Mini Review

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Freeze tolerance in wood frogs

Abstract

The wood frogs are a special variety of frogs native to Alaska, Canada and Northern USA. Their ability to tolerate freezing has been regarded as one of the most astonishing natural wonders of the world. These frogs can survive partial freezing of their body. During winter about 60% of their body fluid freezes up, they stop breathing and their heart stops beating. Organ systems become inactive. However, the body remains alive at cellular level. Due to unavailability of oxygen the cells carry out anaerobic respiration to generate energy. Prior to freezing, the frog's liver produces a large amount of glucose molecules which acts as a cryoprotectant and protects the vital organs of the body from freezing. In addition to glucose accumulation many other factors are involved in providing freeze tolerance. With the onset of spring the frozen frogs start thawing, they regain consciousness and recover from the hibernating state within 24 hours of thawing. Unravelling the underlying mechanism behind their freeze tolerance can help to develop a better strategy for cryopreservation of organs for a longer duration in the future.

Keywords: cryoprotectant, anaerobic respiration, thawing, hibernaculum and glycogenolysis

Volume 6 Issue 1 - 2019

Pranab Roy,[1] Prittam Goswami[2]

[1]Institute of Child Health, India
[2]Dept of Biotechnology, Haldia Institute of Technology, India

Correspondence: Pranab Roy, Institute of Child Health, Kolkata, 700017, India, Email pranaboy@rediffmail.com

Received: December 27, 2018 | **Published:** January 16, 2019

Introduction

The wood frogs, *Rana sylvatica* are a special variety of frogs found in the northern part of North America mostly in Canada, Alaska and some parts of northern USA.[1,2] They are generally rusty red, brown, grey or tan in colour and have a dark patch under their eyes also known as the Robber's mask.[3] Their size varies from 51 to 70 mm in length, the females are generally larger than the males.[4] They have received great attention in the scientific world because of their freeze tolerance property. They can survive an average minimum temperature of −14.6±2.8°C with 60-70% of their body liquid being frozen.[5] Under such condition they stop breathing, their heart stops pumping blood and all the other organ systems stop functioning.[6] However with the onset of spring the frozen frogs start thawing, they recover from the zombie state, and their organ systems get activated. This review mainly focuses on unravelling the different mechanism employed by wood frogs to overcome freezing (Figure 1).

Figure 1 depicts a wood frog, *Rana sylvatica* in frozen state.

Unravelling the survival mechanism

For wood frogs, the onset of harsh winter and falling temperature is accompanied by suppression of the immune system and scarcity of food resources.[7,8] When the temperature falls rapidly and approaches the freezing value the wood frogs start searching for a hibernaculum. An ideal hibernaculum site should have a decreased canopy cover, increased leaf litter depth, and greater number of logs and stumps.[9] This will lead to a higher snowpack depth thus providing better insulation and a warmer temperature for the frog to survive and will also provide enough time for the body to produce and distribute cryoprotectant molecules.[10] They generally hibernate in shallow depressions in the soil where they are concealed by layers of leaf litter and snow coverings.[6] Sarcoendoplasmic Reticulum Ca-ATPase or SERCA is an important enzyme found in cardiac and skeletal musclesthat helps in muscle contraction and relaxation by modulating the concentration of Ca^{2+} ions in the myoplasm.[11] The Sarcoendoplasmic reticulum (SR) is a specialized structure present in the cytoplasm that stores calcium ions.[11] The transfer of Ca^{2+} ions from the sarcoplasmic reticulum to the cytosol stimulates muscles contraction, and the active reuptake of Ca^{2+} ions into the Sarcoendoplasmic reticulum facilitated by SERCA stimulates muscle relaxation.[12] The Sarcoendoplasmic Reticulum Ca-ATPase 1 or SERCA1, coded by the gene ATP2A1 is present in the muscles of wood frogs and other animals.[13] It catalyses the hydrolysis of ATP to provide energy required for the translocation of Ca^{2+} ions from the cytosol to the sarcoendoplasmic reticulum leading to muscle contraction.[14] This enzyme gets inactivated at a low temperature thus causing muscle stiffness.[15,16] According to recent studies the Sarcoendoplasmic Reticulum Ca-ATPase 1 (SERCA 1) extracted from the skeletal muscles of wood frogs showed a unique 7 amino acid substitution.[17] This leads to a lower activation energy of the enzyme and a 1.5 fold higher calcium ions transfer rate compared to the wild type variants which helps the muscles to contract even at sub-zero temperatures thus helping the frog to find a perfect hibernaculum prior to freezing.[17] The role of these substitutions at proteomic level are yet to be known.

