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Swollen Ampulla as an Indicator of Successful Pregnancy in B6C3F1 Recipient Mice used for Assisted Reproduction

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In vitro fertilization (IVF), embryo cryopreservation, and embryo transfer (ET) are assisted reproductive technologies (ARTs) that are used extensively for the maintenance of mouse models in animal research. Inbred mouse strains with different genetic backgrounds vary in their reproductive performance. Cryopreservation can affect embryo quality and viability, and the genetic background of ET recipients can influence the ET result. In this retrospective study, we analyzed the outcomes of ETs performed in our facility during the last 6 y. We found that B6C3F1 mice with swollen ampullae show almost 3-fold higher pregnancy rates than mice with nonswollen ampullae when either freshly isolated or frozen-thawed embryos are implanted. Implantation of freshly collected embryos in recipients with swollen ampullae led to significantly higher pregnancy rates in comparison to implantation of frozen-thawed embryos, regardless of whether the latter were fertilized in vivo or in vitro. Moreover, we found a significant effect of genetic background on the birth rate; C57BL/6J mice and mice with a mixed genetic background had 34% higher birth rates than did C57BL/6N mice. Within the C57BL/6J group, the birth rates were significantly higher when using fresh in vivo-fertilized embryos, and cryopreservation negatively affected both in vivo- and in vitro-fertilized embryos. The success rate of obtaining one living pup was not significantly different between frozen-thawed and fresh embryos. Overall, a swollen ampulla is a strong indicator for a successful pregnancy, together with the embryo manipulation and genetic background. A better understanding of the factors that affect the reproductive outcome might lead to optimization of the ART protocols and contribute to a reduction in the number of mice used for these procedures.

Abbreviations and Acronyms: ART, assisted reproductive technology; ET, embryo transfer; IVF, in vitro fertilization

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Introduction

Embryo transfer (ET) is a well-established technique in the field of transgenic animal production, maintenance, distribution, and experimentation and a key component of assisted reproductive technologies (ARTs). Transgenic animal models are being produced using old and new techniques and being distributed between animal facilities in the forms of animals and fresh or frozen material. The ET of 2- to 4-cell stage fresh or cryopreserved embryos into the infundibulum of the oviduct is the main technique for the rederivation and revitalization of new mouse lines imported into our facility.

The principle of 3Rs according to the Directive 2010/63/EU[10] requires less breeding and maintenance of unused animals in the animal facilities. Therefore, increasingly more transgenic lines are being cryopreserved. To determine whether cryopreservation process was successful, a probe of frozen embryos is thawed. To preserve transgenic lines that have poor reproductive outcomes after normal mating and in order to rederive strains that we receive from other institutions as frozen sperm, we perform in vitro fertilization (IVF) of mature oocytes by using fresh or frozen-thawed sperm. This allows us to produce more embryos while using fewer mice.

The establishment of new techniques for the production of new transgenic lines increases the need for breeding efficiency and optimization of the ET process. Many factors affect the success of ET. In the present study, we examined the following: the cryopreservation and, thus, the use of freshly isolated or frozen-thawed embryos, embryo fertilization after normal mating or through IVF, the genetic background, and the selection of recipient females with regard to signs of pseudopregnancy.

Although the cryopreservation is a well-established technique, it still implicates damages in embryos through freezing and formation of ice crystals.[33] Recently published studies comparing reproductive data generated by using fresh and frozen-thawed material for ET show significantly lower rates of success for thawed materials, requiring the use of more embryos to produce one living pup.[13] Strain-dependent susceptibility to damage by freezing should be considered in planning cryopreservation and ET.[49]

In the case of IVF, the genetic background of the gametes also influences the fertilization rate.[24,47,49] Although IVF has provided a clinical option for human infertility, it has been linked to deficiencies in children as compared with children who were conceived naturally.[7,9,23] Furthermore, studies in mice have shown that in vitro embryo culture slows development and negatively affects embryo metabolism and epigenetic stability.[1]

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Inherited genetic differences cause different responses to components of ART, including superovulation, fertilization ability, production of living pups, and maintenance of the pregnancy and the litter after birth.[2,34] Embryo development and survival depend on the genetic background of the embryos transferred into the recipient mothers.[3,38] In our facility, embryo donors are either wild-type or transgenic female mice, with the latter possibly having both higher variability than wild-type mice and possibly lower capacity of the embryo to survive after ET.[15,30] With regard to foster mothers, genetic differences render female mice preferable for ET.[31,38] A direct connection between the genotype of the recipient murine uterus and pregnancy rates and pre- and postnatal survival of offspring has been published.[11,43]

Research on human uterus and uterus-based reproductive disorders has led to use of the mouse oviduct as research model.[44] Modern imaging techniques and mouse lines with fluorescent reporters provide a realistic representation of the regional and cellular heterogeneity of the mouse oviduct.[18] The present study is focused on the mouse ampulla, which is the swollen part of the oviduct present in an ovulating mouse at postcoital day 0.5 and the site of fertilization. A swollen ampulla is important both for oocyte and embryo donors and for recipient females. Previous electron microscopy studies have shown that injuries to the ampulla result in fewer pregnancies.[53] In addition, a swollen ampulla is a good indicator that donor females have reacted to the hormonal manipulation, and are producing large numbers of oocytes.[20] Correlation studies of pseudopregnancy and the estrous cycle have shown that females that remain in diestrus and have a copulatory plug after mating with vasectomized males show higher pregnancy and birth rates than do females that lack these features.[37] Cellular changes in the oviduct structure during estrus result in more ciliated cells during diestrus[39]; these ciliated cells have the primary role of supporting the movement of the cumulus oocyte complex.[51] Furthermore, the interaction of these factors may initiate critical communication between the ampulla and the spermatozoa in preparation for fertilization.[52]

Thus, the swollen ampulla could be considered as an approval checkpoint for the successful pregnancy and pseudopregnancy for ET.[12,30,41] However, only a few studies have studied the relationship between a swollen ampulla and a successful pregnancy.[41] The goal of our study was to analyze the correlation between a swollen ampulla in B6C3F1 pseudopregnant females and the success of pregnancy and to evaluate the influence of cryopreservation, fertilization, and genetic background on ET success.

