2006

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Summary and Implications

Successful freezing, or cryopreservation, of embryos could greatly impact the pork industry, serving as a tool for conservation of valuable germplasm and enhancing biosecurity for transfer of genetic material. Pig embryos are very sensitive to cooling and few reports have shown successful developmental rates following freezing. The objectives of this study were to determine the efficiency of freezing pig embryos using a microdroplet vitrification method and to investigate in vitro development of embryos from Chinese Meishan and occidental white crossbred females following cryopreservation at different stages of embryonic development. Preliminary studies using the microdroplet vitrification method for cryopreservation and embryo transfer into recipient females resulted in the birth of normal, live piglets indicating the effectiveness of this procedure. Rates of expanded blastocyst formation did not differ between Meishan and white crossbred nonfrozen, control embryos (98 and 95%, respectively). Developmental rates were significantly higher for control embryos than vitrified embryos from both Meishan and white crossbred females at the expanded blastocyst stage ($P < 0.001$), but not at the hatched blastocyst stage. Following collection of embryos from Meishan and white crossbred females, cryopreservation and in vitro culture, the percentage of cryopreserved embryos alive after 24 hours of culture was higher for Meishan (72%) than white crossbred (44%; $P < 0.001$) embryos. However, development of thawed, cryopreserved embryos that survived 24 hours of culture was not different for Meishan and white crossbred embryos at the expanded (64%) or hatched (22%) blastocyst stages. The optimal stages to vitrify pig embryos using the microdroplet method range from late compact morula to early expanded blastocyst. Our results suggest that Meishan embryos have a higher capacity to survive the freezing process than white crossbred embryos, independent of embryo stage.

Background and Introduction

There are approximately 940 million swine in the world today and a large portion of the human population includes pork as an

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important source of protein in the diet. Cryopreservation of porcine embryos could greatly impact the pork industry by serving as a tool for conservation of valuable germplasm and enhancing biosecurity for transfer of genetic material. However, pig embryos are very sensitive to cooling and ice crystallization during the freezing process. Therefore, the efficiency of cryopreservation is much lower in pig embryos than in embryos from other species.

To avoid ice crystal formation in the freezing medium, the vitrification method appears to be the most promising technique for cryopreservation of swine embryos. During vitrification, a type of glass is formed in the freezing medium preventing embryos from being subjected to cellular damage associated with ice crystal formation. Porcine embryos have been successfully frozen at the hatching and hatched blastocyst stages. At these stages however, the zona pellucida, a protective coating surrounding the embryo that is similar in function to the shell of a chicken egg, is unable to act as a barrier against infectious organisms. Cryopreservation also has been successful with embryos from the 4-cell to early blastocyst stages. However, the cryopreservation procedure used in those studies requires creating a hole in the zona pellucida to remove the lipid content after centrifugation. This manipulation disrupts the intact zona pellucida, increasing the susceptibility of embryos to disease transmission.

Transfer of zona-intact embryos that were frozen using the open pulled straw (OPS) method and in combination with cytoskeletal stabilization prior to vitrification, produced live piglets. Scientists at the Agricultural Research Service (ARS) of the United States Department of Agriculture (USDA) in Beltsville, Md. reported that the OPS method improved the survival rate of pig embryos vitrified prior to hatching from the zona pellucida. In addition, other investigators have shown that it is possible to freeze porcine embryos at the compact morula stage and produce live offspring using the OPS freezing method without cytoskeletal stabilization pretreatment. Using a modified microdroplet method, described previously to vitrify bovine oocytes, researchers at the National Livestock Breeding Center in Japan successfully produced piglets from vitrified compact morula and early blastocyst stage embryos. In this study, the pregnancy rate of embryo transfer recipients was 40% and the percentage of live piglets born per embryo transferred was 10%. The advantage of this method is that it does not require chemical pretreatment or manipulation of the zona-intact embryos.

There is much interest in studying reproductive differences between Chinese Meishan and occidental breeds of swine. Meishan females are more prolific than females from white crossbred lines (four to five more pigs per litter). Factors that contribute to the increased litter sizes of Meishan females include ovulation rate, embryonic survival and uterine capacity. Investigators from France reported that Meishan embryos could withstand the vitrification process better than embryos from hyperprolific Large White females. However, the mechanisms underlying this difference have not been investigated. The comparison of embryonic development after cryopreservation between embryos from different genotypes can aid in the discovery of important factors for embryonic survival after vitrification. Thus, the objectives of this study were: 1) to determine the efficiency of freezing pig embryos using a microdroplet vitrification method, 2) to examine *in vitro* development of Meishan and white crossbred embryos following cryopreservation, and 3) to determine the importance of embryonic stage for Meishan and white crossbred embryos on survival rates after cryopreservation by microdroplet vitrification method.