Wood frogs accumulate very high level of glycogen in their body during autumn.[5] During winter, as the temperature in the hibernaculum approaches the extracellular freezing point (-2 C to -1.9 C) the body starts to freeze this leads to the initiation of glycogenolysis at a great pace in the hepatocytes of the wood frogs.[6] Freezing exposure increases enzymatic activities in the liver. In an experiment carried out by K.B. Storey and J.M Storey it was observed that on exposure to freezing conditions the total phosphorylase content of the liver increased by 520%, the amount of active phosphorylase increased from 37% to 80%.[6] In addition, the activities of enzymes such as glycerol-3-P dehydrogenase, glucose6-P dehydrogenase, 6-phosphogluconate dehydrogenase and glucose 6-phosphatase also increased by 140-160%.[6] With freezing exposure considerable increase in the activities of glucose 6-phosphatase and phosphorylase were also observed in the leg muscles of wood frogs.[6] The enzymes Phosphorylase and Glucose 6 Phosphatase are directly involved in the glycogenolytic pathway as depicted in Figure 2. Their hyperactivity increases the rate of glycogenolysis, thus increasing the production of glucose molecules.[18] This large amount of glucose is mostly synthesized in the liver, and it is then distributed throughout the body through the blood.[6] An over expression of glucose transporters on the surface of the liver cells are observed, and the number of glucose transfer sites was found to be 8.5 fold higher in autumn than in summer this leads to a higher transfer rate of glucose in the blood.[19] Within hours from initiation of freezing, the glucose concentration in the core organs rises by about 100 folds.[16,20] It was found to be 10 fold higher in the heart and 3.3 fold higher in the liver.[6] Glucose acts as the sole cryoprotectant in wood frogs.[21] Prolonged exposure to sub-zero temperatures causes cellular shrinkage as the water from the cells are drawn out due to ice formation in the extracellular spaces, leading to cell death and tissue damage.[22] A liquid freezes only when its vapour pressure becomes equal to that of its solid form.[23] The addition of non-volatile solutes to a liquid reduces its vapour pressure which in turn reduces its freezing point.[24] In the same way, the presence of glucose molecules in the blood depresses the freezing point thus reducing the intracellular ice formation. Increased concentration of glucose in the blood also causes organ dehydration which reduces freezing injury caused due to intercellular ice formation.[25]

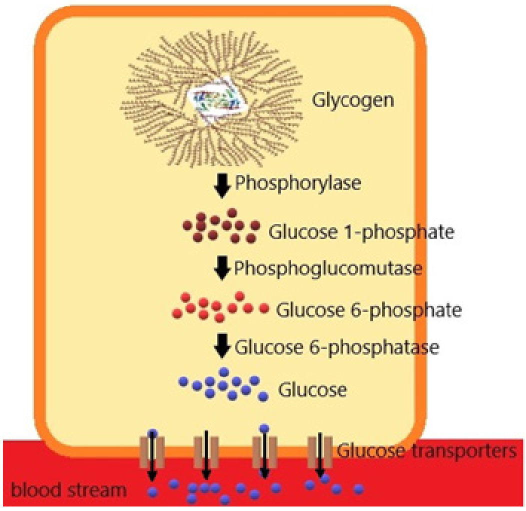

Figure 2 It depicts the process of glycogenolysis that takes place in the hepatocytes of wood frogs. Glycogenolysis leads to the formation of a large amount of glucose molecules which is then transferred to the blood stream through the glucose transporter proteins. The glucose molecules are then transported to the rest of the body through the blood stream.

The insulin extracted from the pancreas of *Rana sylvatica* was compared with the insulin extracted from freeze intolerant American bullfrogs, and it was observed that the primary structure of the insulin from wood frogs differed from that of bullfrogs at A12 (Threonine to methionine substitution), A23 (Asparagine to Serine substitution), B5 (Tyrosine to Histidine substitution) and B13 (Glutamic acid to Aspartic acid substitution) respectively.[26] These substitutions lead to an impaired protein structure.[26] As a result the insulin formed fails to activate protein phosphatase 1 which is responsible for regulating the rate of glycogenolysis through the inactivation of phosphorylase A.[26] As phosphorylase A is not inactivated the rate of glycogenolysis is maintained leading to a high concentration of glucose in the blood.[26] However, the structure of the glucagon was found to be conserved and it didn't show any aberration in its activity.[26] When the wood frogs were dissected at -4 C ice crystals were found surrounding the internal organs, no bleeding, breathing or heart beats were observed.[6] As the organ systems stop functioning it leads to the accumulation of waste products such as urea in the extracellular regions.[27] The urea molecules act alongside glucose and stabilizes cells by reducing water loss.[12] In addition to glucose and urea, a Xylomannan based high molecular weight antifreeze glycolipid (AFGL) is also present in wood frogs.[5] It has a high hysteresis value i.e it can inhibit ice growth by binding to the surface of the nucleating ice crystals, and it can also prevent the incursion of ice into the cells.[5,28]