Materials and Methods

Animal husbandry. The cryopreservation and ET procedures were planned and performed at the Central Unit for Animal Research and Animal Welfare Affairs of the University Hospital in Düsseldorf. All procedures and maintenance of the mice were carried out in accordance with the Directive 2010/63/EU[10] after approval by the State Office for Nature, Environment and Consumer Protection (LANUV, State of North Rhine-Westphalia, Germany). The procedures were approved by the competent supervisory authority as part of the breeding program.

Mice were housed at 20 to 24 °C room temperature and 55 ± 5% humidity on a 12:12-h light:dark cycle (lights on 0600) with food (ssniff Spezialdiäten GmbH, Soest, Germany) and ozone-treated and acidified water ad libitum. The mice in our facility are housed either in open or individually ventilated cages that contain wood fiber LAS bedding HW300/500 (Altromin, Lage, Germany) and are located in different microbiological units with variable microbiological status. Gamete donor male and female mice were obtained either from quarantine rooms with an inferior hygiene status for rederivation purposes or from specific pathogen-free rooms (SPF barrier) that were usually free of all agents listed in Table 3 of the FELASA recommendations[36] and of *Staphylococcus aureus*, *Proteus* spp., *Klebsiella* spp., *Bordetella bronchiseptica*, *Bordetella hinzii*, *Pseudomonas aeruginosa*, *Muribacter muris*, and dermatophytes. Nonpathogenic intestinal flagellates, mouse norovirus, *Helicobacter* spp., *Rodentibacter* spp. and *S. aureus*, *Proteus* spp., *Klebsiella* spp., and *M. muris* were tolerated in some housing areas as either single infections or coinfections. Health monitoring follows FELASA recommendations[36] and is performed quarterly by examination of resident mice and of BALB/c sentinel mice that are exposed to soiled bedding.

Mice used in this study. Genetically engineered mice with C57BL/6J, C57BL/6N, or mixed and undefined genetic backgrounds were used as embryo, oocyte and sperm donors. Embryo donors were mated normally (fertilization in vivo) and sacrificed for embryo collection. Oocyte and sperm donors were euthanized, and the gametes were harvested for the IVF procedure. B6C3F1 females produced by mating C57BL/6J females and C3H males served as recipient mothers.

Superovulation and embryo collection. The oocyte and embryo donors, which were between 4 to 16 wk of age, were superovulated as described previously[16] with pregnant mare serum gonadotropin (Intergonan 240 IU/mL, MSD Tiergesundheit, Unterschleißheim, Germany) and human chorionic gonadotropin (Predalon 5000 IU, Essex Pharma GmbH, Waltrop, Germany) 48 h later, followed by mating with fertile male mice for embryo harvest. The initial hormone dose was selected based on the literature and was adjusted to optimally suit the genetic background of the strains, based on pilot dose-response testing in our laboratory (C57BL/6J, 7 IU; C57BL/6N, 5 IU; mixed background: 7 IU or 5 IU based on the proportions of C57BL/6J or C57BL/6N respectively). The morning after human chorionic gonadotropin or on day 1.5 after human chorionic gonadotropin administration, superovulated mice were euthanized and their oviducts were extracted. Oocytes were harvested by tearing the ampulla open in IVF medium,[27] or embryos were flushed from the oviducts at the 2-cell stage by using M2 medium (Sigma-Aldrich, Munich, Germany). Isolated embryos were washed 2 times in M2 medium, and those with intact zona pellucida and well-formed blastomeres (equal in size and shape with no or little fragmentation) were either used for cryopreservation or transferred into recipient females.

IVF. The IVF with fresh or frozen sperm was performed using media that was prepared as follows.[50] Most of the ingredients were purchased from Sigma-Aldrich. Glucose was purchased from Carl Roth (Karlsruhe, Germany) and BSA from Merck (Darmstadt, Germany). All incubation dishes were prepared on the morning of the IVF session and allowed to equilibrate for 30 min in a bench-top CO_2 incubator (Sanyo, Osaka, Japan) at 37 °C with 5% CO_2. For collection of sperm, blood and fat were removed from the cauda epididymides by using a tissue paper. Sperm were then collected from the epididymis by cutting the tissue with fine spring scissors and releasing the sperm clumps into the sperm collection dish in a 90 µL drop of preincubation medium. For frozen sperm, the straw was thawed for 10 min into a 37 °C water bath and the contents suspended in the center of a 90 µL drop of preincubation medium. The preincubation medium was prepared as described previously[40] and all the reagents were purchased from Sigma-Aldrich. The dish was then returned to the incubator, either for 60 or 30 min, respectively, for fresh and frozen-thawed sperm to allow motile spermatozoa

to swim out of the tissue, recover maximum mobility, and undergo capacitation to acquire the ability to fertilize the oocytes.

The swollen ampullae of the superovulated females were teared open, and the cumulus-oocyte complexes were released into a 200 µL or 90 µL drop of IVF medium for fresh or frozen-thawed sperm, respectively. The IVF medium is a modified human tubal fluid medium with more calcium.[27] After the required incubation times, 5 µL fresh or 10 µL frozen-thawed sperm were added into the IVF drop with the cumulus-oocyte complexes, and the dishes were incubated for 4 to 6 h to allow fertilization to occur. After 2 washing steps in 150 µL high-calcium IVF medium, all cells were transferred into a fresh drop of 150 µL high-calcium IVF medium and incubated overnight. The next morning, the 2-cell IVF embryos were washed twice in M2 medium, and those with intact zona pellucida and well-formed blastomeres that were equal in size and shape and had no or little fragmentation were either transferred directly into recipient females or cryopreserved and thawed for ET at a later time point.

