Cryopreservation, it will chill you.

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Abstract— Tardigrades are micro-animals capable of undergoing cryptobiosis, a freezing process that allows them to survive in damaging environments. Scientists have taken advantage of this capacity for cryopreservation, conserving cells and tissues in time using different techniques such as vitrification for embryos, encapsulation-dehydration for plants or profound hypothermia in surgery. The mechanism is ruled by thermodynamics processes that follow a loss of water by a chemical potential gradient.

Key words— Cryopreservation, Cryptobiosis, Encapsulation-Dehydration, Freezing, Tardigrade, Recrystallisation, Vitrification

1. Introduction

Cryopreservation is the fact of freezing cells or tissues to preserve them in the future. Did you know that Darth Vader used it with Han Solo in one of the Star Wars movies? This method drags many years of researches related to the thermodynamic process of freezing and is based on some organisms like tardigrades. Moreover, it can be extended to many fields such as plants storage, surgery or in vitro fecundation.

2. History

The phenomena of cryptobiosis, a metabolic state of inactivity in which some organisms enter in response to adverse conditions [1], has been studied since the second half of the XVI century. At those times, biologist realized that this period of inactivity could vary from hours to years, being caused by a huge variety of stimuli: extreme temperature, unavailability of food or water, desiccation or oxygen deficiency [2], [3]. Cryptobiosis includes three physiological states, anhydrobiosis, osmobiosis and cryptobiosis [2].

An important discovery in relation to cryptobiosis, in 1702, was the identification of an organism capable of adapt in many adverse environments [4]. These organisms were called Tardigrades, and were first described in 1773 [5]. They are now known for being able to survive -at least for a few minutes- at temperatures from -272ºC to 151ºC; and for resisting extreme pressures as vacuum; dehydration and radiation [4]. In those cases of extreme environments the metabolism of these creatures reduces to 0.01% of the normal activity, and the water content of the body also decreases to 1% of the normal volume [5].

From the study of phenomena like cryptobiosis a new idea crossed the mind of some scientist: cryopreservation. The history of cryopreservation starts in the early XX century, when James Lovelock suggests that the damage caused in blood cells when frozen could be due to osmotic stress [3]. Here, several studies were done in this area of frozen living cells and it was in 1949 when Ernest John Christopher Polge accidentally discovered the cryoprotective properties of glycerol on fowl sperm. This meant the start of a new field of study which soon began to grow exponentially [6]. From that point, many of those researches were performed in order to learn a way of freezing sperm cell, so that in vitro fertilization became possible.

In 1953 Jerome K. Sherman was successful in freezing and thawing human sperm, and on the same year he founded the first sperm bank. Despite all of the discoveries taken place in the XX century, it was not until 1964 that the term cryobiology was invented and it was defined as a science. In the 80s, several discoveries were done in relation to insemination and pregnancy. For example, in 1986, an Australian biologist called Christopher Chen was successful in the freezing of human oocytes, and this meant a huge step in the fertility field [6].

3. Tardigrade's biochemistry

As tardigrade was one of the first organisms found in relation to cryptobiosis, investigating its genome was crucial to know more about that topic. After sequencing its DNA, it was found that their genome was the most different one from any other animal. They only share about the 17% of their genomic sequence with other species [7].

In a study carried out with different species of tardigrades from different environments, the main points that an organism has to overcome in order to survive cryptobiosis were defined. These critical points are the process of freezing, the energy needed to enter this physiological process and end of the cryptobiosis event. A too rapid freezing could induce physical damage; it is also possible that the organism has not enough energy to deal with this process, so it would die. The ending of the frozen state is also important, as if there has been any damage it should be repaired before 'coming back to life' [8].

In order to survive in extreme environmental conditions, tardigrades are capable of carrying out some preparative steps like losing most of their water, -around the 95%- synthesizing cell protective components and reducing dramatically their metabolism [9]. One of the components that are known to have a cryoprotective role in tardigrades is the ice nucleating protein. These proteins are

important to control the freezing of the body of tardigrades in order to prevent sudden and damaging ice formation [10].

There exist factors which may affect the survival of tardigrades during cryptobiosis. These are similar to the ones affecting these organisms during desiccation and are caused mainly by reactive oxygen species (ROS). ROS can cause damage in macromolecules when the tardigrade is in a cryptobiotic state and, as we have already mentioned, it has to be repaired before the organism leaves this state [11].

As we have said before, another aspect that is important when cooling down is the velocity by which the negatives temperatures are reached. In a recent research, it has been demonstrated the correlation between this velocity and the rate of survival. A possible explanation for this correlation is that, when the temperatures decreased gradually, the organism has time enough to produce all the substances required. On the other hand, when it happens suddenly, there is no way of producing all the compounds [8].

In general terms, the process of cryptobiosis needs such a complicate mechanism, in which many substances and specific conditions are required. That makes all the organisms capable of doing it an important center to focus on for future researches.

4. THERMODYNAMICS OF THE PROCESS

The intracellular medium of living cells is composed essentially by liquid water, and its solidification by freezing is lethal most of the times. However, it is also possible to preserve cells for long periods of time in a frozen viable state. This process slows and even stops some biochemical reactions, but other ones are accelerated at the same time [12].

