

# “Survival of Mouse Embryos Frozen to -196 ° and -269 °C” (1972), by David Whittingham, Stanley Leibo, and Peter Mazur

In 1972, David Whittingham, Stanley Leibo, and Peter Mazur published the paper, “Survival of Mouse Embryos Frozen to -196 ° and -269 °C,” hereafter, “Survival of Mouse Embryos,” in the journal *Science*. The study marked one of the first times that researchers had successfully cryopreserved, or preserved and stored by freezing, a mammalian embryo and later transferred that embryo to a live mouse who gave birth to viable offspring. Previously, scientists had only been successful cryopreserving single cells, like red blood cells. Mammalian embryos, on the other hand, were more difficult to cryopreserve because they are more complex and therefore more easily weakened or destroyed by the formation of ice within its cells. Whittingham, Leibo, and Mazur’s work provided a successful model for mammalian embryo cryopreservation, a technology that later expanded to cryopreserve more complex embryos, such as human embryos.

At the time of the publication of “Survival of Mouse Embryos,” Whittingham was studying mammalian embryo development at Cambridge University in Cambridge, England. In 1971, Whittingham was one of the first to cryopreserve mouse embryos, but those embryos only briefly survived and did not successfully develop into offspring. Leibo and Mazur, who worked as staff biologists studying the fundamentals of cryopreservation at Oak Ridge National Laboratory in Oak Ridge, Tennessee, invited Whittingham to their laboratory to replicate his original experiment. However, once there, the team could not achieve Whittingham’s original results. Leibo and Mazur, who had studied cryopreservation in the past, helped Whittingham refine the freezing process of mouse embryos. Together, the team achieved the first successful mammalian birth from a frozen embryo, the results of which they published in their article.

“Survival of Mouse Embryos,” is a report of the authors’ 1972 experiment and is broadly divided into four sections. In the introduction, the authors begin by explaining that previous attempts at freezing multicellular mammalian systems had failed until Whittingham’s 1971 study, and that it was only by applying some previously unapplied methods of cryopreservation that the team was successful in 1972. In the methods section, the authors explain their techniques of freezing and thawing the embryos, while detailing the temperatures and rates they used to freeze the embryos. They then discuss the different factors, including temperatures, cooling and warming rates, and storage times that resulted in the highest survival of embryos before transfer to live mice. Lastly, the authors briefly discuss potential future applications of their work for research in fields like agriculture or medicine.

In the introduction section, Whittingham, Leibo, and Mazur begin by discussing the fact that few researchers had previously been able to cryopreserve any complex structure derived from a mammal, such as organs and tissue, due to a lack of understanding of the fundamentals of cryobiology. Until 1971, the authors claim that most attempts to freeze embryos to temperatures lower than -20 °C had failed. Then, Whittingham’s 1971 study successfully froze mouse embryos with eight cells to -79 °C for thirty minutes. Temperatures between -20 and -79 °C are about -4 to -110 °F.

The authors then explain that in order to successfully cryopreserve embryos to lower temperatures, the embryos must be frozen at rates slow enough for water to move out of the cell before it crystallizes into ice. If ice forms within the cell itself, it may injure or destroy the cell completely. When cryopreserving cells, scientists suspend cells in liquid solutions with salt concentrations that are similar to the liquid inside the cell. As the temperature decreases, pure water in the solution outside

of the cell crystallizes and turns into ice, leaving behind a solution with a higher salt concentration because it contains less water. Water inside the cell will diffuse out of the cell towards the solution with the higher salt concentration as part of the process of osmosis. However, if cells are cooled too quickly, water inside the cell does not have enough time to move out, causing that water to freeze and crystallize into ice within the cell. Ice that forms within membranes or organelles can be harmful and even lethal because it can disrupt their normal structures and break up the contents of the cell to the point where they no longer function.

Mazur and Leibo first identified the risk of ice formation discussed within the introduction of, "Survival of Mouse Embryos," in a previous paper. Mazur and Leibo's 1972 paper, "A Two-Factor Hypothesis of Freezing Injury: Evidence for Chinese Hamster-Tissue culture Cells," was the one of the first to identify and describe the different risks associated with freezing cells too rapidly or too slowly. According to Mazur, prior to the publication of that 1972 paper, researchers largely believed that rapid cooling was safer for cells because it minimized the cells' exposure to higher salt concentrations, which can weaken cell membranes and cause the cell to burst. While that was still a risk during cryopreservation, Mazur and Leibo realized that, because they also needed to avoid ice formation within a cell that forms during rapid cooling, researchers should use intermediate cooling rates to protect against both factors that can injure the cells. Mazur and Leibo's understanding of the fundamentals of cryopreservation enabled the team to successfully cryopreserve mouse embryos in "Survival of Mouse Embryos."

