

Developmental Potential of In Vitro Fertilization-Derived Mouse Zygotes Following Vitrification: Effects of Superovulation Method

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Cryopreservation of pronuclear stage embryos from superovulated mice is beneficial to safeguard genetically modified (GM) mouse strains as well as the efficient production of novel GM mouse strains. C57BL/6J female mice were superovulated with either anti-inhibin serum (AIS) or equine chorionic gonadotropin (eCG), and the resulting oocytes were inseminated via in vitro fertilization (IVF) to produce pronuclear stage embryos. A subset of fresh embryos was cultured in vitro to assess their developmental potential to the blastocyst stage, and the remaining embryos derived from either AIS or eCG superovulation methods were cryopreserved via vitrification and subsequently assessed to determine both in vitro and in vivo developmental competence. The percentage of IVF-derived fresh embryos that developed to 2-cell (92.9 ± 4.1 compared with 92.4 ± 4.2) and the blastocyst stage (91.9 ± 3.84 compared with 91.8 ± 7.9) from eCG and AIS, respectively, was not different ($P = 0.89$). The percentage of the vitrified pronuclear embryos that were intact after warming for eCG (93.08 ± 5.96) and AIS (88.0 ± 6.63) mice was different ($P = 0.039$). However, the percentage of IVF-derived vitrified warmed zygotes that developed to 2-cell (82.23 ± 7.18 compared with 83.9 ± 7.22) and the blastocyst stage (71.35 ± 7.76 compared with 74.52 ± 5.57) from eCG and AIS, respectively, was not different. Vitrified pronuclear embryos produced after either AIS or eCG-administered mice were in vitro cultured to 2-cell and surgically transferred into CD-1 surrogate mothers to compare pregnancy rates and live birth rates. There were no differences in percent pregnancy rates between eCG (85.7) and AIS (85.7) superovulation methods. Similarly, there were no differences in the percentage of live offspring between eCG (27.6) and AIS (23.2) superovulation methods. This study suggests that eCG and AIS superovulation methods yield similar in vitro and in vivo embryonic development rates following vitrification, and thus, AIS may be preferred, especially for the strains with difficulty in obtaining large quantities of oocytes or embryos for the production of GM mice or genome banking.

Abbreviations and Acronyms: AIS, anti-inhibin serum; ART, assisted reproductive techniques; COC, cumulus-oocyte complex; eCG, equine chorionic gonadotropin; FHM, flushing holding media; GM, genetically modified; IVF, in vitro fertilization; KSOM, potassium simplex optimized medium; LN₂, liquid nitrogen; PBS, phosphate buffered saline

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Introduction

The development of highly efficient gene editing technologies for the creation of genetically modified (GM) mouse models has been accelerated over the past decade.¹ Various assisted reproductive techniques (ART), such as in vitro fertilization (IVF), embryo culture and cryopreservation, and embryo transfer, have significantly helped with the creation of these genetically important mouse models.² Cryopreservation ensures the future use of these unique lines as a cost-effective and practical method by safeguarding mouse models of human disease from natural disasters, genetic contamination, and infectious diseases.^{1–3} Furthermore, transporting cryopreserved germplasm rather than live animals is more humane and cost-effective and facilitates collaborative research among biomedical researchers without a lengthy quarantine period.^{2,4,5} Although pronuclear-stage embryos used for GM mouse production are traditionally obtained from superovulated and naturally mated donor mice, IVF can also be used for this purpose. After being created, a large

number of IVF-derived embryos from desired mouse strains can be cryopreserved and stored in liquid nitrogen (LN₂) for many years until they are used for future biomedical purposes at any given time. Recently, for the production of genome-edited rodent strains, pronuclear-stage embryos have been used to integrate nucleases such as CRISPR-Cas9 via microinjection⁶ or electroporation.^{7–9}