The physical events which take place in this process of freezing depend on the cooling velocity. If it is slow, water will be lost quickly enough by exosmosis to concentrate the intracellular solutes in order to eliminate supercooling and maintain the chemical potential of water inside the cell at equilibrium with the potential of the extracellular medium. As a result, there is a dehydration of the cell and its intracellular medium does not freeze. On the other hand, if the cell is cooled at a high velocity it will reach equilibrium by freezing intracellularly as it will become increasingly supercooled [12].

The rate of water loss during this process can be described by different equations proposed. The first one associates this rate of loss of cytoplasmic water with the chemical potential gradient expressed as a ratio of vapor pressure [12]:

$$dV/dt = \left(L_p A R T \ln \frac{P_e}{P_i}\right)/V_i^o \quad (1)$$

V: Volume of cell water. **A:** Surface area. **t**: Time.
R: Gas constant. **T**: Temperature. V^o_i: Water molar volume.
L_p**:** Permeability coefficient for water.
P_e**,** P_i**:** Vapor pressure of extracellular and intracellular water.

This equation derives from the combination of two simple principles. One is the application of Fick's Law to the rate of water loss of a cell, which is given by the permeability constant of water (L_p), the cell membrane area (A) and the osmotic pressure gradient between extracellular and intracellular media ($\pi_i - \pi_e$). As the osmotic pressure is a function of the vapor pressure, we can combine these two equations to get equation 1:

$$dV/dt = L_p A(\pi_i - \pi_e) \quad (2) \quad \pi v = RT \ln P^o/P \quad (3)$$

The change of vapor pressure with temperature can be calculated from Clausius-Claperyon equation and Raoult's law [12]:

$$\frac{d \ln\left(P_e/P_i\right)}{dT} = \frac{L_f}{RT^2} - \left[\frac{N_2 V_i^o}{(V+N_2 V_i^o)V}\right] dV/dT \quad (4)$$

N_2**:** Osmoles of intracellular solute.
L_f**:** Latent heat of fusion of ice.

Finally, L_p is related to temperature by the application of the Arrhenius equation, which gives the dependence of a rate constant (in this case L_P) on the absolute temperature T, in relation to the activation energy of the process:

$$L_P = L_P^g \exp[-E/R(1/T) - (1/T_g)] \quad (5)$$

L_p^g **:** Permeability coefficient of water at a temperature T_g.
E: Activation energy.

There are two important variables which essentially condition the survival of cells that have been frozen. Firstly, the appearance of intracellular ice crystals depends on the rate of the cooling process. Rapid cooling rates produce small crystals, and the higher the cooling rate is, the smaller the crystals will be. However, these small ice crystals are thermodynamically unstable, so they will have a tendency to aggregate during warming to form larger crystals [12].

This process is known as recrystallization, and it is related to the second variable which conditions the survival of cells: the rate of subsequent warming. Slow warming will be harmful to frozen cells as it provides them time enough so the process of recrystallization occurs. But, how does recrystallization condition the survival of cells?

Even though this question has not yet been clearly answered, it has been proven that there exists a correlation between this process and cell death in yeast, plant cells [13] and some other tissue culture cells [14]. Some studies report that "the presence of intracellular ice during warming is innocuous but it may lead to a change in another unknown component which then causes injury" [15].

5. APPLICATIONS

5.1 Surgery

A type of light cryopreservation is profound hypothermia. It is used in humans while operating in complex cardiovascular surgical procedures. It is based on the fact that cooling sufficiently the organism may effectively stop all cerebral activity and is able to provide protection for all the organs considered vital. The temperature to reach is 10-14ºC around and it is made by a heart-lung machine that makes the blood to pass through a cooling chamber. After the intervention, the blood is gradually warmed using the same apparatus [16].

5.2 Plants storage

Cryopreservation is applied to species that are unable to be stored by other means and are sensitive to the medium (i.e. dehydration). Furthermore, endangered species can be conserved in such a way that their imminent extinction is evaded. All the process is useful as it is value-for-money, a small place to store is needed and contamination by microorganisms is avoided [17].

The most popular plant material for cryopreservation is the so called encapsulation-dehydration of shoot tips, based on the production of artificial seeds. These shoots need to be pregrown in an enriched liquid medium and it needs to be partially desiccated to freeze as it facilitates the cryopreservation [18]. Apical meristems are commonly used in this process as they contain a relatively small vascular system [17].

5.3 Cryopreservation of embryos

Fertilized eggs are called embryos [19]. Embryo cryopreservation is considered very useful for pregnant mothers that used in vitro fertilization, as they can save the embryos and are able to return for another pregnancy some years later. Moreover, the ones that are left over could be donated and used by another couple willing to have a baby or it is even possible to provide them to investigation or destroy them (as the Spanish Law of assisted reproduction techniques marks) [20]. The current process followed to freeze is denominated vitrification. Through it, the water molecules of embryos are removed and replaced with cryoprotectant and then introduced into liquid nitrogen. This quick change of temperature (-12.000 degrees Celsius per minute) is going to create a *glass* state, where embryos are vitrified. It also reduces the possibility of intercellular ice crystals to be formed, thus protecting the future baby [18].

6. CONCLUSIONS

The cryopreservation discovered in the process of cryptobiosis in micro-animals such as tardigrades has made it possible to make progress in the storage of living beings or their tissues in order to maintain them through time. The method requires a great loose of water from the organism, mechanism that is naturally based on a chemical potential gradient, thus Clausius-Claperyon equation and Raoult's law apply. Many applications have been developed thanks to this behavior, as the cryopreservation of embryos during in vitro fecundation.

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