lines show rats in which there was a failure to restore vital functions (*n* = 6) (rewarming occurred at a rate of 0.6°C min-1). (b) Green lines indicate rats that survived hypothermic conditions (n = 12) (rewarming occurred at a rate of 1.2°C min-1 (left group) and 0.6°C min-1 (right group)

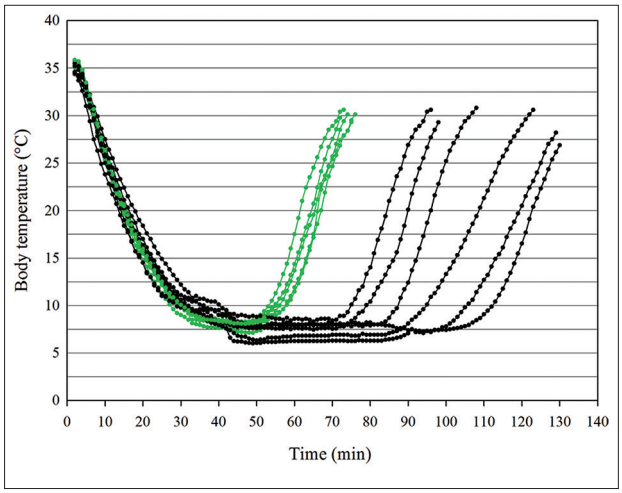

Figure 5: Rectal temperature change in rats cooled to 8°C. Green lines indicate rats that survived hypothermic conditions ($n = 6$) (clinical death duration did not exceed 30 min); black lines indicate rats in which vital functions were not restored ($n = 6$) (clinical death duration exceeded 45 min)

60 min. Increasing time spent in a state of clinical death led to decreases in the number of surviving animals. Observed difference in the maximum duration allowed in our study and the abovementioned work may be linked to the additional use of the hypoxia–hypercapnia method by Andzhus and Hozich prior to the cooling of the animals, which could have increased their resistance to ultra-low temperatures.[21]

A similar approach has adopted Niazi (1957) who used gas mixtures with an increased CO_2 concentration during lung ventilation of monkeys cooled between 4°C and 9°C. In his experiments, animals restored activity after a 2-hour period of clinical death.[22] During prolonged clinical death under ultra-deep hypothermic conditions, metabolic processes slow, but do not fully stop. This leads to energy and oxygen depletion in the organism. Hypoxia that develops during hypothermia leads to direct damage of the brain and heart through disruption of the NA/K pump, accumulation of intracellular Ca, edema, and acidosis. Also, it leads to the accumulation of reduced oxygen equivalents in the mitochondrial electron transport chain, which can stimulate increased formation of reactive oxygen species and oxidative stress process that causes lipid, protein, and nucleic acid dysfunction.[23]

At the same time, tolerating hypoxia–hypercapnia prior to, or during, initial stages of cooling in rats activates defence mechanisms similar to those that exist in hibernating animals.[24] Evoked hypoxia–hypercapnia leads to the accumulation of γ-aminobutyric acid, which is the main inhibitory mediator of the central nervous system. Suppression of neuronal activity increases brain tolerance to hypoxia, decreases oxygen consumption and contributes to survival during hypothermia. Overall, it allows the body to withstand longer periods in a state of clinical death involving ultra-deep hypothermia.[25]

HR dynamics observed in our study show that there is a gradual decrease in heart rate following a temperature drop. When temperature dropped to below 13°C, ECG tracings showed in most cases a decrease in the amplitude of heart contractions, a gradual smoothing of the *P* waves, inversion of the QRS complex, and arrhythmia of ventricular origin. There are data demonstrating that an increase of catecholamine is observed in the blood during cooling in experiments involving moderate hypothermia of 33–34°C.[26,27] We believe that it is not enough to compensate for heart rate decreases under intensive cooling conditions used here. During rewarming, a slight increase in HR was observed compared to cooling step, which is in accordance with previous studies.[3] The arrhythmia arising at the time of rewarming quickly stabilized, however, in some rats, single extrasystoles of the ventricle persisted until they were removed from the water. We suppose that the occurrence of more serious cardiac arrhythmias during cooling and rewarming steps is prevented by lung ventilation which was turned off or started after reaching a rectal temperature of 10°C. It protects the heart from hypoxia, which occurs during the natural respiratory arrest at a temperature of 16.2 ± 0.6°C.[28]

It should be noted that in nature, the problem of reversible ultra-deep hypothermia has successfully been solved. In a state of deep dormancy, the body temperature of the Yakut ground squirrel (Spermophilus undulatus) decreases to 0°C.[29] The animals can remain for 3 weeks in a state in which their heart rates slow to 3–4 beats min^{-1}. The similarity of morpho-physiological changes found in hibernators and non-hibernating animals entering a low-temperature state indicates the presence of common mechanisms that affecting a switch to a low metabolic rates under certain conditions.[28,30-33]

Artificial deep and ultra-deep hypothermia is of particular interest for medicine, since it can be used in intensive surgical treatment of seriously injured or ill (e.g., aortic aneurysms) patients to protect organs during circulatory arrest.[34,35] Emergency Preservation and Resuscitation Technology involves the use of a cold aortic flush to replace blood with saline solution and induce a deep hypothermic state at 10°C for up to 2 h, and facilitates the repair of traumatic injuries.[36,37] Whether hypothermic temperatures can be lowered further than 10°C is the subject of debate. Our

data suggest the possibility of the successful revitalization of rats subjected to cooling to temperatures as low as 3°C, without pharmacological support. Deeper hypothermia may contribute to a further decrease in metabolic activity by an additional 50–70%. Oxygen consumption of the brain decreases 1.8–3.5 fold (depending on the temperature interval) every 10°C.[38,39] The overall metabolic rate of hibernating animals at near-zero temperatures decreases to 1/10–1/100 of the normal physiological levels.[40] Also, decreasing hypothermic temperatures to below 10°C will not significantly prolong the duration of reversible clinical death, according to our data. Theoretically, it may increase the protection of organs against ischemia and decrease possible complications that occur after rewarming.

CONCLUSIONS

The obtained results of this work suggest the following:

1. The probability of the successful recovery of vital functions in deeply cooled rats does not depend directly on the minimum temperature reached (in the range of 1–8°C).
2. The rate of rewarming of deeply cooled rats is not the defining factor for the recovery of vital functions.
3. The successful recovery of vital functions of deeply cooled rats depends on the duration of clinical death. If the duration of clinical death did not exceed 35 min, all animals were able to recover from cooling. The reanimation of rewarmed animals was not observed when the duration of clinical death exceeded 45 min.

The limitation of this study was the emphasis on the time limiting boundaries of reversible clinical death during ultra-deep hypothermia. According to our data, the period from 35 to 45 minutes was borderline in terms of the organism's survival. In borderline group, at the stage of rewarming, in addition to death and recovery, some animals showed a delayed death in a period from several hours to 2 days. It was accompanied by shortness of breath and frostbite signs on extremities. The difference in survival in borderline group probably depends on the individual resistance to hypothermia of a particular animal and does not fit all or nothing boundaries we aimed for. We consider that another limitation of this work is the lack of complex assessment of cognitive functions in rats subjected to ultra-deep hypothermia which could be a subject of further research.

Ethical approval and consent to participate

All animal procedures performed in this study were approved by the Biological Safety and Ethics Committee (Institute ICB RAS) in accord with Directive 2010/63/EU.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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