Previously cryobanked embryos can also be used as a source to create novel mutant mouse models for human diseases, thereby avoiding the need for live animal maintenance or ongoing animal purchase and providing significant logistical and financial help.^{9–13} When choosing a type of genome biobanking, it is important to determine the appropriate cells or germplasm for cryopreservation. Sperm are abundant, easy to collect, and relatively simple to cryopreserve; however, this germplasm should only be considered when there is only one mutation of interest.² Embryos allow the preservation and subsequent reanimation of the entire genome of the mouse after simple embryo transfer.¹⁴ When collecting embryos for cryopreservation, the goal is to collect as many embryos per donor animal as possible to decrease the total number of animals needed for these techniques, satisfying one of the 3Rs (Replacement, Reduction, Refinement).¹⁵ To achieve this goal, superovulation methods

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are used to stimulate the ovaries to produce as many quality oocytes or embryos as possible.¹⁶ There are 2 main approaches currently used in mice to increase the number of mature oocytes for ovulation: equine chorionic gonadotropin (eCG) and anti-inhibin serum (AIS). eCG acts like a follicle-stimulating hormone (FSH) in mice, causing oocytes to mature.^{16,17} eCG, like FSH, acts primarily by binding to FSH and LH receptors in various animal species, including mice, stimulating follicular growth and inducing ovulation. Meanwhile, inhibin is secreted by the granulosa cells of tertiary follicles and acts on the pituitary gland to decrease the amount of FSH produced, ultimately decreasing the number of oocytes available for ovulation.¹⁸ When AIS is administered, it binds to inhibin, blocking it from creating a negative feedback loop and increasing the amount of available FSH and subsequent mature oocytes.¹⁸

Cryopreservation is a multistep process that can impose a significant adverse effect on cells and tissues. Therefore, obtaining quality fresh oocytes is required to produce quality embryos after cryopreservation.^{19–21} While eCG superovulation has been traditionally used for several decades, there is little information on the effects of cryopreservation on embryos derived from the AIS superovulation method.^{22–24} There have been reports^{25,26} demonstrating the ability to obtain a larger quantity of oocytes from AIS superovulation than eCG. However, little research has investigated the quality of these oocytes or embryos, particularly after cryopreservation. To test this, C57BL/6J female mice were superovulated with either AIS or eCG, and the resulting oocytes were in vitro fertilized to generate pronuclear stage embryos. We first compared the quality of fresh embryos generated from either AIS or eCG superovulation by in vitro 2-cell development and blastocyst formation.

As for the cryopreservation, in addition to slow freezing (0.3–0.5°C/min typically performed with 1.5 M cryoprotectant (eg, DMSO), Rall and Fahy²⁷ succeeded the preservation of mouse embryos by ultrarapid cooling (5000–20000°C/min) or vitrification with the use of cryoprotectant concentration reaching about 7 M. Vitrification largely eliminated harmful intracellular ice crystal formation and damages caused by hypothermia, and it is simpler and quicker (approximately 10 minutes) than slow freezing (approximately 90 minutes) to complete each batch. Thus, it has become a practical alternative for embryo and oocyte cryopreservation over the past decades. We therefore performed an experiment to compare embryonic development rates of IVF-derived zygotes of the AIS or eCG superovulation method following vitrification. Surgical embryo transfers were performed on vitrified embryos into surrogate females to determine and compare if viable pups could be successfully developed after eCG or AIS superovulation methods.

Materials and Methods

Animals. This study was conducted at a facility accredited by AAALAC International and under an IACUC-approved protocol at the University of Missouri. C57BL/6J females (7–8 week old; $n = 37$) and males (12–14 week old) C57BL/6J ($n = 8$) mice were obtained from in-house breeding stock for oocyte for embryo donors. CD1 mice (8–12 week old [$n = 14$]; Charles River Laboratories, Wilmington, MA) were used as surrogates. There was no exclusion criterion other than the age of the animal. The housing environment was maintained at $22 \pm 2^\circ\text{C}$, with a relative humidity of 30% to 70% on a 14:10-hour light:dark cycle (lights on 7:00 central standard time). Recipient mice were single-housed in standard polypropylene shoebox cages (7.25 in [length] $\times$ 11.75 in [width] $\times$ 5 in [height]; Allentown, Inc., Allentown, NJ), and oocyte and sperm donor mice were

group housed in groups of 8–10 in large shoebox caging (10.5 in [length] $\times$ 19 in [width] $\times$ 6 in [height]) on aspen bedding and had unrestricted access to a commercial rodent diet (Formulab Diet 5008; LabDiet, Richmond, IN) and water. Colony health was evaluated every 3 months through sentinel exposure to dirty bedding. All sentinels were seronegative for mouse hepatitis virus, minute virus of mice, mouse parvovirus, parvovirus NS-1, Theiler murine encephalomyelitis virus, murine rotavirus, *Mycoplasma pulmonis*, and Sendai virus. PCR testing was negative for fur mites and pinworms.

