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A Study on the Production of 2-Cell Stage Embryos in C57BL/6Mlac Mice with Adult Age

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Abstract

Background and Objectives : The embryo bank unit was established in the National Laboratory Animal Center, Mahidol University, Thailand. The primary role of the embryo bank unit is to preserve various mouse strains. The assisted reproductive technologies for the embryo bank unit included superovulation, *in vitro* fertilization, *in vivo* embryo production, vitrification and thawing, *in vitro* culture, embryo transfer, and cesarean section. In the embryo bank unit, female mice aged 8-10 weeks were used for *in vivo* embryo production of 2-cell stage embryos, while older mice were terminated from the colony. To optimize animal use, we focus on producing 2-cell stage embryos from adult C57BL/6Mlac female mice through *in vitro* embryo production, followed by vitrification, thawing, *in vitro* culture, and embryo transfer. This study aims to determine the percentage of superovulation (% superovulation), the percentage of *in vitro* fertilization (% *in vitro* fertilization), the percentage of surviving 2-cell stage embryos after vitrification and thawing (% survival), the percentage of development to blastocysts after *in vitro* culture (% development), and the percentage of newborn after the embryo transfer (% newborn).

Methodology : C57BL/6Mlac mice were bred in the embryo bank unit. Mice were maintained under routine husbandry procedures in the animal room with strict hygienic conventional, controlled temperature at 22±3ºC, 30-70% humidity, regulated light conditions, a standard mouse diet, and 5-7 ppm choline reverse osmosis water. Mice were kept in plastic cages and stainless-steel lids. The sterile bedding used consisted of corn cobs and dried water hyacinth. Eleven 17-week-old C57BL/6Mlac female mice were used for oocyte production following the routine superovulation protocol. C57BL/6Mlac female mice were induced to superovulation through an intraperitoneal (IP) injection of 10 IU pregnant mare serum gonadotropin (PMSG) at 2:00-3:00 PM, followed by an IP injection of 10 IU human chorionic gonadotropin (hCG) 48 hours later. Nineteen to twenty-one hours after the hCG injection, female mice were euthanized by cervical dislocation. Oviducts were removed and placed in M2 medium. Cumulus oocyte complexes (COCs) were released from the ampulla under a stereomicroscope. COCs of one female mouse were kept in EmbryoMax® Human Tubal Fluid (HTF) medium covered with mineral oil in a CO_2 incubator (37ºC, 5%CO_2,

95%humidity). Two 12-week-old C57BL/6Mlac male mice were used for fresh sperm collection. A 5 µl of sperm solution was transferred into a COCs drop by micropipette. The sperm and COCs were incubated in a CO_2 incubator overnight. The next morning, the 2-cell stage embryos with normal morphology were counted to determine the % *in vitro* fertilization. The 2-cell stage embryos were collected and pretreated with a Holding medium (Bovine serum albumin, M2 medium) for 3 minutes and frozen by the vitrification method using a 35EG solution (35% v/v ethylene glycol, Bovine serum albumin, Polyvinylpyrrolidone, Trehalose dehydrate, M2 medium) in a 0.25 ml straw tube at room temperature. The 0.25 ml straw tube was placed over liquid nitrogen for 3 minutes, plunged into liquid nitrogen directly, and stored in a liquid nitrogen tank for 6 months. These straw tubes were taken out of the liquid nitrogen tank, warmed at room temperature for 20 seconds, and then warmed at 37ºC water for 20 seconds. The 2-cell stage embryos were found under a stereomicroscope and thawed using a 0.3 M Trehalose, a 0.15 M Trehalose, a 0.075 M Trehalose, and a Holding medium. The surviving 2-cell stage embryos were counted to determine the % survival and then divided into two groups for quality testing through *in vitro* culture and embryo transfer.

Main Results : Nine 17-week-old C57BL/6Mlac female mice responded to the 10 IU PMSG/hCG superovulation, with the % superovulation was 81.8 (9/11 females). After superovulation and overnight *in vitro* fertilization, the number of 2-cell stage embryos was 130, the number of oocytes was 38, and the number of abnormal oocytes was 137. The % *in vitro* fertilization was 42.6 (130/305). The 2-cell stage embryos were kept in a liquid nitrogen tank for 6 months. After thawing, 110 of 2-cell stage embryos were observed under a stereomicroscope, 23 embryos were dead. A total of 87 2-cell stage embryos remained alive after thawing, the % survival was 66.9 (87/130). In Group 1, 29 out of 47 embryos developed into blastocysts, the % development was 61.7 (29/47). In Group 2, 40 embryos were used for embryo transfer into 4 recipients. On the day of birth, the number of newborns was 2, 2, and 2 in the 1st, 2nd, and 4th recipients, respectively, the 3rd recipient gave birth but consumed all the newborns. The % newborn was 15.0 (6/40) after embryo transfer. At 4 weeks old, 2 male and 4 female immature C57BL/6Mlac mice were counted at weaning.