Cryopreservation and thawing. In vivo- and in vitro-fertilized 2-cell embryos were cryopreserved in M2 medium with 1.5 M of 1,2-Propandiol (Sigma-Aldrich) supplemented with 0.1 M sucrose (VWR, PA). For the cryopreservation process, the isolated embryos were washed 2 times in M2 medium and then transferred in a drop of cryo medium and allowed to sink to the bottom of the dish. The IVF embryos were first transferred into a drop of 0.05 M sucrose and allowed to sink in 0.1 M sucrose. After that, they were transferred in a straw (MTG Technologies, Bruckberg, Germany) that was sealed on one side with a metal ball, and on the other side the flagellum was sealed by heat. All straws were attached on a controlled-rate freezer (Labotect Cryo Unit - LCU, Labotect, Göttingen, Germany) for slow freezing. The straws were cooled at a rate of 5 °C/min from room temperature to 0 °C and were held there for 10 min. Then they were frozen down at a cooling rate of 1 °C/min to -7 °C and held there for 10 min for seeding. After ice formation, they were frozen at a cooling rate of 0.4 °C/min from -7 to -32 °C and held there for 10 min. After this time, the straws were transferred in liquid nitrogen.

In vivo- and in vitro-fertilized (IVF) embryos were thawed by removing each straw from liquid nitrogen and holding it in the air for at least 40 s. After removal of the metal ball and the flagellum, the contents of the straws were flushed into a dish by using a syringe containing the thawing medium (200 µL of 0.1 M sucrose diluted in M2). After 5 min, 200 µL of the thawing medium was added to the dish. After another 5 min, a second aliquot of thawing medium was added. Five minutes later, the embryos were collected and washed twice in M2. The thawed embryos were then transferred into recipient females.

ET. Recipient B6C3F1 females, 8 to 16 wk of age, were mated at 1400 h to vasectomized B6C3F1 males with proven sterility to induce pseudopregnancy. Only females with a visible vaginal plug were used the following morning for the ET.[22]

ET was performed after the female mice had been injected intraperitoneally with ketamine (100 mg/kg body weight; Ketavet, Zoetis, Berlin, Germany) and xylazine (10 mg/kg body weight; Rompun, Bayer HealthCare, Leverkusen, Germany). To provide analgesia, recipients received subcutaneous carprofen (5 mg/kg body weight; Rimadyl, Pfizer, Germany). Before the surgical procedure, the eyes were covered with eye ointment (Bepanthen, Bayer, Leverkusen, Germany), and the skin was shaved and disinfected with alcohol dorsolumbar. The numbers of embryos that were available for ET after superovulation or thawing varied depending on the number of plugged females found on that day. In general, no less than 5 and no more than 20 embryos were transferred unilaterally into the left oviduct, as described in detail elsewhere.[22] A glass capillary tube was inserted into the swollen infundibulum, and the embryos were placed in the ampulla, which was visually evaluated as swollen when it was rounded, glassy, and at least 2-fold larger than the normal-size ampulla seen in anestrual and nonovulating mice. At this point, the success of the transfer was indicated by the presence of a small bubble left in the oviduct behind the embryos. During surgery, the mice were kept on a warm plate (37 °C) until they had recovered from anesthesia, at which point they were placed in individually ventilated cages (Zoonlab, Castrop-Rauxel, Germany) in which they gave birth and had weaned their pups. Embryo recipients were fed breeding rodent chow (ssniff Spezialdiäten GmbH, Soest, Germany). The birth date and litter size were noted as soon as the mice gave birth.

Statistical analysis. The dependent variables used in this study were pregnancy rate, defined as the number of recipients that gave birth to the total number of ET recipients; birth rate, calculated as the ratio of pups born to the total number of transferred embryos; and success rate, the mean number of transferred embryos needed to produce at least one living pup.

Data for recipients with or without swollen ampullae refer to results of ETs during the last 5 y (2018 to 2022) at our institution, whereas data on ET birth and success rates are from 2017 to 2022 (6 y). Normality was determined graphically by Q-Q plots. Three-way, two-way, or one-way analyses of variance (ANOVA) were performed to assess the effect of fertilization method (normal mating or IVF), embryo isolation (freshly isolated or frozen-thawed), ampulla condition (swollen or nonswollen), and genetic background (C57BL/6J, C57BL/6N, or mixed) on the dependent variables (pregnancy rate, birth rate, and success rate). Post hoc analysis consisted of simple main effects testing when the ANOVA revealed a significant interaction and main effects testing when there was no significant interaction. Data are presented as mean values ± SEM%. Statistical significance of $P \leq 0.01$ is represented graphically as dagger (†) and $P \leq 0.05$ as single asterisk (*). Statistical analysis and graphs were conducted using IBM SPSS Statistics 25 software (IBM Corporation, Armonk, NY) and GraphPad Prism version 9.0.0 for Windows (San Diego, CA), respectively.

Results

Among 1,050 plugged B6C3F1 ET recipients housed in our facility during the last 5 y, 72 ± 2% had a swollen ampulla, and 28 ± 2% did not have a swollen ampulla. Regardless of the category of embryos transferred, 56 ± 2% of females with swollen ampulla gave birth, as compared with 19 ± 3% of those without a swollen ampulla (Table S1).

Effect of ampulla condition and cryopreservation on pregnancy rate. Pregnancy rate is defined as a percentage of ET recipients that gave birth relative to the total number of ET recipients. Of the total number of ETs, 57 ± 3% of 388 females that received fresh embryos and 39 ± 2% of 662 that received cryopreserved embryos gave birth. Of 388 B6C3F1 plugged females, 76 ± 2% had a swollen ampulla and 67 ± 4% of these gave birth. The remaining females did not have a swollen ampulla (25 ± 2%). Nevertheless, 24 ± 5% of these gave birth. Among the recipients that received frozen-thawed embryos, 70 ± 2% had a swollen ampulla and 30 ± 2% did not. 49 ± 3% and 17 ± 3% respectively gave birth (Table S1).