As physical activity and breathing stops the body cells have to rely only on endogenous sources of energy for survival. The cells switches to anaerobic respiration for energy which leads to the accumulation of lactic acid in the tissues.[6] Creatine phosphate reserves present in the leg muscles are also depleted in order meet the demand for energy.[6] Wood frogs can remain frozen for about 193days at -6.3 C with 100% chances of survival.[5] As spring sets in, the atmospheric temperature rises causing the ice to melt. It takes about 4hours for the wood frog's body to defrost completely.[6] However, it takes about a day for the wood frog to regain consciousness and sensitivity.[9] Recent studies unravelled the upregulation of 5genes in wood frogs in response to freezing.[29] A 457 bp long novel gene was found to be expressed in the liver, gut, heart, lung, brain and bladder of wood frogs with the initiation of freezing.[30] The gene was found to exclusively present in certainfreeze tolerant animals and wasn't found to be homologous to any pre-existing genes present in the gene bank database.[30] The gene was designated as Fr10, and the putative protein was found to carry a hydrophobic N terminal containing a nuclear export signal (NES signal).[30] It was hypothesized to be involved in the movement of certain freezing induced mRNAs from the nucleoplasm to the cytoplasm where they can be translated into freeze tolerance proteins.[30] A second novel gene li16, was also depicted to be upregulated in response to anoxia stress.[29] It codes for a 115 amino acids long protein that is suggested to provide endurance to organs during ischaemia.[29] The Aat gene, coding for ADP/ATP translocase was observed to be upregulated prior to freezing.[31] ADP/ATP translocase stimulates the exchange of ADP and ATP between the cytoplasm and the mitochondrial matrix. It provides a constant supply of ATP required for the process of glycogenolysis that takes place in the cytoplasm.[31] Upregulation of the genes coding for fibrinogen alpha and fibrinogen gamma was also observed during freezing, they are suggested to be involved in clotting to prevent bleeding loss from the ice damaged and ruptured parts of the body during thawing.[24] In general, the physiology of wood frog is quite distinct from other amphibians or mammals which gives them the advantage of survival in extreme cold temperature.

Drawbacks

Although the anti-freezing strategy employed by wood frogs proved to be effective, it has got some limitations. Survival rate appears to be dependent on the degree of extracellular freezing, frogs that were exposed to -30 C had 50% of their body frozen and they failed to recover to their normal state.[32] Temperature fluctuations in the natural environment can expose the wood frogs to multiple cycles of freezing and thawing.[33] This may lead to the generation of reactive oxygen species which can cause immense tissue damage due to oxidative stress.[34]

Conclusion

Global shortage of organs is a major healthcare challenge. As estimated by Global Observatory on Donation and Transplantation in 2010, only 10% of the need for organs are being met.[35] It has been suggested that ignoring all restrictions organ replacement can prevent more than 30% of all deaths in the US.[36] Every year 50,600patients are added to the US transplant waiting list.[37] The above problem can be solved in two steps. The first step is to collect enough organs to meet the global need and the second step is to preserve these organs until they are transplanted.[37] It is the second step that seems to be arduous. Although several techniques such as machine perfusion and organ cryopreservation have been developed the maximum preservation time is very low which increases the average cost of transplantation to about 1 million dollars.[37–44] Mike D. Taylor, Professor at Carnegie Mellon University said "At least 45 species survive bitterly low temperatures including the Arctic wood frog. That's why we think translating the natural mechanisms of survival can affect organ preservation. We can manipulate organs with cocktails from species of nature and extend storage from one or two days to one or two weeks, which would be a game changer in transplants".[45] Unravelling the science behind the freeze tolerance of wood frogs can lead to the development of a better strategy for preserving human organs for a longer time thus making it possible to be easily shipped from one place of the world to another which would also reduce transplantation cost.[22] Disease transmission rates in organ transplantation are 10,000 times higher compared to blood transfusion.[46] Increasing the preservation time would allow thorough screening of organs for transmissible diseases prior to transplantation . This would greatly improve the donor matching process and organ reuse which could save millions of lives throughout the world.[47,48]

Acknowledgments

None.

Conflicts of interest

The authors declare that there is no conflict of interest.

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