Conclusions : Adult C57BL/6Mlac female mice can be used to produce the 2-cell stage embryos by *in vitro* embryo production consisting of the 10 IU PMSG/hCG superovulation and the *in vitro* fertilization. The 2-cell stage embryos are resistant to vitrification and suitable for use in the embryo bank unit. Although the % *in vitro* fertilization and the % newborn were low, there are sufficient numbers of newborns to establish a new colony. Additionally, it promotes the use of laboratory animals for maximum benefit at the National Laboratory Animal Center.

Keywords : mice ; 2-cell stage embryos ; *in vitro* fertilization ; vitrification ; embryo transfer

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Introduction

The National Laboratory Animal Center, Mahidol University, Thailand, established the embryo bank unit with the primary function of preserving laboratory animal strains and preventing their loss due to various factors, such as genetic drift, infectious outbreaks within the colony, natural disasters, and the high cost of sustained live laboratory animal production. In our laboratory, the *in vivo* embryo production method for 2-cell stage embryos of the C57BL/6Mlac mice typically utilizes female mice aged 8–10 weeks only. Mice older than this age range are often culled due to efficiency issues with this method. This practice conflicts with the principles of the 3Rs (Replacement, Reduction, and Refinement). To ensure the embryo bank unit utilizes the full potential of its animals, it is necessary to study and find alternative methods for 2-cell stage embryo production using mice older than 10 weeks. This approach aligns with the 3Rs principles and further supports the development of advanced skills in assisted reproductive technologies.

The *in vitro* embryo production protocol consists of superovulation and *in vitro* fertilization. Superovulation is used in mouse production to induce the ovulation of multiple oocytes from a limited number of female mice (Luo *et al*., 2011). The standard protocol of superovulation was induced by PMSG and hCG. The effectiveness of superovulation in mice depends on several factors, including the method of administration, the time interval between the hormone administration, the dose of hormone, the mouse strain, and the age of female mice (Byers *et al*., 2006; Nakagata & Takeo, 2019; Takeo *et al*., 2019). *In vitro* fertilization relates to the fertilization of oocytes with capacitated sperm in a tissue culture dish. This technique can produce large numbers of embryos without using many males. These embryos can be used for cryopreservation in the embryo bank unit (Taft Robert, 2017). The success of *in vitro* fertilization largely depended on the mouse strains (Sztein *et al*., 2000). The efficacy of superovulation and *in vitro* fertilization is generally better in outbred strains than in inbred strains (Golkar-Narenji *et al*., 2012). Inbred, outbred, and hybrid mouse strains with adult age were used for several oocyte collections by superovulation protocol (Hasegawa *et al*., 2016). In C57BL/6J mice, immature females have been used in studies of embryo production by superovulation, *in vitro* fertilization, vitrification and thawing, and embryo transfer (Byers *et al*., 2006).

In this study, we focused on producing 2-cell stage embryos from 17-week-old C57BL/6Mlac female mice through *in vitro* embryo production, which includes superovulation and *in vitro* fertilization, followed by vitrification, thawing, *in vitro* culture, and embryo transfer. This aim is to improve the animal use at the embryo bank unit, the National Laboratory Animal Center.

Methodology

1. Experimental design

The experimental design in this study was conducted according to the embryo banking process, involving the following sequential steps: Initially, oocytes were collected from female mice, and sperm from male mice were collected to produce 2-cell stage embryos using *in vitro* fertilization. Subsequently, the 2-cell stage embryos were cryopreserved utilizing the vitrification method. The vitrified embryos were then warmed to assess the survival rate. The survival 2-cell stage embryos were evaluated for their developmental potential in both *in vitro* culture and *in vivo* development (Figure 1).