Superovulation and oocyte collection. Superovulation was performed as previously described.²⁶ Each female mouse was intraperitoneally injected with 7.5 IU of eCG (Cat. no. hor-272-a; ProSpec Bio USA, East Brunswick, NJ) or 100 μL of AIS plus 3.75 IU eCG (CARD HyperOva; Cosmo Bio USA, Carlsbad, CA) followed by intraperitoneal injection of 7.5 IU human chorionic gonadotropin (hCG; cat. no. hor-007; ProSpec Bio USA, East Brunswick, NJ) 48 post-AIS or eCG. The AIS had a titer of 1:1 000 000 as defined by the final dilution of the antiserum required to bind 50% of 125I-labeled bovine 32-kDa inhibin. Animals were euthanized via cervical dislocation 14–15 hours following the hCG injection. The oviducts were dissected out from each previously superovulated donor mouse. Cumulus-oocyte complexes (COCs) were collected as described below. Researchers were not blinded to the treatments.

IVF and embryo culture. Fresh sperm were used to create IVF-derived pronuclear stage embryos. Male mice were euthanized via cervical dislocation immediately before IVF. The cauda epididymis was dissected out, quickly washed in phosphate buffered saline (PBS), and cleaned from any blood and fat on a clean tissue paper. The epididymis was placed into preequilibrated mineral oil next to a drop of mouse sperm capacitation media (FERTIUP; Cosmo Bio USA, Carlsbad, CA), and a small cut was made in each epididymis using a sterile 28-gauge needle. The sperm clot was then pulled into the FERTIUP drop and allowed to capacitate for 30 minutes before IVF was performed in 35-mm Petri dishes containing 90 μL FERTIUP and CARD media under mineral oil in an incubator at 37°C containing 5% CO_2 as previously described.²⁸ Dissected oviducts containing the oocyte clutches from each donor female were placed into mineral oil next to CARD IVF media droplets. The clutches of COCs from each donor were visualized under a stereomicroscope, and a 28-gauge needle was used to release the COC into a 90- μL CARD drop. Once all COCs were placed within a CARD droplet, fresh capacitated sperm (approximately 3.5×10^5 motile sperm/mL) were gently added into each CARD drop. The Petri dishes containing the sperm and oocytes were placed back into the incubator and allowed to incubate for approximately another 6 hours. The fertilized oocytes were washed 3 times in flushing holding media (FHM) containing bovine serum albumin (BSA) (4 mg/mL) and transferred into potassium simplex optimized medium (KSOM) amino acid embryo culture drops^{29,30} under mineral oil in an incubator at 37°C containing 5% CO_2 to evaluate 2-cell next day and blastocyst development rates 3.5 days after IVF.

Zygote vitrification and warming. Cumulus-free IVF-derived zygotes were first exposed to 15% ethylene glycol (EG) in PBS containing 20% fetal calf serum (FCS) for 5 minutes at room temperature. They were then transferred into a solution containing 30% EG + 0.5 M sucrose solution in PBS containing 20% FCS for about 30 seconds. The zygotes were then placed within 0.25-mL French straws, heat sealed, and immediately plunged into LN_2 for long-term storage. To recover cryopreserved zygotes, straws were removed from LN_2 and placed into a 37°C warm

water bath for 10 seconds. Once warmed, the contents of each straw were preloaded with 1 M sucrose, and the vitrification solution (VS) was expelled onto a culture dish and held for 5 minutes before being moved into an FHM washing solution and transferred into KSOM media for in vitro culture to assess further development competence.

