

“A Two-Factor Hypothesis of Freezing Injury: Evidence from Chinese Hamster Tissue-Culture Cells” (1972), by Peter Mazur, Stanley Leibo, and Ernest Chu

In 1972, Peter Mazur, Stanley Leibo, and Ernest Chu published, “A Two-Factor Hypothesis of Freezing Injury: Evidence from Chinese Hamster Tissue-culture Cells,” hereafter, “A Two-Factor Hypothesis of Freezing Injury,” in the journal, *Experimental Cell Research*. In the article, the authors uncover that exposure to high salt concentrations and the formation of ice crystals within cells are two factors that can harm cells during cryopreservation. Cryopreservation is the freezing of cells to preserve them for storage, study, or later use. Mazur originally suggested the two factors in a 1970 paper, but that article was based on evidence from simple yeast cells. By using hamster cells in 1972, Mazur, Leibo, and Chu confirmed that Mazur’s two-factor hypothesis applied to more complex mammalian cells. The article dispelled the widely accepted notion that rapid cooling rates were safest for all cells, and instead showed that each kind of cell had a different optimal cooling rate depending on the solution in which it froze.

At the time of publication, all three authors worked in the biology research division of Oak Ridge National Laboratory in Oak Ridge, Tennessee. Mazur joined in 1959 after completing a postdoctoral fellowship at Princeton University in Princeton, New Jersey. Chu joined the laboratory upon graduating from Yale University in New Haven, Connecticut. Leibo joined the laboratory in 1962 as part of his graduate training at Princeton University in Princeton, New Jersey, and was later hired as a staff biologist at the laboratory. The three began collaborating in 1969, when they first started studying hamster tissue-culture cell responses to freezing and thawing. “A Two-Factor Hypothesis of Freezing Injury” was the culmination of their various collaborative projects at the laboratory, where they worked to understand how cells behaved during cryopreservation.

“A Two-Factor Hypothesis of Freezing Injury” discusses the study Mazur and his team performed to confirm that the harmful factors identified by the two-factor hypothesis for other organisms were applicable to mammalian cells. The article has four main sections. In the introduction, the team explains the uses of cryopreservation and their goal of determining whether the two-factor hypothesis Mazur had formed studying simpler cells was applicable to more complex mammalian cells. In their materials and methods section, Mazur and his colleagues explain how they prepared cells, measured rates of freezing and thawing, and counted cell survival to determine which cooling rates led to the highest survival. In the results section, the authors show that intermediate rates of cooling resulted in the highest survival for cells, but that the optimum cooling rate was different for each kind of cell and depended on the salt concentrations within the solutions in which the cells froze. In the discussion, the team explains that the result of intermediate cooling rates yielding the highest survival supports the idea that injury to cells during freezing is caused by two different factors, salt concentration and ice crystals, that rely on cooling rates in opposite ways.

Mazur and colleagues begin the introduction by discussing some of the uses of cryopreservation and how the process works. Freezing viable cells for later use can save space in laboratories and remove the hassle of trying to maintain live colonies of different organisms. Freezing also enables clearer observation of cells through microscopes because researchers can observe them when the cells are unmoving, but still alive. When researchers cryopreserve cells, they suspend the cells in test tubes containing solutions of water and dissolved salts. As temperatures drop, the water surrounding the cell begins freezing first. As those solutions cool, pure water separates from dissolved salts as it crystallizes into ice. Therefore, as ice forms, the salt concentration of the unfrozen solution

increases. The solution outside the cells then has a higher salt concentration than the solution inside the cells. The imbalance of salt concentrations within and outside of the cell pushes water molecules within the cell to leave, moving towards the higher salt concentrations to maintain equilibrium as part of a process called osmosis. The movement of water in and out of the cell changes depending on how quickly the cell cools and is critical as to whether the cell survives freezing or not. The authors then discuss the relationship between cooling rates and the two factors of injury in the next part of the introduction.