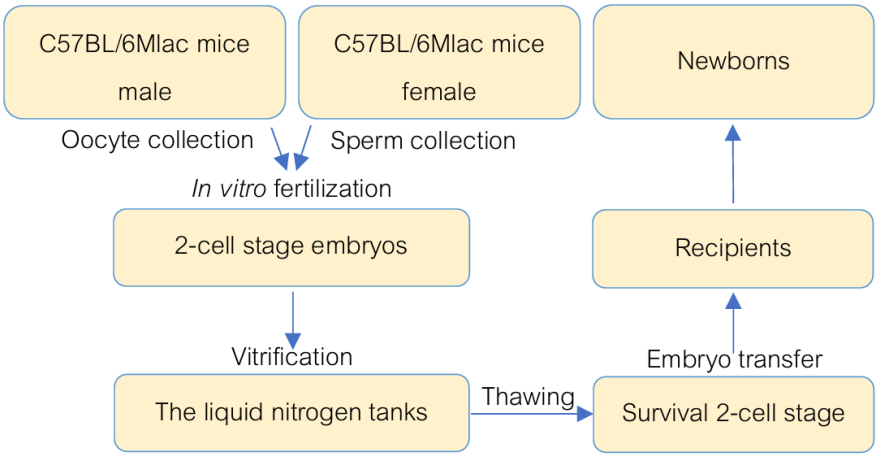

Figure 1 Flow chart summarizing the experimental design of this study

2. Animals

C57BL/6Mlac mice were bred in the embryo bank unit at the National Laboratory Animal Center. The protocol of this study was approved by the National Laboratory Animal Center - Animal Care and Use Committee (NLAC-ACUC), Mahidol University (Protocol No. RA2024-22). All animal procedures were conducted at the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) International. C57BL/6Mlac mice were maintained under routine husbandry procedures in the animal room with strict hygienic conventional, controlled temperature at 22±3ºC, 30-70% humidity, regulate light conditions (lights on from 5:00 AM to 7:00 PM), a standard mouse diet (082G, Perfect Companion®, Thailand), and 5-7 ppm choline reverse osmosis water (*ad libitum*). Animals were kept in plastic cages and stainless-steel lids. The sterile bedding used consisted of corn cobs and dried water hyacinth.

3. In vitro embryo production

Eleven 17-week-old C57BL/6Mlac female mice were used for oocyte production by the routine superovulation protocol. Female mice were IP injected with 10 IU of PMSG (Folligon®, Intervet, Germany) at 2:00-3:00 PM, and 48 hours later, followed by 10 IU of hCG (Chorulon®, Intervet, Germany). Nineteen to twenty-one hours after the hCG injection at 9:00-11:00 AM, female mice were euthanized by cervical dislocation. The abdominal skin of female mice was sterilized with 70% alcohol, cut the abdominal skin, and the abdominal cavity was opened. Oviducts were removed (Figure 2a) and placed in a 50 µl of 37ºC M2 medium (M7167, Sigma-Aldrich, USA) on a 35 mm sterile plastic dish. COCs were released from the ampulla by a 21G needle under a stereomicroscope. COCs of one female mouse were kept in a 150 µl of HTF medium (MR-070-D, Sigma-Aldrich, USA; IVF dish) covered with mineral oil (M5310, Sigma-Aldrich, Canada) in a CO_2 incubator (Figure 2b).

To prepare fresh sperm, sperm were collected using a modified method described by Sztein *et al*. (2000). Two 12-week-old C57BL/6Mlac male mice were used for the sperm collection. Male mice were euthanized by cervical dislocation. The cauda epididymis was removed and placed in a 35 mm sterile plastic dish containing 250 µl of HTF medium (sperm dish) covered with mineral oil in a CO_2 incubator. It was then cut seven times using a 21G needle and equilibrated in the CO_2 incubator for 10 minutes (Figure 2b).

In vitro fertilization was accomplished using a modified method described by Sztein *et al*. (2000). A 5 µl of sperm solution was transferred into a COCs drop by micropipette. The sperm and COCs were incubated in a CO_2 incubator overnight (Figure 2c-e). The next morning (9:00 AM), the 2-cell stage embryos with normal morphology were counted to determine the % *in vitro* fertilization. The 2-cell stage embryos were then washed three times in a 50 µl of M2 medium and kept in a 150 µl of M16 medium (M7292, Sigma-Aldrich, USA), covered with mineral oil, in a CO_2 incubator. C57BL/6Mlac female mice with >15 oocytes/embryo were counted for the % superovulation.