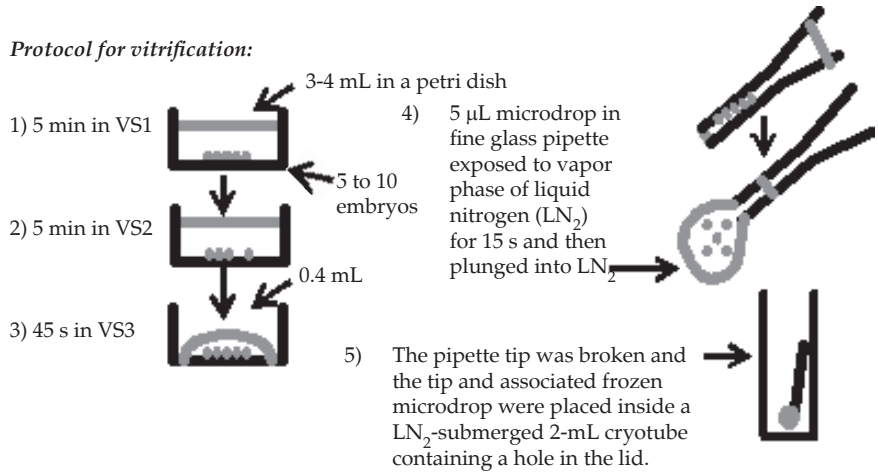
Materials and Methods

Efficiency of Microdroplet Method

The microdroplet vitrification procedure was used with some modifications from the original published protocol described by scientists at the National Livestock Breeding Center in Japan. Embryos from white crossbred females on day 5 following estrus (day 0 = onset of estrus) were flushed from donor reproductive tracts at surgery using Beltsville Embryo Culture Medium (BECM). After flushing, the embryos were selected, cryopreserved by the microdroplet protocol, and stored in liquid nitrogen (-196^{o}C) for approximately one hour (Figure 1). Immediately prior to embryo transfer, the embryos were thawed using a four-step procedure (Figure 1). Two white crossbred recipients on day 5 of the estrous cycle received either 24 compact morulae or 24 compact morulae/blastocysts. The recipients were checked for return to estrus over 30 days and allowed to gestate until farrowing.

Comparison of Development between Meishan and White Crossbred Embryos Following Cryopreservation

Embryo collections were performed at the USDA, ARS, Roman L. Hruska U.S. Meat Animal Research Center in Clay Center, Neb. and transferred for four hours at 39^{o}C to the Department of Animal Science at the University of Nebraska–Lincoln. First parity Meishan sows (n = 11) and white crossbred gilts (n = 13) were observed for estrus every 12 hours and naturally mated at 12 and 24 hours after the onset of estrus within breed using two different boars. Females were slaughtered between day 4.5 and 6 after estrus and embryos were collected. Compact morula and blastocyst stage



VS1: BECM + 5% EG + 0.57 M sucrose
VS2: BECM + 2.5% EG + 0.27 M sucrose + 1% polyethylene glycol (PEG)
VS3: BECM + 40% EG + 0.36 M sucrose + 2% PEG

Protocol for thawing:

1) 5 min in WS1 → 2) 5 min in WS2 → 3) 5 min in WS3 → 4) 5 min in WS4

WS1: BECM + 5% EG + 0.57 M sucrose
WS2: BECM + 2.5% EG + 0.29 M sucrose
WS3: BECM + 0.3 M sucrose
WS4: BECM

Figure 1. Vitrification and thawing aspects of the microdroplet protocol for cryopreservation of pig embryos. VS = vitrification solution. WS = warming solution. BECM = Beltsville embryo culture medium.

embryos from each female within breed were randomly allocated either directly into the culture system to serve as controls (68 Meishan and 48 white crossbred embryos) or to undergo vitrification, storage in liquid nitrogen (-196°C) for one hour, thawing, and placement into culture (101 Meishan and 78 white crossbred embryos). Embryos from each treatment were cultured in 50 µl drops of modified Whitten's medium + 1.5% BSA under oil at 37°C in a 5% CO_2 in air environment and scored for development at 24, 48 and 72 hours of culture. Embryos were considered to have survived if they advanced a stage in development following 24 hours of culture and did not show signs of lyses or degeneration. The percentages of expanded and hatched blastocysts were calculated based on the number of surviving embryos only.