Embryo transfers. Female CD-1 mice were mated with vasectomized males, and plug-positive females were identified to serve as surrogates ($n = 7$ per treatment group). Mice were anesthetized using a ketamine + xylazine cocktail (90 mg/kg + 10 mg/kg body weight IP) and administered an analgesic (flunixin meglumine, 2.5 mg/kg body weight SC) to prevent postoperative pain and stress. The surgical site was clipped and cleaned and, once in a surgical plane of anesthesia, a midline dorsal incision was made through the skin. The skin incision was moved right of the midline, and the muscle was incised, and the ovary was located. A stereomicroscope was used to locate the opening of the infundibulum. Once located, a pipette tip containing eight 2-cell embryos was inserted into the opening of the infundibulum, and the embryos were expelled. The same procedure was repeated on the left side, and the skin incision was closed with wound clips. The wound clips were removed a week following surgery.

Statistical analysis. Statistical analysis was performed using SigmaPlot version 14.0 (Systat Software, San Jose, CA), and P values < 0.05 were considered significant. To determine if the superovulation method impacted fresh zygote and blastocyst development rates, a 2-way ANOVA was used. A 2-way ANOVA was also used to compare differences in percentage of intact zygote, 2-cell, and blastocyst development rates in vitrified-warmed embryos, as well as pregnancy and live pup development rates.

Results

A primary experiment was performed on the fresh IVF zygotes' in vitro embryonic development potential between the eCG and AIS superovulation methods. Figure 1 shows the percentage of IVF zygotes that developed into 2-cell and blastocyst stage produced from oocytes retrieved from either the eCG or

AIS superovulation method. There was no difference between eCG and AIS superovulation methods regarding 2-cell (199/214 [92.9%] compared with 281/304 [92.4%]; $P = 0.27$) or blastocyst (183/199 [91.96%] compared with 258/281 [91.81%]; $P = 0.89$) stage embryo development. A later study aimed at determining postwarming morphologic integrity of vitrified IVF zygotes in vitro 2-cell and blastocyst formation rates between eCG and AIS superovulation methods. The zygotes were considered morphologically intact if their plasma membrane was intact, not fragmented, and the cytoplasmic content remained. Figure 2 shows the percentage of intact zygotes following vitrification and warming, 2-cell embryo development, and percentages of 2-cell embryos that were successfully cultured to the blastocyst stage between eCG and AIS superovulation methods.

While 260 IVF zygotes were produced from eCG ($n = 14$), 425 IVF zygotes were produced from the AIS ($n = 10$) superovulation method. Significantly more intact zygotes after vitrification and rewarming were recovered from eCG than the AIS group ($93\% \pm 5.96\%$ compared with $88\% \pm 6.63\%$; $P = 0.039$). Of the 260 zygotes vitrified, 242 were successfully recovered intact from eCG ($93.08\% \pm 5.96\%$), and of the 425 zygotes vitrified, 374 were from the AIS superovulation group ($88\% \pm 6.63\%$). Of those intact zygotes, 199 developed into 2-cell embryos in eCG ($82.23\% \pm 7.18\%$) and 314 in the AIS superovulation method ($83.9\% \pm 7.22\%$). As for the blastocyst development rate, 142 and 234 2-cell embryos for the eCG ($71.35\% \pm 7.76\%$) and the AIS ($74.52\% \pm 5.57\%$) superovulation methods developed into the blastocyst stage. There were no significant differences between 2-cell ($P = 0.326$) or blastocyst development rates between treatment groups ($P = 0.612$). Finally, to determine if the superovulation method impacted pregnancy establishment and subsequent live pup birth rates, the percentage of pregnancy and the number of live pups from each surrogate dam and superovulation method were determined. For each treatment, a total of 7 surgical embryo transfers were performed, and no significant difference was found between the eCG (6/7 [85.7%]) and AIS (6/7 [85.7%]) treatment groups (Figure 3). While surrogate dams in the eCG group delivered a total of 31 live pups, an average of