The conditions of the ampullae and embryos (swollen/nonswollen and fresh/frozen-thawed) are factors that affected

the pregnancy rate, as determined by 2-way ANOVA that revealed a significant interaction effect (ampulla × embryo, $F(1,451) = 3.67$, $P = 0.046$). Simple main effects analysis revealed that mice with swollen ampullae show higher pregnancy rates than those with nonswollen ampullae when they receive either freshly isolated embryos (swollen, 67 ± 4%; nonswollen: 24 ± 5%) or frozen-thawed embryos (swollen, 49 ± 3%; nonswollen, 17 ± 3%) ($F(1,451) = 97.69$, $P = 0.0001$). Mice with swollen ampullae also had higher pregnancy rates when implanted with freshly isolated versus frozen-thawed embryos ($F(1,451) = 17.43$, $P = 0.0013$) (Figure 1).

Effect of ampulla condition, cryopreservation, and fertilization method on pregnancy rate. We further analyzed the effect of fertilization method on the ET outcome. Of the plugged recipients, 78 ± 3% of 296 and 64 ± 4% of 92 females that received fresh in vivo- or in vitro-fertilized embryos, respectively, and 72 ± 3% of 452 and 67 ± 4% of 210 that received frozen-thawed in vivo- or in vitro- fertilized embryos also had a swollen ampulla. The remaining, 22 ± 3% and 36 ± 4% of recipients that received fresh in vivo- and in vitro-fertilized embryos, as well as 28 ± 3% and 33 ± 4% of recipients of frozen-thawed embryos produced by normal mating and IVF, respectively, did not have swollen ampulla (Tables S2 and S3).

Our analysis did not reveal a significant interaction (ampulla x embryo x fertilization method) in 3-way ANOVA ($F(1,459) = 0.27$, $P = 0.603$). Simple main effects analysis showed a significant effect for ampulla condition ($F(1,445) = 98.02$, $P = 0.0001$). Under normal mating, mice with swollen ampullae showed higher pregnancy rates than mice with nonswollen ampullae, regardless of whether freshly isolated embryos (swollen, 65 ± 4%; nonswollen, 24 ± 5%) or frozen-thawed embryos were implanted (swollen, 51 ± 3%; nonswollen, 21 ± 4%) (Figure 2). Similarly, after IVF, mice with swollen ampullae showed higher pregnancy rates than mice with nonswollen ampullae, regardless of whether they had been implanted with freshly isolated embryos (swollen, 78 ± 6%; nonswollen, 25 ± 9%) or frozen-thawed embryos (swollen, 46 ± 5%; nonswollen, 10 ± 4%) (Figure 2).

Effect of the genetic background on birth rate. To assess the relative contribution of the genetic background on the birth rate (calculated as the ratio of born pups to the total number of transferred embryos), the litters of all the lines transferred during a 6 y period were analyzed independent of their fresh or frozen-thawed status or fertilization method. This analysis considered only litters of ET recipients with a swollen ampulla.

Genetic background had a significant effect on birth rates ($F(2,899) = 4.88$, $P = 0.008$) (Figure 3). Pairwise comparisons of ETs revealed that C57BL/6J mice (21 ± 1%; $n = 529$) had higher birth rates than C57BL/6N mice (16 ± 2%; $n = 151$) ($P = 0.003$). Mice with a mixed genetic background (22 ± 2%; $n = 125$) also had higher birth rates than C57BL/6N ($P = 0.027$).

To assess whether fresh and frozen-thawed embryos, in vivo- or in vitro-fertilized, support development to term and to determine whether genetic background influences development, embryos of the genetic backgrounds mentioned above were transferred into recipient females with a swollen ampulla. C57BL/6J mice showed no significant effects on 2-way ANOVA for fertilization method × embryo interaction ($F(1,525) = 0.005$, $P = 0.944$). However, significant main effects were detected for both embryo condition ($F(1,525) = 15.25$, $P = 0.001$) and fertilization method ($F(1,525) = 5.12$, $P = 0.024$). After normal mating, C57BL/6J mice had higher birth rates when implanted with freshly isolated embryos (28 ± 2%; $n = 183$) versus frozen-thawed embryos (19 ± 2%; $n = 189$). Similarly, after IVF, mice showed higher birth rates when implanted with freshly isolated embryos (23 ± 3%; $n = 60$) as compared with frozen-thawed embryos (14 ± 2%; $n = 97$) (Figure 4).

In contrast to C57BL/6J, C57BL/6N mice did not show a significant interaction for fertilization method × embryo based on 2-way ANOVA ($F(1,237) = 0.240$, $P = 0.625$), and no significant main effects were found (fertilization: $F(1,237) = 0.444$, $P = 0.507$; embryo: $F(1,237) = 2.69$, $P = 0.102$). After normal mating, these mice had similar birth rates when implanted with freshly isolated embryos (19 ± 2%; $n = 103$) or frozen-thawed embryos (15 ± 3%; $n = 68$). Similarly, after IVF, birth rates did not

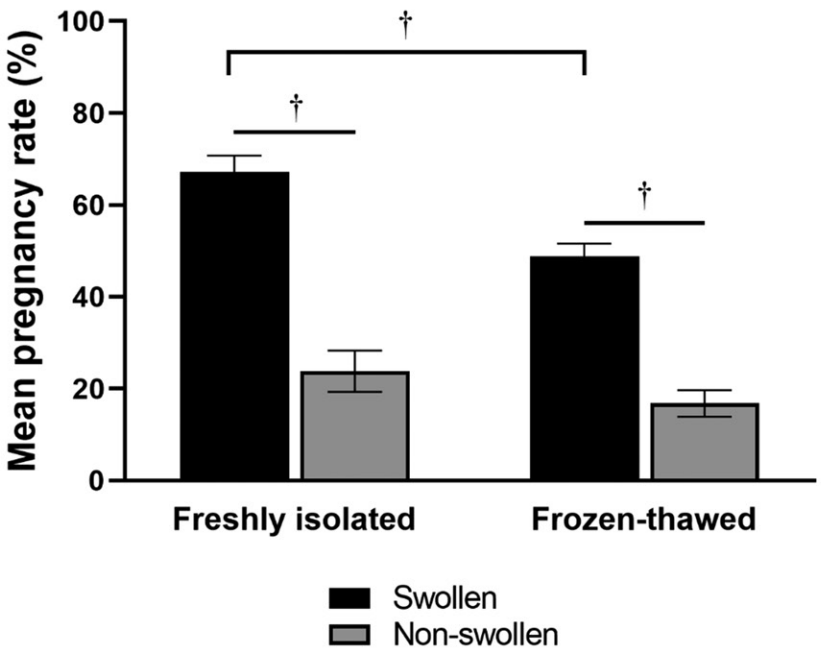

Figure 1. Mean pregnancy rates for mice with swollen and nonswollen ampullae implanted with freshly isolated and frozen-thawed embryos. Data are shown as mean ± SEM%. †$P \leq 0.01$.