Importance of Embryonic Stage on Survival Rates after Vitrification

A total of 93 blastocysts/early expanded blastocysts from four Meishan (n = 42) and seven white crossbred (n = 51) females were compared with 56 compacted 8-cells/early morulae from four Meishan (n = 26) and four white crossbred (n = 30) females to determine survival rates following cryopreservation. Following the thawing procedure, embryos were cultured for 24 hours as described above and survival rates were determined.

Statistical Analysis

Data were analyzed with a non-parametric X^2 test using the CATMOD procedure of the Statistical Analysis System. The percentages at different stages were compared between groups and were considered statistically significant if $P < 0.05$.

Results

Efficiency of Microdroplet Method

We performed two embryo transfers, placing 24 embryos into the uterine horn of each white crossbred recipient on day 5 of the estrous cycle. On day 21 of gestation, the female that received 24 compact morulae returned to estrus. The recipient that received a combination of compact morulae/blastocysts (n = 24) produced six live offspring. These piglets were healthy and exhibited a normal phenotype (See cover picture).

Comparison of Development between Meishan and White Crossbred Embryos Following Cryopreservation

The retrieval rates from the cryovials for both breeds were above 92% (Figure 2). The survival rate was higher for Meishan (72%) than white crossbred (44%; $P < 0.001$; Figure 2) embryos. However, *in vitro* developmental rates of embryos that initially survived cryopreservation to the expanded (64%) or hatched (22%) blastocyst stages were not different between breeds (Figure 3). Developmental rates to the expanded blastocyst stage were higher for control embryos than frozen embryos from both breeds ($P < 0.001$), but no breed difference was observed for development to the hatched blastocyst stage (Figure 3). Rates of expanded blastocyst formation did not differ between Meishan and white crossbred control embryos (98 and 95%, respectively), but more Meishan control embryos developed to the hatched blastocyst stage (22% for Meishan vs. 9% for white crossbred; $P < 0.05$).

Importance of Embryonic Stage on Survival Rates after Vitrification

The survival rate was much higher for embryos of both breeds

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vitrified at the blastocyst/early expanded blastocyst stage (74% for Meishan and 47% for white crossbred) than at the compacted 8-cell/early morula stages (31% for Meishan and 4% for white crossbred; $P < 0.001$; Figure 4). Once more, a lower tolerance to vitrification was observed for white crossbred embryos than Meishan embryos, independent of the initial embryonic stage ($P < 0.001$). Cryopreservation decreased expanded blastocyst formation equally for both breeds (Figure 4).

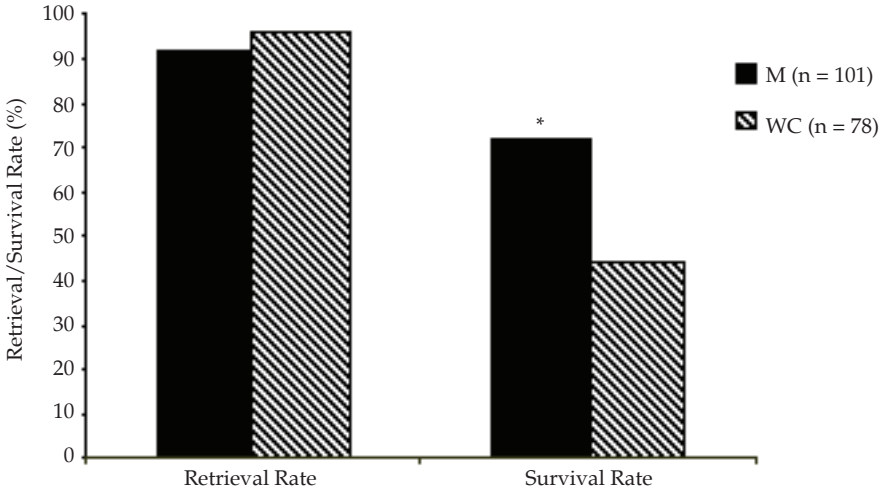

Figure 2. Comparison of embryo retrieval from cryovials (retrieval rate) and survival rate following cryopreservation of Meishan (M) and white crossbred (WC) embryos. Embryos were considered to have survived if they advanced a stage in development following 24 hours of culture. * $P < .001$ vs. WC.