In the second part of the introduction, the authors discuss how observing cell survival at different cooling rates could determine whether injury in hamster tissue-culture cells resulted from the two factors in their hypothesis, which include exposure to high salt concentrations or ice formation within the cell. James Lovelock, a scientist who investigated cell responses to freezing in England in the 1950s, was one of the first to distinguish the risk associated with cell exposure to higher salt concentrations in 1953. High salt concentrations can dehydrate the cell before its activity is paused due to freezing. High salt concentrations can also reduce the stability of cell membranes, making the cells more susceptible to rupture, or bursting.

Mazur, Leibo, and Chu continue on to explain that, around the time they published "A Two-Factor Hypothesis of Freezing Injury," most researchers believed that a rapid rate of cooling was the best way to ensure survival for any kind of cell because it minimized the cell's exposure time to high salt concentrations. However, in his 1970 paper, Mazur challenged that belief when he indicated that rapid rates of cooling resulted in ice formation within a yeast cell. Though rapid cooling does reduce the amount of time cells are exposed to high salt concentrations, it also gives water inside the cell less time to move out through osmosis. As a result, Mazur had previously found that the water ends up crystallizing into ice within the yeast cell. The formation of ice crystals within a cell is harmful and potentially lethal because they can disrupt the cell's normal structures to the point where they no longer function. Mazur claims that slowing the rate of cooling can prevent ice formation within the cell. Therefore, Mazur explained that an intermediate rate of cooling would be best to protect cells against both of the factors that cause harm. While Mazur demonstrated those results in his earlier 1970 paper based on freezing yeast cells, the authors suggested they expected to see similar results in a mammalian cell. If the hamster cells they used had better survival rates at intermediate rates of cooling, it could be concluded that they were influenced by the same two factors of injury that Mazur observed in yeast cells.

In the materials and methods section, the authors describe the different cell treatments and controls they utilized to determine whether hamster tissue-culture cells were damaged by the two hypothesized factors of injury. The team prepared various samples of the hamster tissue-culture cells in solutions containing different kinds of salts. The authors prepared multiple samples with each kind of solution, keeping one sample of each kind of solution as a control. A control is a sample kept as a constant, unchanging standard to which to compare experimental data in order to ensure that the effect came from the experimental manipulations rather than other factors. Therefore, the researchers did not freeze the control samples, and instead, they kept the controls at a constant temperature throughout the experiment. The researchers froze the rest of the samples at slow, intermediate, or rapid rates until reaching the appropriate freezing temperatures. After freezing, the team temporarily stored the samples, then thawed them at rapid or slow rates. Once thawed, they plated samples onto petri dishes containing nutrients that facilitated cell growth. After nine days, they counted the cell colonies that had grown on the petri dishes to determine which cooling and thawing rates led to highest levels of mammalian cell survival.

In the results section of their article, Mazur, Leibo, and Chu disclose that cell survival was highest at intermediate cooling rates. Survival rates were consistently low with slower cooling rates, showing that slow cooling rates were not effective for preserving viable cells. Survival rates were slightly better when cooling rates were rapid, but still low and thus relatively ineffective for preserving cells. However, the authors note that slower warming rates were much more detrimental to cells that had been cooled rapidly. They explain that ice crystallized more frequently within rapidly cooled cells, and if warming was too slow, resulted in recrystallization.

In the discussion, the team outlines how their results support the theory that injury to rapidly and slowly cooled cells is caused by the two identified factors. First, the researchers' conclusion that

cell survival was highest at intermediate cooling rates supports the hypothesis that different injuries happen at the two extremes of rapid cooling and slow cooling. Mazur, Leibo, and Chu note that rapid cooling is risky because it can lead to the formation of intracellular ice, and slow cooling can lead to damage caused by long exposure to high salt concentrations in the solutions surrounding the cell. Intermediate cooling rates protected against both factors the best. Second, rapidly cooled cells that were warmed slowly were injured much more than slowly cooled cells that were warmed rapidly. That further supports the idea that different effects cause injury at slow and rapid cooling rates. Thus, the authors validate that the two factors leading to cell injury in a yeast cell, including exposure to high salt concentrations and the formation of ice crystals within cells, also apply to mammalian hamster cells.