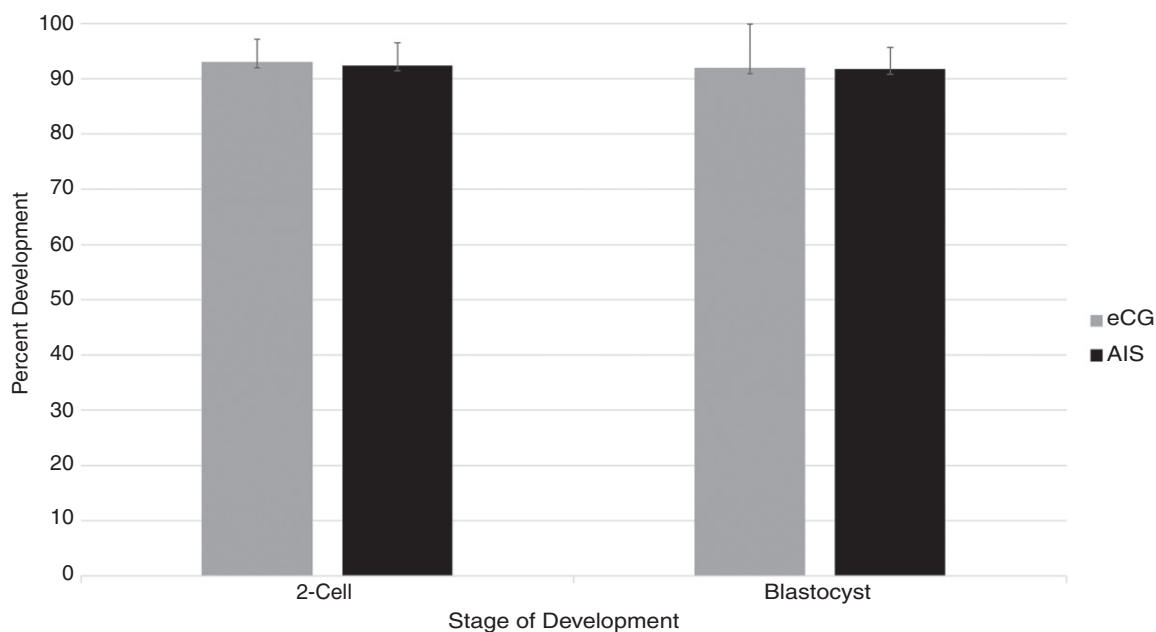


Figure 1. The Percentage of Fresh Zygotes That Developed to 2-Cell ($P = 0.89$) and Blastocyst ($P = 0.27$) Obtained from Either eCG ($n = 8$) or AIS ($n = 5$) Superovulation. The results are presented as average percentages across groups. AIS, anti-inhibin serum; eCG, equine chorionic gonadotropin.