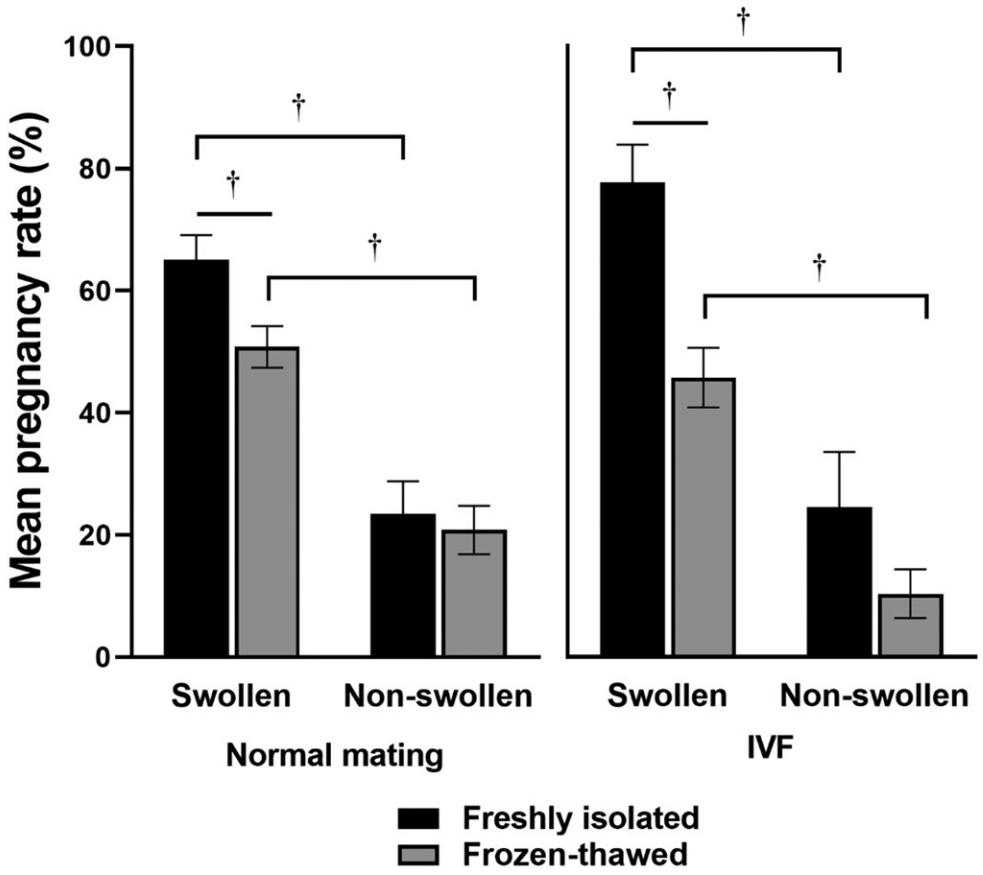

Figure 2. Mean pregnancy rates after normal mating and IVF for mice with swollen and nonswollen ampullae implanted with freshly isolated and frozen-thawed embryos. Data are shown as mean ± SEM%. †$P \leq 0.01$.

differ between C57BL/6N mice implanted with freshly isolated embryos (18 ± 5%; n = 18) or frozen-thawed embryos (11 ± 3%; n = 52) (Figure 4).

Mice with a mixed genetic background showed a significant fertilization method × embryo interaction based on 2-way ANOVA ($F(1,128) = 5.84$, $P = 0.017$). Simple main effects showed no significant difference in birth rates between normal mating (24 ± 4%; n = 26) and IVF (33 ± 6%; n = 16) when freshly isolated embryos are used (P = 0.214). However, their birth rates differed significantly between in vivo- (24 ± 4%; n = 42) and in vitro-fertilized (12 ± 4%; n = 41) frozen-thawed embryos (P = 0.018) (Figure 4).

Table 1 summarizes the raw data for the success rate (the mean number of transferred embryos needed to produce at least one living pup) of all 3 line groups with a C57BL/6J, C57BL/6N, or mixed genetic background. The genetic background or fertilization method did not influence the lowest number of transferred embryos necessary to produce one pup.

Discussion

The technique of ET in mice is one of the most important ARTs. Many factors affect the outcome of the ET, with the recipient female, as the embryo carrier, of great importance. Different strains of recipients have been used during the years based on their reproductive performance and ability to care for their offspring. In our facility, the B6C3F1 mice are used due to their good mothering instincts[48] and low intraperitoneal fat, which makes the infundibulum easier to find and the ET without complications to perform. To our knowledge, this is the first study to examine the ET success in B6C3F1 hybrid mice with regard to a swollen ampulla of the oviduct. Both fresh and frozen-thawed embryos are routinely transferred into recipient females. Embryos are produced by either after normal mating or IVF. We examined both factors in ET recipients with and without swollen ampullae. Finally, we compared birth and success rates in genetically engineered strains with different genetic backgrounds.

