

# Plasma Membrane Integrity of Swine Embryos Following Cryopreservation

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## Abstract

Despite the onset of livestock embryo freezing technologies in the 1980's, swine have proven to exhibit challenges due to their extreme sensitivity to cooling. However, studies suggest that Meishan embryos have a higher capacity to survive the vitrification process compared to white crossbred embryos. First we will determine the timing of post-thaw embryonic death during *in vitro* culture. This research will also examine the plasma membrane of pre-implantation embryos to determine if differences in structural proteins are key to the higher survival rate of Meishan embryos. By determining how Meishan embryos can better survive cryopreservation, further advances may be made in embryo preservation methodologies for all species. This can have implications on preservation of maternal germplasm, global genetic transport, breeding line regeneration or proliferation, and conservation of resources for the genetic rescue of certain species.

## Introduction

Cryopreservation of embryos from livestock species has made significant advances in recent decades; however, application to swine has lagged since porcine embryos are intrinsically very sensitive to cooling and ice crystallization. While freezing techniques have been adapted to embryos from cattle, goats, sheep, and other animals, the proportionately large number of cytoplasmic lipid droplets in porcine embryos has been detrimental to the practical success of their cryopreservation. Lipid reduces the cryotolerance of the embryos by causing irreversible damage to the plasma membrane structure at temperatures between 10 and -5°C (1).

Several concepts have been introduced to circumvent this problem and increase post-thaw survival rates. These include delipidation before freezing, pre-treatment cytoskeletal stabilization, and removal of the zona pellucida (1, 2). Though these procedural alterations are effective at improving post-thaw survival rates, they also compromise embryo viability and increase the risk of disease transmission during embryo transfer (2). Vitrification has emerged to be the most successful technique for the cryopreservation of swine embryos. Vitrification with suitable cryoprotectants enables water to harden like glass, without crystal formation, effectively eliminating the cellular damage associated with ice crystals. The Open Pulled Straw (OPS) method provides more rapid rates of cooling and can be performed with the zona pellucida intact, which enhances general quality control practices for embryo transfer. Live piglets have also been produced using a modified microdroplet method to vitrify embryos without chemical pretreatment or manipulation of the zona pellucida (3). While these improvements have certainly impacted porcine embryo cryopreservation, vitrification methods are still not as successful as in other livestock species (4). Further studies of factors affecting embryo survival after vitrification and thawing are required for widespread application of cryopreservation in swine.

There is great potential for these answers to be gained from studying the reproductive differences between Chinese Meishan and white crossbred lines of swine. Meishan sows are more prolific, averaging litter sizes 30-40% greater than white crossbred females (5). Reproductive performance of Meishan females is significantly greater than contemporary lines due to increased ovulation rate, embryonic viability and prenatal survival, and uterine capacity. In 2000, French researchers reported that Meishan blastocyst stage embryos had a 40% greater post-thaw viability than those from Large White hyperprolific gilts following vitrification in phosphate buffered saline-based cryoprotectant dilution media (6).

## Objectives

- To examine *in vitro* embryonic development of Meishan and white crossbred embryos following cryopreservation.
- To determine differences in timing of embryonic death during *in vitro* development of Meishan and white crossbred embryos following vitrification.
- To analyze post-thaw structural membrane integrity of Meishan and white crossbred embryos.

## Materials and Methods

### Embryo collection, preservation, and culture:

- Embryos were flushed from donor reproductive tracts at day 5 or 6 following estrus (onset of estrus = day 0).
- The vitrification and thawing protocols are shown below (Figure 1).
- Following thawing, embryos from each genotype were cultured in 50  $\mu$ L drops of modified Whitten's medium + 1.5% BSA under oil at 37°C in a 5% CO<sub>2</sub> in air environment.
- The embryos were scored for development at 24, 48, and 72 h of culture. Embryos were considered to have survived if they advanced a stage of development following 24 h of culture without exhibiting signs of lysis or degeneration.

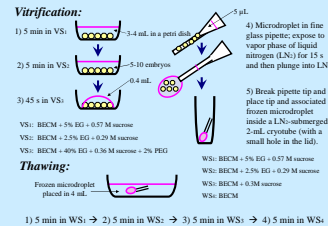


Figure 1. Protocols for vitrification and Thawing of Porcine Embryos. VS = vitrification solution. WS = warming solution. BECM = Beltsville embryo culture medium. EG = ethylene glycol. PEG = polyethylene glycol.