4. Vitrification and thawing

This study used vitrification and thawing procedures following our routine laboratory protocols. The 2-cell stage embryos were washed three times in M2 medium, pretreated with 37ºC Holding medium [Bovine serum albumin (A9647, Sigma-Aldrich, USA) in M2 medium] at room temperature for 5 minutes, and then frozen using the vitrification method. This involved using a 35EG solution [35% v/v ethylene glycol (E9129, Sigma-Aldrich, USA), Bovine serum albumin, Polyvinylpyrrolidone (PVP40, Sigma-Aldrich, China), Trehalose dehydrate (T0167, Sigma-Aldrich, USA), and M2 medium] in a 0.25 ml straw tube at room temperature. The 0.25 ml straw tube was inserted into a 1 ml syringe. The 0.25 ml straw tube was filled with 1 cm of 35EG, 1 cm of air, 0.5 cm of 35EG, 1 cm of air, and 1.2 cm of 35EG (Figure 3a). The embryos were loaded in 1.2 cm of 35EG under a stereomicroscope

(Figure 3b), filled with 1 cm of air and 2 cm of 0.3 M Trehalose (Trehalose dehydrate, Bovine serum albumin, M2 medium), respectively. The 0.25 ml straw tube was sealed with sigillum wax (Figure 3c), and labeled with the strain, embryo stage, and number of embryos. The 0.25 ml straw tube was placed 1 cm above liquid nitrogen for 3 minutes, plunged into liquid nitrogen directly, and stored in a liquid nitrogen tank for 6 months.

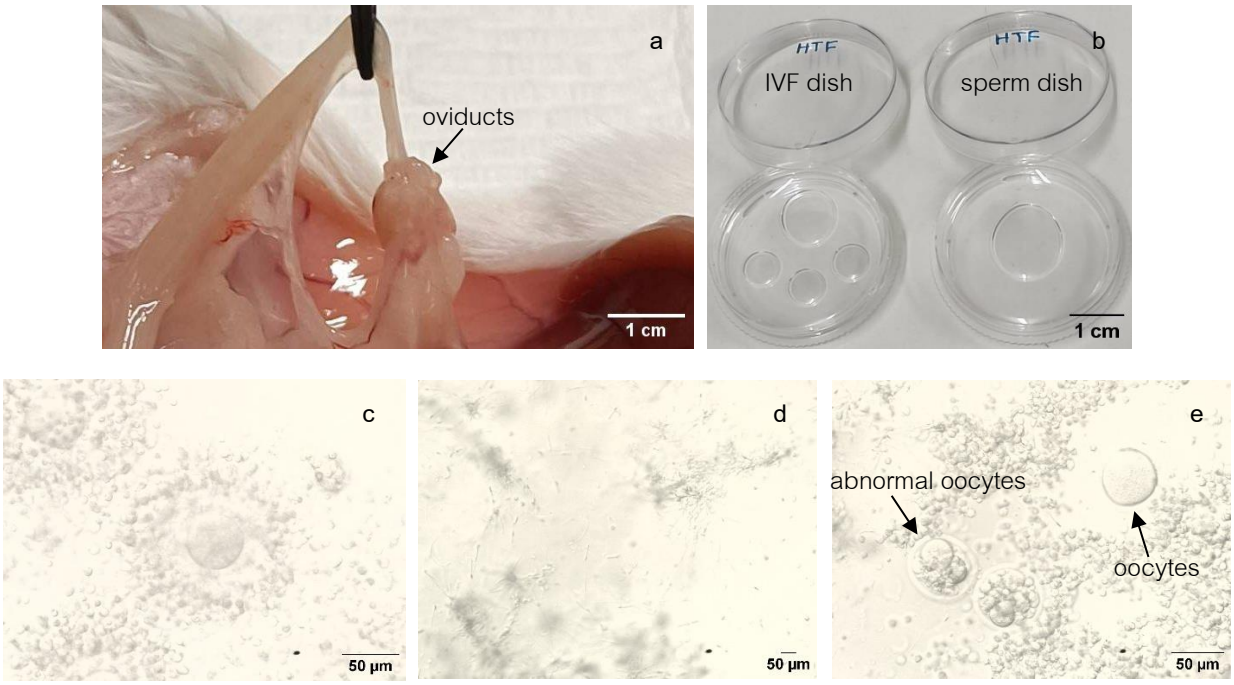

Figure 2 The *in vitro* embryo production, oviducts (a), a preparation of IVF and sperm dish (b), COCs (c), sperm (d), and COCs and sperm in IVF dish (e)

Figure 3 The preparation of a 0.25 ml straw tube, before embryo loading (a), embryos were loaded in 1.2 cm of 35EG using a mouth pipette (b), and after embryo loading (c).