Previous efforts of our group to increase plug formation rate involved determining the estrous stage of prospective foster mothers or adding soiled bedding material from vasectomized males to the female cages for synchronization before mating, but these approaches did not improve plug formation. Multilateral hormonal manipulation of recipients also failed to improve the pseudopregnancy rate, mating efficiency or embryonic development.[19] Our results show that the success rate for frozen-thawed in vitro-fertilized embryos (IVF) lies between 4.0 and 4.5, which is lower than previously published results but still comparable to mice that only receive eCG.[19] Achieving a desired target success rate requires consideration of the side effects of stress and burden due to animal handling. Thus, in our laboratory, before every ET procedure, a group of female mice, without prior treatment or synchronization, are mated with the vasectomized males, and all females with plugs are used for ET, regardless of their ampullae status.

Despite the importance of plug formation as a critical factor for selecting pseudopregnant females,[6] it is inadequate as a sole criterion[22] because it does not necessarily correlate with either maintenance of the pregnancy or a positive outcome.[4,21] Thus, in our animal facility, even though a copulatory plug has been considered the only sign of successful pseudopregnancy, we

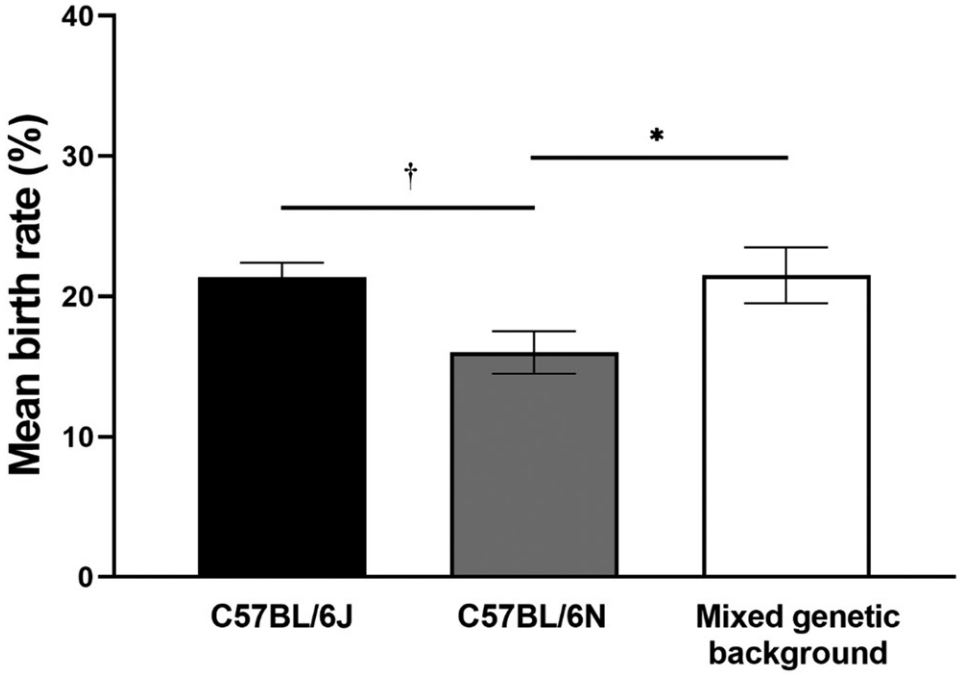

Figure 3. Mean birth rates for mice with different genetic backgrounds (C57BL/6J, C57BL/6N, and mixed). Data are shown as mean ± SEM%. *$P \leq 0.05$. †$P \leq 0.01$.

have also recorded the swollen status of the ampulla during ET the last 5 y.

Our study showed that 72% of 1,050 plugged recipient mice had a swollen ampulla, and the remaining 28% did not. Additionally, the existence of the swollen ampulla led to higher pregnancy rates compared to the plugged females with no swollen ampulla, as also described previously.[41]

When fresh embryos were transferred into females with swollen ampullae, our recipient B6C3F1 hybrid mice exhibited a 65% pregnancy rate, similar to another study that reported 50 and 69% pregnancy rates for FVB/N and ICR strains, respectively.[41] A previous study graded the ampulla as 1, corresponding to nonswollen, and 2 to 4, corresponding to swelling.[41] Our data show a clear relationship between pregnancy rate and the status of the ampulla. In FVB/N mice, pregnancy rates were 27, 44, and 80% when ampullae swelling grades were 2, 3, and 4, respectively.[41] In that study, stage 1 ampullae were associated with a pregnancy rate of 13%, whereas we found pregnancy in 25% of mice with nonswollen ampullae. Therefore, the importance of a swollen ampulla to pregnancy may vary among different recipient mouse strains. The strong dependency we saw for B6C3F1 and the FVB/N in another study,[41] does not appear to be equal for all strains; for the outbred strain ICR, the success rates for stages 2, 3, and 4 ampullae were 62, 72, and 74%, respectively, and for stage 1, no swollen ampulla, were still as high as 63%.[41] Thus, ICR mice appear to be better choices as embryo recipients. Endogenous hormone concentrations, physical factors of ampulla and oviduct, and physiologic events regulate the time of copulation (early in the afternoon, during the night, or even in the morning before plug check), together with the time and duration of mating with the vasectomized males, determine the appearance of the swollen ampulla and the degree and duration of swelling. These factors will potentially help to maintain the pregnancy and eventually lead to birth.[18,19,45]

Strains and hybrids that are used for fostering should be repeatedly tested. Making direct and accurate comparisons is difficult when comparing the work of different groups, as the data are generally based on different mouse lines and strains, ages, genetic backgrounds and protocols.[25] We define pregnancy rate as the percentage of recipients that delivered pups, in contrast to using the number of females that had positive abdominal palpation 2 wk after ET.[41] Although our data were obtained from a different mouse strain (B6C3F1), these foster mothers received both wild-type and transgenic embryos in varying numbers each time. Seasonal variations may have affected the reproductive performance of the mice.[14]