### Differential live/dead staining of embryos:

- Following vitrification and thawing, embryos will be stained at 6 and 12 h of culture using a differential live/dead stain.
- Embryos from both lines will be placed in 50  $\mu$ L drops of dye solution containing Dulbecco's Phosphate Buffered Saline (DPBS), 0.05 mg/mL ethidium bromide (EtBr), and 0.005 mg/mL fluorescein diacetate (FDA). The embryos will be left in the dark for at least 3 min to allow for FDA cleavage of the acetate radical and dye accumulation inside the cells.
- Embryos will be viewed using a fluorescence microscope under UV epilluminescence.
- The live embryos stain green and the dead embryos appear red/orange (Figure 2).

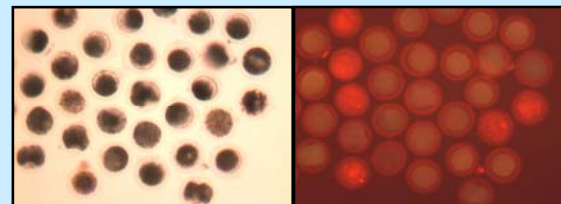


Figure 2. Differential Staining of Live and Dead Porcine Oocytes. The image of porcine oocytes on the left was taken with brightfield microscopy at 20X magnification. The picture on the right are the same oocytes visualized with UV epilluminescence after being stained with EtBr and FDA. Note the six dead oocytes shown here in red. The green oocytes are still viable, but the green color fades quickly as they remain under UV light.

### Immunocytochemistry:

- At 6 h post-thawing, embryos from both lines will be fixed in 100  $\mu$ L drops of paraformaldehyde solution.
- Embryos will be incubated with RNase prior to being stained with propidium iodide (1:1000 dilution) and mounted to glass slides using diluted (1:10) poly-L-lysine solution.
- After drying, the embryos will be analyzed under fluorescent microscopy for structural integrity.

## Results

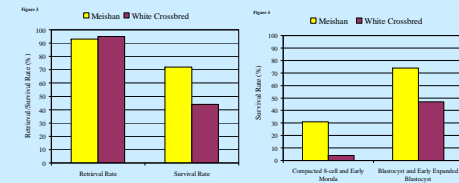


Figure 3. Comparison of Embryo Retrieval and Survival Rates Following Cryopreservation of Meishan and White Crossbred Embryos. Retrieval rate was similar in these two lines of swine, but survival rate strongly favored the Meishan embryos (72% vs. 44%). Adapted from Montagner et al. (3).

Figure 4. Effect of Initial Embryonic Stage on Survival Rates Following Cryopreservation of Meishan and White Crossbred Embryos. Regardless of breed, survival rates were much higher for blastocyst and early blastocyst embryos compared to compacted 8-cell/early morula stage embryos, and survival rates of Meishan embryos (31% and 74%) were markedly better compared to white crossbred embryos (4% and 47%). Adapted from Montagner et al. (3).

- Differential staining of embryos is expected to reveal increased post-thaw signs of degeneration and lysis earlier in white crossbred embryos than Meishan embryos following vitrification.
- Examination of membrane integrity is expected to show greater morphological changes in white crossbred embryos compared to Meishan embryos as a result of cryopreservation.

## Future Experiments

- To compare levels of structural plasma membrane proteins between Meishan and white crossbred embryos.
- To investigate the effect of lipid content on post-thaw plasma membrane integrity of Meishan and white crossbred embryos.
- To perform a reciprocal nuclear transfer between Meishan and white crossbred embryos to examine the effect on *in vivo* survival rates in divergent lines.

## Conclusions

Meishan embryos show a higher tolerance for cryopreservation with increased post-thaw survival rates compared to those of white crossbred swine. Since porcine embryos are more difficult to freeze than other species, the identification of a line with greater post-thaw viability is intriguing. Since the underlying mechanisms for these differences are yet unknown, comparisons between embryos of these two genotypes can uncover factors related to increased post-thaw embryo viability that can greatly advance present cryopreservation technologies.

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