For thawing, the 0.25 ml straw tube was removed from the liquid nitrogen tank, warmed at room temperature for 20 seconds, then warmed in a 37ºC water bath for 20 seconds. The 0.25 ml straw tube was cut with sterilized scissors, and the embryos, along with 35EG and 0.3 M Trehalose, were released into a 50 µl of 37ºC 0.3 M Trehalose on a 35 mm sterile plastic dish. The embryos were observed under a stereomicroscope and thawed using a 50 µl of 37ºC 0.3 M Trehalose for 3 minutes, a 50 µl of 37ºC 0.15 M Trehalose for 3 minutes, a 50 µl of 37ºC 0.075 M Trehalose for 3 minutes, and a 50 µl of 37ºC Holding medium for 5 minutes. The surviving 2-cell stage embryos were counted for the % survival. The surviving 2-cell stage embryos were divided into 2 groups for quality testing by the *in vitro* culture and the embryo transfer.

5. In vitro culture

Seven to ten of survival 2-cell stage embryos were washed in a 50 µl of 37ºC M2 medium 3 times, washed in a 50 µl of 37ºC M16 medium 3 times, and cultured in a 150 µl of M16 medium in a CO_2 incubator. Seventy-two hours later, blastocysts were counted under a stereomicroscope. The blastocysts were counted for the % development.

6. Embryo transfer

The outbred strains of Mlac:ICR mice were used as recipients and vasectomized males. Embryo transfer was performed by the routine in our laboratory, based on the methods described by Velazquez *et al*. (2018) and Lamas *et al*. (2020). Four 8-week-old female mice were paired for overnight mating with a 12-weeks-old vasectomized male. Female mice with vaginal plugs were used as recipients. Recipients were anesthetized by an intraperitoneal injection of ketamine (50 mg/kg BW, Hameln Pharmaceuticals, Germany) and xylazine (10 mg/kg BW, L.B.S. Laboratory CO., LTD, Thailand). After the animals show no pain reflexes, cover the eyes with Terramycin™. The surgery involves making a 1 cm incision along the middle dorsal side, creating a hole in the peritoneal wall to expose the right ovary fat, and removing the ovary fat from the abdominal cavity. Serafin clips were applied to the ovary fat, and the oviduct and ampulla were identified under the stereomicroscope. The surviving 2-cell stage embryos were washed three times in M2 medium using a mouth pipette (pulled glass capillary). Embryos were prepared in a mouth pipette by first pulling a small air bubble, followed by a small amount of M2 medium, another small air bubble, and then 10 embryos in the M2 medium (Figure 4a). The hole was marked close to the ampulla by a 27G needle, insert a mouth pipette into the hole, and the embryos along with two air bubbles were placed into the ampulla (Figure 4b). The ovary fat was returned to the abdominal cavity, and the skin was sutured using autoclip. Recipients were subcutaneously injected with tramadol™ (8 mg/kg BW, T.P. Drug

Laboratories CO., LTD, Thailand) and kept individually on the warm plate at 37ºC until recovery from anesthesia. The newborns were naturally born on days 19-20 after embryo transfer.

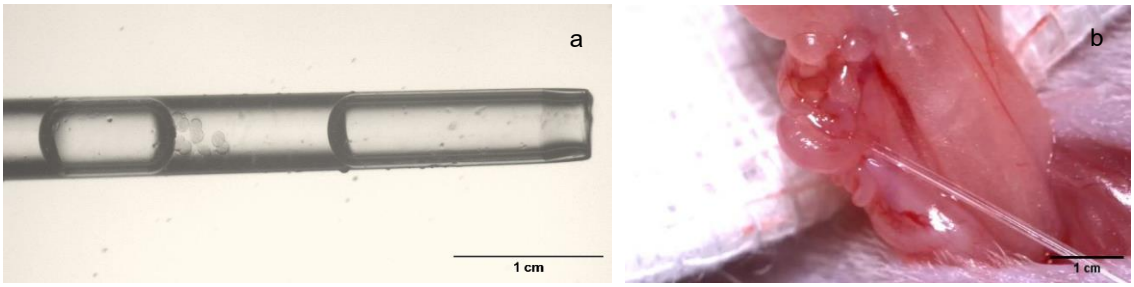

Figure 4 The preparation of a mouth pipette with embryos for embryo transfer (a), and embryo transfer in Mlac:ICR mice (b).