In our animal facility, fresh as well as frozen-thawed embryos are transferred into female mice to rederive new mouse lines and document the success of the cryopreservation process. Despite the fact that the cryopreservation process is constantly being improved, it still can have detrimental effects. As shown in Figure 1, the pregnancy rate of recipients with swollen ampullae that received fresh embryos (65%) was significantly higher than that of recipients that received frozen-thawed embryos (51%). This result agrees with other published studies that compared the pregnancy rates between fresh and frozen-thawed embryos.[13] As shown in Table S2, the pregnancy rate of the recipients that received fresh embryos lie between 24% (nonswollen ampulla) and 65% (swollen ampulla) and, in the case of frozen-thawed embryos, between 21 and 51%. Previous research does not differentiate between swollen and nonswollen ampullae and had pregnancy rates ranging from 38 to 94% with fresh embryos and 38 to 63% with frozen-thawed embryos.[13] We found that the birth rate ranged from 19 to 28% for fresh embryos and 15 to 24% for frozen-thawed embryos. Another study found a higher frequency of born offspring when using fresh embryos (30 to 42%) but a rate similar to ours when using frozen-thawed embryos (18 to 24%).[13] When fresh IVF embryos from lines with C57BL/6J background were implanted, the birth rate of our B6C3F1 recipients was 23%, which is comparable to the percentage found by another group (21%), although they used the potentially superior IVF strain for ET.[25]

Our data showed that the pregnancy rate of recipients with swollen ampullae, that received fresh embryos that were

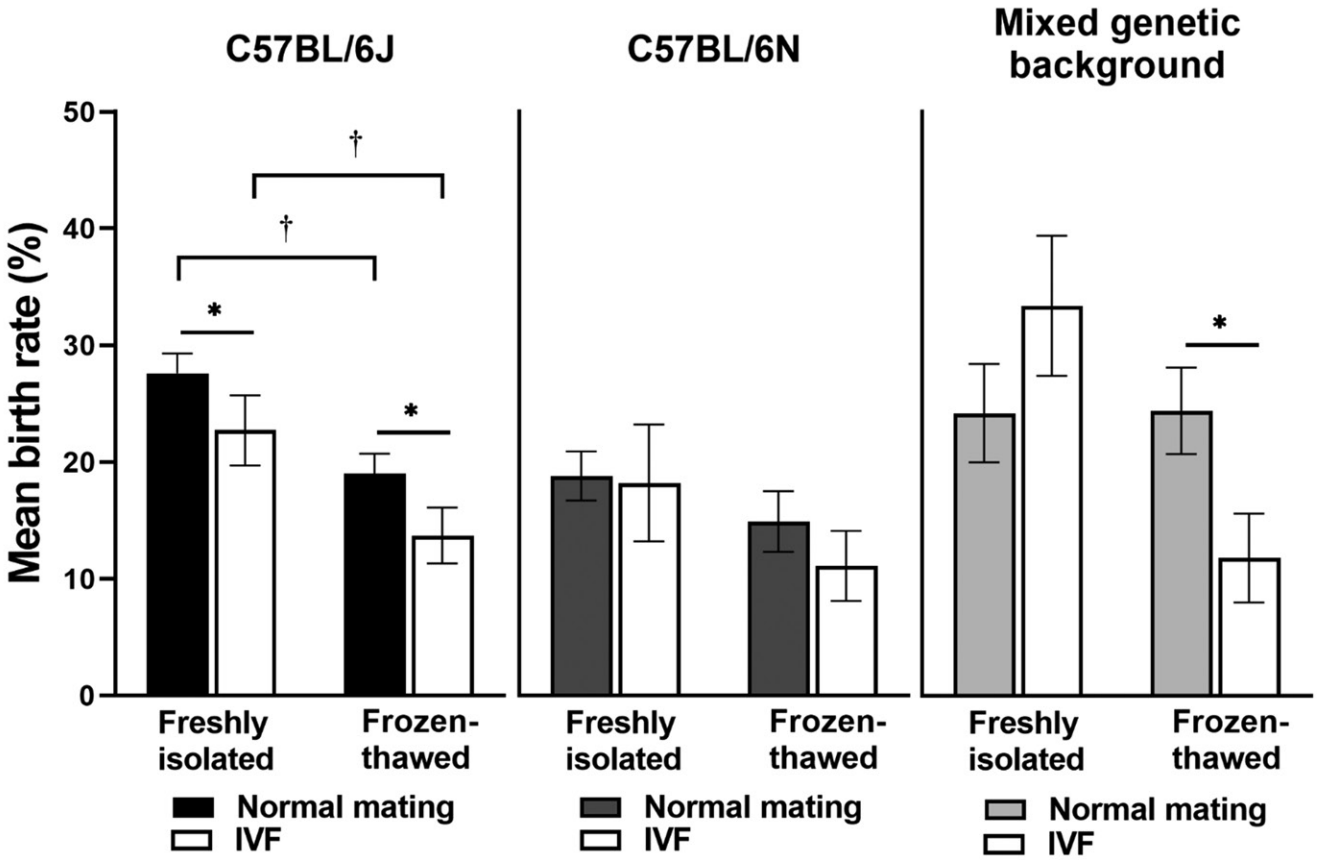

Figure 4. Mean birth rates of normal mating and IVF after implantation of freshly isolated and frozen-thawed embryos for mice with different genetic backgrounds (C57BL/6J, C57BL/6N, and mixed). Data shown as mean ± SEM%. *$P \leq 0.05$. †$P \leq 0.01$.

fertilized in vitro was 78%; this finding is in accord with another study that found a pregnancy rate of 74 to 79% when fresh IVF embryos were transferred only into recipients with swollen ampullae.[35] Our finding embryo survival rates fell after IVF can be inferred from a previous study that showed the detrimental effects of in vitro culture on early embryo development.[1] Consistent with that study,[1] our birth rate also fell after incubation of the embryos in IVF media. Nevertheless, we found that the same number of in vivo- and in vitro-fertilized embryos, 3.3 versus 3.0 embryos, respectively, is required in our ETs to produce one pup, in contrast to a study in which twice as many IVF embryos (6.0) than normal embryos (3.2) were needed to achieve the same outcome.[1]

Our observations indicate that the in vitro-fertilized embryos were more fragile, less stable, and more susceptible to the cryopreservation process, as compared with fresh in vivo derived embryos. Our data suggest that the combination of the IVF manipulation and the freezing may reduce embryo quality and resistance to survival 2-fold, thus leading to lower survival in the uterus (Figure 2). Mouse lines with poor reproductive performance may benefit from IVF; however, IVF and embryo culture can negatively affect the quality of the embryos due to reactive oxygen species produced in the culture media that cause oxidative stress.[17] In agreement with previous work,[5] we provide additional evidence that manipulation of gametes and fertilized embryos during IVF affect the quality of the transferred embryos and, eventually, the ET success.