In the methods section of "Survival of Mouse Embryos," Whittingham, Leibo, and Mazur detail how they experimented with a range of cooling rates, cooling temperatures, storage durations, and thawing techniques to determine the ideal conditions for producing viable embryos. The researchers state that, after obtaining the embryos, they slowly cooled them at rates ranging from 0.3 °C per minute to 2 °C per minute. They ultimately cooled the embryos at each of the different rates to eventual temperatures of -78 °C, -196 °C, or -269 °C. The team added another variable for storage time, and stored the embryos at each temperature for periods ranging from one minute to eight days. As one final variable in their experiment, the authors explain that they then thawed samples using room temperature air, water baths, ice baths, or ethanol baths at different rates.

Then, the authors washed and transferred the embryos to small concave glass discs called watch glasses so they could observe the embryos through microscopes and separate the embryos that survived from the ones that did not. Next, Whittingham, Leibo, and Mazur state that they transferred all the viable embryos to media that contained vital nutrients and let the embryos grow for one to four days until they developed into blastocysts. Blastocysts are embryos with cells that have begun to differentiate into distinct parts, including an inner cell mass that will develop into the fetus and an outer layer of cells that will develop into the placenta. In mice, blastocysts contain about sixteen to forty cells. Whittingham, Leibo, and Mazur defined embryo survival as successful development into blastocysts.

In the results section, the authors report that slow cooling and slow thawing rates yielded the highest survival of embryos. Rapid thawing appeared to be detrimental for reasons the authors say they did not understand at the time. The temperatures embryos were held at did not greatly affect chances of survival. Additionally, the amount of time the researchers stored the embryos, from one to eight days, did not drastically affect overall survival, either.

Whittingham, Leibo, and Mazur then discuss the results of transferring blastocysts to female surrogate mice. Out of all the recipient mice, 65 percent became pregnant. The authors state that the rate was within the normal range of pregnancy rates for healthy mice. Additionally, the rate of pregnancy of mice receiving frozen-thawed embryos in, "Survival of Mouse Embryos," was very similar to the rate of pregnancy in mice that received embryos that had never been frozen, as reported in a different study. As a result, the authors conclude that non-pregnancies were the result of the foster mother's physiology rather than a lack of viability of the frozen-thawed embryos. Furthermore, the embryos developed into normal fetuses which eventually developed into healthy live offspring, indicating that the cryopreserved embryos were completely viable.

In the discussion section, Whittingham, Leibo, and Mazur consider the ways their results could apply to different fields of science. The authors declare that their results indicate that freezing could

enable scientists to store different strains of mice with different mutations. Researchers studying certain strains of mice could thaw and transfer the embryos to surrogate mothers as needed in order to reestablish the mutant strains rather than having to constantly keep live mice in their laboratory for research. The authors then suggest that the procedures they used could be applicable to agriculture. They indicate that the ability to cryopreserve domestic animal embryos could facilitate worldwide sharing of livestock with desirable genetic backgrounds for agricultural purposes. That would make selective breeding easier, enabling livestock producers to introduce more desirable traits into their own populations. Lastly, the authors suggest that the same principles for cryopreserving mouse embryos would apply to larger, more complex embryos.

Since the publication of “Survival of Mouse Embryos,” researchers have learned to cryopreserve embryos of many mammalian species, including humans. Cryopreservation is frequently used in laboratory research and agriculture as predicted by Whittingham, Leibo, and Mazur. For example, researchers use cryopreservation as a contemporary use of conservation. Many conservation facilities store frozen embryos containing genetic information of endangered species with the goal of one day using those embryos to reestablish the species. Additionally, researchers also use cryopreservation as a form of assisted reproduction technology in humans. One of the first human infants developed from a once frozen embryo was born in 1984. Since then, using frozen embryos to facilitate in vitro fertilization has become a common practice around the world. According to a study done at the University of Adelaide in Adelaide, Australia, frozen embryos are just as likely to result in a live birth when transferred to a mother as fresh, never frozen embryos.

“Survival of Mouse Embryos” also showed that there was potential to store embryos at low temperatures for long periods of time without affecting their viability. As of 2020, the longest an embryo has been frozen before being transferred into a woman and developing into a healthy infant is twenty-four years. The ability to freeze embryos and store them for later use has practical advantages. Saving embryos allows people who may have conditions that affect their egg supply to wait until they are ready for pregnancy. The extra time before pregnancy provided by cryopreservation also enables potential parents to test the embryos for genetic diseases before pregnancy. Whittingham, Leibo, and Mazur’s success in cryopreserving mouse embryos served as an example for other researchers aiming to cryopreserve more complex mammalian embryos, including human embryos, thereby advancing the field of cryopreservation.

## Sources

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