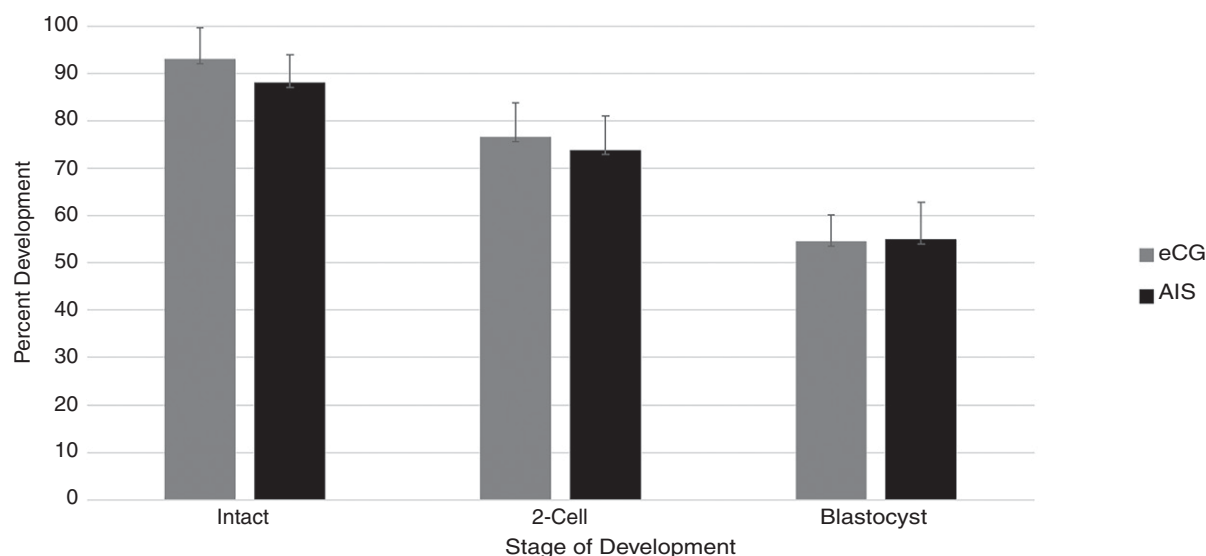


Figure 2. The Percentage of Intact Vitri-fied and Warmed Zygotes, 2-Cell Embryo Development from Intact Zygotes, and Blastocyst Development from 2-Cells Obtained from Either eCG (n = 14) or AIS (n = 10) Superovulation. Significantly more intact zygotes were recovered from the eCG group ($P = 0.039$), while there were no significant differences between 2-cell ($P = 0.326$) or blastocyst development rates between treatment groups ($P = 0.612$). The results are presented as average percentages across groups. AIS, anti-inhibin serum; eCG, equine chorionic gonadotropin.

5.2 pups per litter (Figure 4), the AIS group resulted in 26 live pups, with an average of 4.3 pups per litter. No implantation sites were observed for either group on necropsy. There were no significant differences in live pup birth rates between the groups ($P = 0.577$).

Discussion

The conventional method of superovulation in mice has been the administration of eCG and subsequent administration of hCG. Recently, AIS in combination with eCG has been introduced as an ultra-superovulation method that yields

significantly higher numbers of oocytes compared with eCG administration only.^{17,31}

Several previous studies^{25,26,31–34} compared the efficacy of AIS on commonly used inbred mouse strains and obtained up to 2–3 times greater numbers of oocytes and comparable live birth rates to superovulation using eCG. In addition, our previous study showed that AIS produces oocytes having a thinner zona pellucida than those produced using eCG, but all other morphologic characteristics, such as perivitelline space, oocyte diameter, and subcellular organelles (microtubule integrity, F-actin, cortical granules, and mitochondrial distribution), as well as in vitro embryonic developmental potential, were similar. Although the

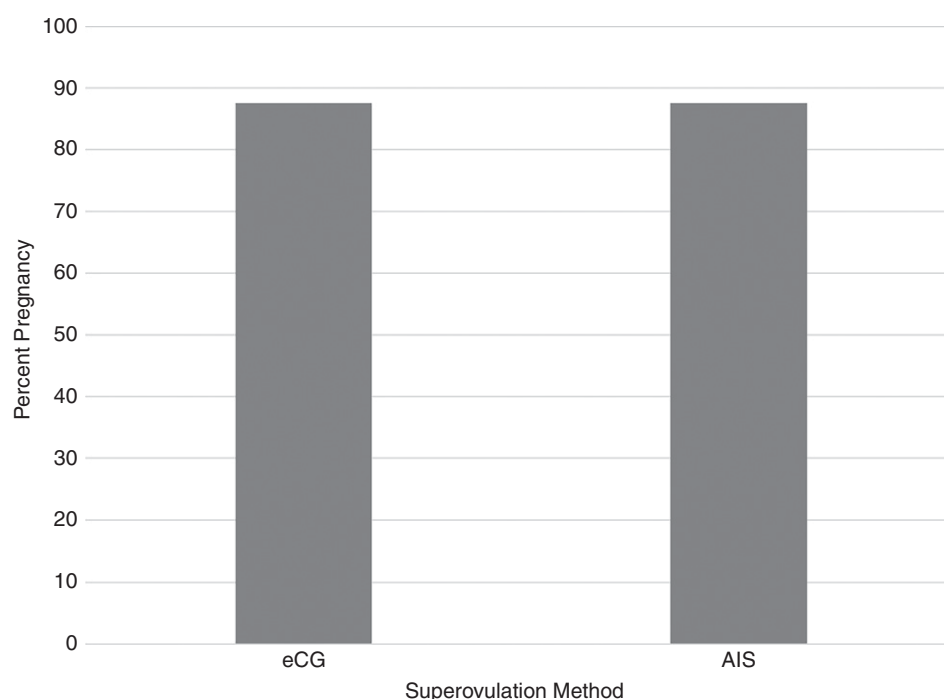


Figure 3. Pregnancy Rates after Transfer of 2-Cell Embryos Derived from Either eCG (n = 7) or AIS (n = 7)-Induced Superovulation Method. Pregnancies are presented as the percentage of animals that became pregnant following embryo transfer in each group ($P = 1.00$). AIS, anti-inhibin serum; eCG, equine chorionic gonadotropin.