Within the discussion section, the team also discusses probable implications the study has for the field of cryobiology. In the article, the authors recount that the dominant belief among most scientists who used cryopreservation at the time had been that all cells had an optimum cooling rate of one degree Celsius per minute. Their results were not consistent with that belief. Instead, the team found different optimum cooling rates range depending primarily on the solutions used. The team advises other scientists to be wary of protecting cells against the two factors of injury.

“A Two-Factor Hypothesis of Freezing Injury” encouraged scientists to reconsider the way they approached cryopreservation by pinpointing the risks of cryopreservation and outlining how to protect against them. Additionally, Mazur, Leibo, and Chu showed that intermediate cooling rates yield the highest survival rates in frozen complex cells, which served as evidence that the hypothesis was applicable to more complex mammalian cells in addition to the less complex cells with which it had already been supported. In a 2009 article discussing the history and challenges of cryopreservation, a group of researchers in the 2000s claimed that the two-factor hypothesis was one of the most notable advances in the field of cryopreservation. They asserted that it provided a foundation for future cryopreservation experimentation to advance.

As of 2020, researchers use cryopreservation in a wide variety of fields. Gene banks store cryopreserved genetic material of endangered species for conservation purposes. Research labs store strains of species of interest being studied to save space. In hospitals, cryopreservation of human embryos is a common practice used for human reproductive assistance such as in-vitro fertilization. Along with researcher David Whittingham, Mazur and Leibo published another study in 1972, in which they documented one of the first successful mammalian births from frozen embryos using mice. “A Two-Factor Hypothesis of Freezing Injury” provided a baseline to facilitate those advancements in the use of cryopreservation.

Sources

1. Baust, John G., Dayong Gao, and John M. Baust. “Cryopreservation: An Emerging Paradigm Change.” *Organogenesis* 5 (2009): 90-6. <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2781087/> (Accessed February 28, 2020).
2. Indiana University-Purdue University Indianapolis. “Mazur Obituary in Knoxville News Sentinel.” Department of Physics, 2016. <https://www.physics.iupui.edu/~starman/Starman/Cryobiology/Mazur/Obit-Knoxville-Mazur.htm> (Accessed February 28, 2020).
3. Krause, Carolyn. “Stopping Biological Time.” *Oak Ridge National Laboratory Review* 10 (1977): 30-7.
4. Lovelock, James E. “The Hemolysis of Human Red Blood Cells by Freezing and Thawing.” *Biochemica et Biophysica Acta* 10 (1953): 414-26.
5. Mazur, Peter. “Cryobiology: The Freezing of Biological Systems.” *Science* 168 (1970): 939-49. <https://www.physics.iupui.edu/~starman/Starman/Cryobiology/Mazur/Two-Factor-Hypothesis-Scanned.pdf> (Accessed February 28, 2020).
6. Mazur, Peter, Stanley P. Leibo, and Ernest H.Y. Chu. “A Two-Factor Hypothesis of Freezing Injury: Evidence from Chinese Hamster Tissue-Culture Cells.” *Experimental Cell Research* 71 (1972): 344-55.
7. Mazur, Peter, John Farrant, Stanley P. Leibo, and Ernest H. Y. Chu. “Survival of Hamster Tissue Culture Cells After Freezing and Thawing: Interactions Between Protective Solutes and

- Cooling and Warming Rates.” *Cryobiology* 6 (1969): 1-9.
8. Rieger, D. and T.B. Pool. “Stanley Paul Leibo (1937–2014): Scientist, Mentor, and Friend.” *Reproductive Biomedicine Online* 29 (2014): 14-6. [https://www.rbmojournal.com/article/S1472-6483\(14\)00262-4/fulltext](https://www.rbmojournal.com/article/S1472-6483(14)00262-4/fulltext) (Accessed February 28, 2020).
 9. Whittingham, David G., Stanley P. Leibo, and Peter Mazur. “Survival of Mouse Embryos Frozen to -196 ° and -269 °C.” *Science* 178 (1972): 411-4. <https://www.osti.gov/servlets/purl/4604285> (Accessed February 28, 2020).