Another study also examined the birth and success rates and studied the influence of the genetic background, differentiating the C57BL/6 strain between C57BL/6J and N.[35] We found that C57BL/6J and C57BL/6N had birth rates of 23 and 18%, the other study found birth rates of 31 and 24%.[35] Other studies have also reported higher birth rates when using fresh in vivo-fertilized embryos but they used the ICR as the recipient strain[15,30]; as mentioned above, ICR mice might have better reproductive performance when used as an ET recipient,

Table 1. The success rate considering the genetic background of the lines of the present study: C57BL/6J, C57BL/6N, and mixed genetic background

	Success rate for fresh embryos		Success rate for frozen-thawed embryos	
	Normal mating	IVF	Normal mating	IVF
C57BL/6J	3.1 ± 0.2 (121)	3.1 ± 0.5 (36)	4.5 ± 0.4 (116)	4.5 ± 0.5 (47)
C57BL6/N	3.7 ± 0.5 (56)	3.7 ± 0.6 (12)	4.4 ± 0.7 (33)	4.3 ± 0.9 (18)
Mixed	3.9 ± 0.8 (21)	2.7 ± 0.2 (10)	4.1 ± 0.6 (32)	4.0 ± 0.7 (15)

Data presented as mean ± SEM%. Number of litters in parentheses.
Success rate was calculated as the mean number of transferred embryos needed to produce at least one living pup.

possibly due to heterosis that gives some uteri a better environment for embryo survival and growth.[42] In contradiction to our birth rate, our success rate of fresh in vivo-fertilized embryos was comparable to the previously mentioned studies.[15,30] The data suggest that a slightly higher number of C57BL/6N fresh embryos (3.7) is required to achieve success rates comparable to C57BL/6J (3.1). Other investigators also found a success rate of 4.1 for the C57BL/6N strain and 3.3 for the C57BL/6J strain.[35] In another research group, only 2.3 C57BL/6J fresh in vivo-fertilized embryos were necessary to transfer to produce one living pup.[30] Another group published a success rate of 3.0 C57BL/6 embryos,[15] which is similar to our success rates. Other investigators also analyzed the success rate and found that 3.2 C57BL/6 fresh and 5.1 frozen-thawed embryos were necessary to generate one living pup[13]; in our study, we required 4.5 and 4.4 frozen-thawed embryos for C57BL/6J and N, respectively. Regardless of whether fresh or frozen-thawed embryos were used, in our study the overall success of generating one pup was not significantly different between in vivo- and in vitro-fertilized embryos. Our success rate was between 3 and 4 for fresh embryos and between 4 and 5 for frozen-thawed embryos. Our success rates for the mixed background lines also lie within the same ranges, as most of the lines in our facility have at least in a small percentage of C57BL/6J and/or N in their genetic background. However, others have shown that an even higher variability can be expected when using other strains (for example, BALB/c [ET-fresh 12.4, ET-cryo 18.8]).[13]

We found that females with swollen ampullae showed higher pregnancy rates with the transfer of either fresh or frozen-thawed embryos, as was also the case for in vivo-derived and in vitro-fertilized embryos. However, the kind of fertilization seems to play no significant role unless the genetic background of the transferred embryos is considered. As shown in Figure 4, in the C57BL/6J strain, the number of live pups per transferred embryos was lower with IVF, as was also the case for mixed background mice when frozen-thawed embryos were used. In general, our results are comparable to published studies of other groups.[13,25,35,41] Differences may be due to the genetic background of the donor and recipient strains, which may affect embryo survival and development.[29,32,38] Our data were obtained from a heterogeneous population of mice that included various transgenic knockout and knockin lines, some of which were known to have poor reproductive performance and known revitalization difficulties, potentially a totally undefined background, and more variability due to their different genetic modifications, compared to wild type strains, which possibly led to lower survival after ET.[15,30] From a practical point of view, different protocols and handling media between facilities may variably affect embryo quality. Improvement of the superovulation and culture conditions for IVF may produce more stable embryos that will develop into more live pups after ET.

By analyzing so many ETs of different embryo categories, our study provides a comprehensive description of the B6C3F1 mouse as an embryo recipient. Our data on the presence and absence of a swollen ampulla during ET indicate the importance of this structure for a successful ET and show that a swollen ampulla is an important sign of pseudopregnancy and a criterion for eliminating poor candidates for ET. Females without a swollen ampulla could potentially be used as recipients for future ET,[12,28] but this would require another surgery. To date, a noninvasive method of detecting swollen ampullae is not available. Cryopreservation, IVF, and genetics clearly affect embryo viability. Differences between the substrains C57BL/6J and C57BL/6N suggests a need to identify the responsible genetic difference. As previously reported,[8,46] a generalized C57BL/6 strain does not exist.[26] The present work complements existing knowledge from both practical and ethical perspectives. Steps can be taken by which number of mice used and transgenic production success can be improved.

Supplementary Materials

Table S1. Percentages of females with swollen (+) and nonswollen (-) ampullae and pregnancy rates after ET with freshly isolated and frozen-thawed 2-cell embryos.

Table S2. Percentages of females with swollen (+) and nonswollen (-) ampullae and pregnancy rates after ET with freshly isolated and frozen-thawed 2-cell embryos, fertilized naturally after normal mating.

Table S3. Percentages of females with swollen (+) and nonswollen (-) ampullae and pregnancy rates after ET with freshly isolated and frozen-thawed 2-cell embryos, fertilized through IVF.

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