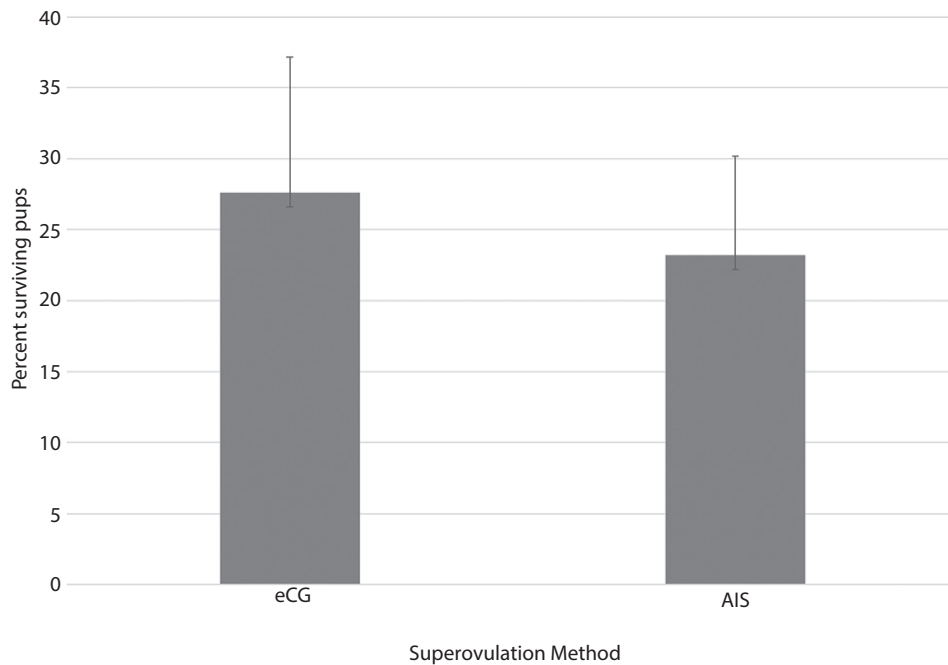


Figure 4. The Percentage of Pups after Transfer of 2-Cell Embryos from Either eCG (n = 7) or AIS (n = 7) Superovulation Method. A total of 16 embryos were transferred into each recipient mouse ($P = 0.577$). AIS, anti-inhibin serum; eCG, equine chorionic gonadotropin.

quantity and associated morphology of oocytes are important, it is also essential to obtain oocytes and embryos that have similar cryosurvival, fertility, and healthy fetal development to term. Thus, it was important to investigate if embryos derived from AIS possess levels of endurance to cryopreservation-induced injuries compared to those derived from eCG. If confirmed, fewer animals can be used to obtain oocytes or embryos for ARTs, thus satisfying one of the 3Rs, reduction, as there are fewer donors needed to achieve the same goal.³⁵

In this study, we generated mouse IVF zygotes from oocytes derived from either eCG or AIS superovulation methods and compared fresh as well as postvitrification in vitro developmental competence. There were no differences in 2-cell and blastocyst development rates between AIS and eCG for either fresh or after vitrification. We obtained slightly higher plasma membrane-intact embryos from eCG superovulation following vitrification, but the subsequent embryonic development of fresh zygotes to the blastocyst stage was indistinguishable and in favor of AIS for vitrified zygotes. In addition, embryonic development rates were similar in fresh IVF experiments, and live pup rates were similar between eCG- and AIS-derived vitrified embryos. Nevertheless, further experimentation using a larger sample size may shed more light on the difference in plasma membrane integrity between the 2 superovulation methods.