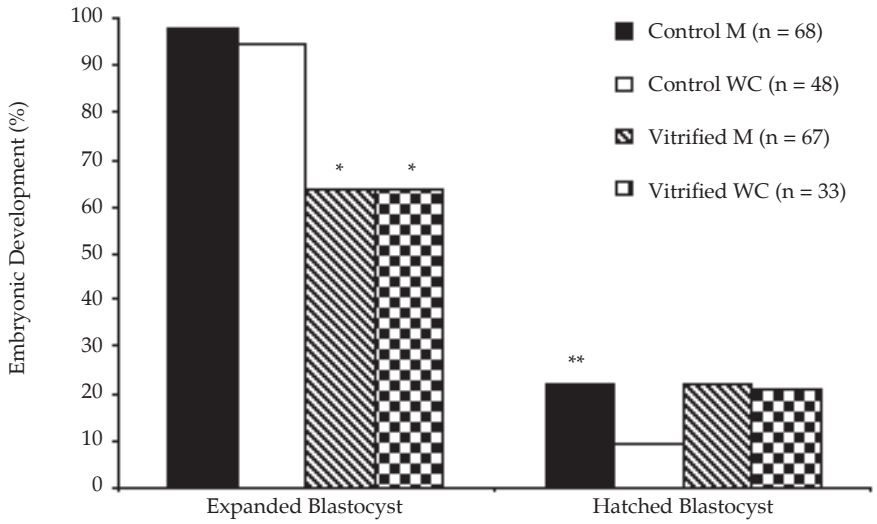

Figure 3. Effect of cryopreservation on development of Meishan (M) and white crossbred (WC) embryos *in vitro*. * $P < .05$ vs. controls. The only breed effect detected was for rates of hatched blastocyst formation in the control treatment. ** $P < .05$ vs. WC.

Discussion

Numerous attempts to freeze pig embryos in the past 30 years have resulted in low success rates, mainly due to the sensitivity of porcine embryos to hypothermia. In the 1990s and the beginning of this decade, several experiments were conducted resulting in protocols that permitted the birth of live and normal piglets from cryopreserved embryos. However, to perform these protocols with satisfactory results embryos must be exposed to cytoskeletal stabilization agents and/or micromanipulation procedures. Our studies suggest that it is possible to produce piglets after vitrification of zona pellucida-intact embryos without cytoskeletal stabilization agents and expands a previous report using a microdroplet protocol. The success of the OPS and microdroplet methods can be attributed to embryos passing through a critical temperature zone more rapidly than with conventional methods.

In several cases of porcine embryo vitrification, the type and concentration of permeable cryoprotectants have varied greatly. Cryoprotectants, such as ethylene glycol, can be highly toxic to embryos. Differences in cytoskeleton makeup may contribute to substantial species variation in sensitivity to cryoprotectants. The deleterious effects can include disruption of microfilaments during equilibration and after thawing can be evidenced as cell lyses and disaggregation, as well as nuclear and plasma membrane damage. A concern with the microdroplet protocol was the amount of time that embryos would be exposed to ethylene glycol. At higher temperatures, bovine embryos are susceptible to ethylene glycol when they are exposed to increased concentrations for extended periods of time (>10% ethylene glycol for over five minutes). The microdroplet protocol includes exposure times of 10 minutes in 10% of ethylene glycol and 45 seconds in the vitrification media containing 40% ethylene glycol, all at 37°C. During the thawing procedure embryos are exposed for more than 10 minutes in 2.5 to 5% ethylene glycol. The birth of live piglets described in this study and by investigators at the National Livestock Breeding Center in Japan suggests that pig embryos have a higher tolerance to ethylene