To date, 2 different VSs have been commonly used to cryopreserve unfertilized and fertilized mouse oocytes. One VS contained 2 M DMSO, 1 M acetamide, and 3 M propylene glycol, or commonly referred to as DAP213, in a cryovial.³⁶ The other VS contained 15% EG + 15% DMSO + 0.5 M sucrose^{36,37} using cryotop as a carrier. However, these methods of vitrification and cryostorage have the disadvantage of exposing the biologic samples to LN₂, which may be contaminated with microbial agents. In addition, cryovials can accumulate LN₂ internally during storage and can explode during the thawing procedure, creating health hazards to personnel. In contrast, we used

0.2-mL French straws to achieve vitrification, thus allowing us to take advantage of having a closed vitrification system in which specimens do not interact with LN₂.

Several studies^{31,38} have demonstrated that unfertilized oocytes recovered via AIS superovulation combined with IVF to produce zygotes can be used to produce various mutant mice by direct microinjection of genome editing reagents. Although freshly produced zygotes are useful, they are not readily available without prior superovulation scheduling, and thus, cryopreservation of zygotes is required for microinjection or electroporation. Vitrified AIS-derived IVF zygotes have been successfully used for the generation of mutant mouse strains, but there is a lack of studies making a direct comparison of the efficiency between AIS and eCG superovulation methods. Goto et al¹⁶ vitrified IVF-derived 2-cell mouse embryos of AIS or eCG superovulation origin and did not find a difference in blastocyst rates, ranging from 86% to 96% from fresh embryos of 4-, 8-, 12-, and 24-week-old oocyte donors. Birth rates were, nonetheless, lower for AIS-derived embryos obtained from 4 weeks (52% compared with 35%) and 8 weeks (60% compared with 43%) of eCG or AIS-administered oocyte donors, respectively. Interestingly, the difference did not reach the level of significance in the case of 12-week-old (58% compared with 49%) and 24-week-old (37% compared with 52%) oocyte donors.

Another study³⁸ vitrified unfertilized MII oocytes derived from AIS or eCG superovulation, and the survival rate of AIS-generated vitrified-warmed oocytes was comparable to that of eCG (99% compared with 92%). Two separate studies^{12,13} conducted by the same group used the DAP213 as a VS in cryovials. While Nakagawa et al¹³ obtained 90.5% cryosurvival using eCG-derived IVF zygotes, Nakagawa et al¹² obtained 87.5% cryosurvival using AIS-derived IVF zygotes. The results from these previous reports are consistent with those of the current study, in which we obtained 93% and 88% intact embryos from eCG and AIS-derived IVF zygotes, after vitrification, respectively.

Mochida and Ogura¹⁴ vitrified IVF-derived zygotes obtained from eCG using 15% EG + 15% DMSO + 0.5 M sucrose as VS via Cryotop and obtained cryosurvival, 2-cell, and blastocyst development rates of 84%, 74%, and 63%, respectively; and we used a similar VS via 0.25-mL French straws and had results that are consistent (93%, 82%, and 58%) with their study for eCG, respectively.

In this study, we chose to work with C57BL/6J mice, as they are the most commonly used background strain for transgenic mice production.²⁹ Our results suggest that AIS superovulation may be preferred in reproductively impaired mouse strains or wild-mouse species, which often poorly respond to eCG and are shown to lead to a greater number of oocytes with AIS.^{32,35,39} For example, AIS has been shown to significantly increase superovulation success in female *Npc1*^{-/-} mice as it increases endogenous FSH by neutralizing inhibin and exogenous FSH, promoting follicle development, demonstrating its effectiveness in rescuing ovulation dysfunctions in female *Npc1*^{-/-} mice.²¹ Thus, AIS superovulation, oocyte or embryo cryopreservation, and subsequent offspring production from unique mouse strains and wild-mouse species would provide wider research opportunities that have not been fully realized, and use of these genetically diverse species may likely expand further.^{11,21,32,33,35,39}

In conclusion, the current study has demonstrated that while AIS superovulation produces greater numbers of oocytes and embryos, the developmental competence is comparable to that derived from eCG after they are subjected to a vitrification procedure. This is important, as fewer animals can be used for ARTs when using AIS, although eCG superovulation may still be preferred in certain situations due to cost. In addition, AIS superovulation may be preferred for reproductively challenging strains and large numbers of embryos needed for genome banking as it produces more embryos per donor mouse compared with eCG superovulation.

Conflict of Interest

The authors have no conflicts of interest to declare.

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Data availability

Raw data can be made available upon request to the corresponding author.

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