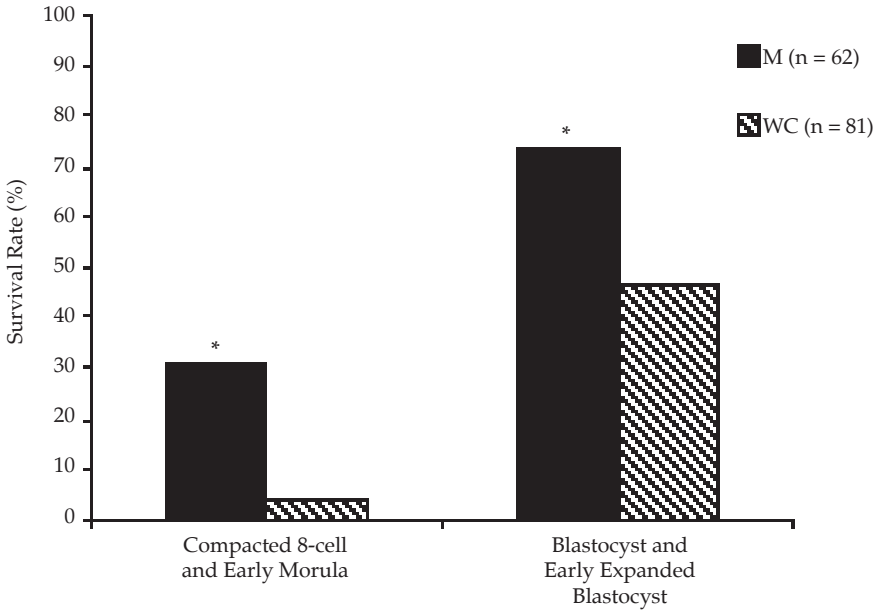

Figure 4. Effect of initial embryonic stage on survival rates of Meishan (M) and white crossbred (WC) embryos after cryopreservation. Embryos were considered to have survived if they advanced a stage in development following 24 hours of culture. Survival rates of all embryos were much higher for blastocyst/early expanded blastocyst than compacted 8-cell/early morula stages, regardless of breed ($P < .01$). The survival rate for M embryos was higher than WC embryos regardless of initial embryonic stage. * $P < .001$ vs. WC.

glycol than bovine embryos. We speculate that this phenomenon is due to a higher amount of lipid in pig compared to bovine embryos at the morula and blastocyst stages.

Only one French study has compared cryopreservation of embryos from females of Meishan and occidental breeds. These scientists, comparing embryos from Large White hyperprolific and Meishan breeds, found that Large White hyperprolific blastocysts (27%) had a lower viability *in vitro* than Meishan blastocysts (67%), when embryos were vitrified with phosphate-buffered saline. However, no difference between breeds (41 and 43%) was detected using Tissue Culture Medium 199 as the base vitrification solution. In the same study, developmental rates of vitrified morulae did not differ for the two breeds (11% for Large White hyperprolific and 14% for Meishan, respectively), although viability rates were low. In contrast, in the present study Meishan embryos survived the vitrification process better than white crossbred embryos at all developmental stages examined; compacted 8-cell, early morula, compact morula, blastocyst or expanded blastocyst. The difference between breeds for embryonic survival was almost 30%, regardless of developmental stage at cryopreservation. However, no difference was observed between breeds for *in vitro* development of embryos that initially survived vitrification to the expanded or hatched blastocyst stages. This finding is intriguing and indicates a unique mechanism present in Meishan embryos compared to embryos from white crossbred females.

The development of an efficient protocol to cryopreserve 8-cell and early morula embryos is important for the practical use of porcine embryo transfer in the field. Cryopreservation at these stages is possible, but these protocols involve micromanipulation, centrifugation and cytoskeletal stabilization. In the present study, the microdroplet method produced unsatisfactory results for survival rate of cryopreserved 8-cell compacted/early morula. The initial stage of the embryo had a strong effect on embryonic survival for both breeds. Approximately 40% more embryos survived when vitrified at the blastocyst/expanded blastocyst stages than at the compacted 8-cell/early morula stages. Consistent with this, porcine morulae are more sensitive to vitrification than blastocysts using the OPS method. It is important to point out that 8-cell pig embryos can show signs of compaction. These embryos can be easily confused with morulae or compact morulae. Therefore, researchers and technicians in the field must diligently evaluate embryos prior to vitrification. An incorrect evaluation could result in decreased survival rates of cryopreserved embryos.

Conclusion

Our study describes a vitrification method for zona pellucida-intact swine embryos that is effective in producing normal, live piglets. The optimal stage to vitrify pig embryos using the microdroplet protocol is at the blastocyst/expanded blastocyst stage. Further, our results suggest that Chinese Meishan embryos have a higher capacity to survive the vitrification process than white crossbred embryos. However, embryos from both breeds that initially survive vitrification have similar developmental capabilities *in vitro*. Improvements in embryo freezing procedures are likely to become increasingly important to the swine industry, especially with the advancement of nonsurgical embryo transfer in swine.

[1]Marcelo M. Montagner was a visiting scholar in the Animal Science Department at the University of Nebraska–Lincoln and recently received his doctorate from the Federal University of Santa Maria in Santa Maria, Brazil. Paulo B.D. Gonçalves is a Professor in the Biotechnology and Animal Reproduction Laboratory at the Federal University of Santa Maria. Ronald K. Christenson is a Research Physiologist at the USDA, ARS, Roman L. Hruska U.S. Meat Animal Research Center in Clay Center, NE. Ginger A. Mills is an Agricultural Research Technician and Brett R. White is an Assistant Professor in the Animal Science Department at UNL.