7. Statistical analysis

Descriptive statistics were used to report the data, including the % superovulation, which was determined by calculating the proportion of 17-week-old C57BL/6Mlac female mice with more than 15 oocytes/embryos from superovulation. The % *in vitro* fertilization was derived from the *in vitro* embryo production protocol, the % survival from the vitrification and thawing protocol, the % development from the *in vitro* culture protocol, and the % newborn from the embryo transfer protocol.

$$\% \text{ superovulation} = \frac{\text{C57BL/6Mlac female mice with >15 oocytes/embryos X 100}}{\text{11 of C57BL/6Mlac female mice}} \quad (1)$$

$$\% \textit{ in vitro} \text{ fertilization} = \frac{\text{2-cell stage embryos X 100}}{\text{a total of embryos}} \quad (2)$$

$$\% \text{ survival} = \frac{\text{surviving 2-cell stage embryos X 100}}{\text{vitrified embryos}} \quad (3)$$

$$\% \text{ development} = \frac{\text{no. of blastocysts X 100}}{\text{no. of } \textit{in vitro} \text{ culture}} \quad (4)$$

$$\% \text{ newborn} = \frac{\text{no. of newborns X 100}}{\text{no. of transferred embryos}} \quad (5)$$

Results

Nine 17-week-old C57BL/6Mlac female mice responded to the 10 IU PMSG/hCG superovulation, with the % superovulation of 81.8 (9/11 females). After superovulation and overnight *in vitro* fertilization, the number of 2-cell stage embryos was 130, the number of oocytes was 38, and the number of abnormal oocytes was 137. The % *in vitro* fertilization was 42.6 (130/305) (Table 1, Figure 5). The 2-cell stage embryos were kept in a liquid nitrogen tank for 6 months. After thawing, 110 of the 2-cell stage embryos were observed under a stereomicroscope. Subsequently, 23 embryos were found to be dead. A total of 87 2-cell stage embryos survived after thawing, the % survival was 66.9 (87/130) (Table 2). The surviving 2-cell stage embryos were divided into 2 groups. Group 1 was used for assessing *in vitro* development, while Group 2 was used for assessing *in vivo* development by transferring 10 embryos per recipient (totaling 4 recipients). The results show that, in Group 1, 29 out of 47 embryos developed into blastocysts; the % development was 61.7 (29/47) (Figure 6). In Group 2, 40 embryos were used for embryo transfer into the right oviducts of 4 recipients. On the day of birth, the number of newborns was 2, 2, and 2 in the 1st, 2nd, and 4th recipients, respectively. The 3rd recipient gave birth but consumed all the newborns. The % newborn was 15.0 (6/40) after embryo transfer in recipients. At 4 weeks old, 2 males and 4 females of immature C57BL/6Mlac mice were counted during the weaning period (Table 3, Figure 7).

Table 1 The performance of *in vitro* embryo production in C57BL/6Mlac mice

Stage of embryos	No. of embryos (11 female mice)	Percentage
2-cell stage embryos	130	42.6
Oocytes	38	12.5
Abnormal oocytes	137	44.9

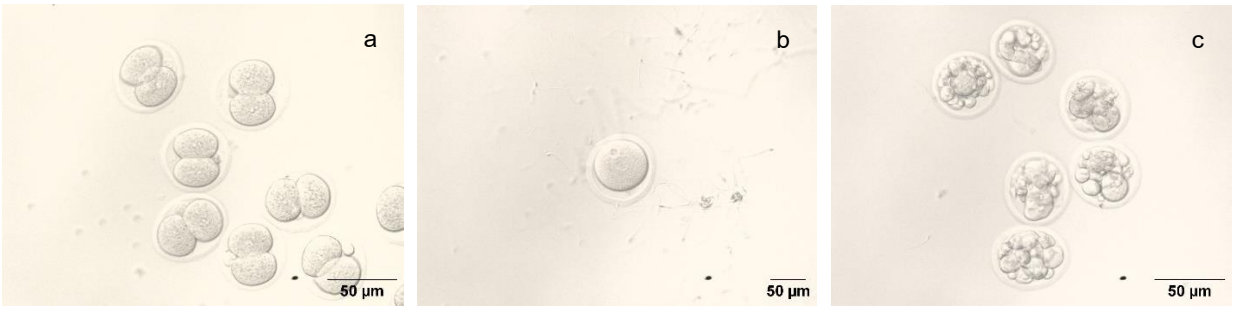

Figure 5 The *in vitro* embryo production, 2-cell stage embryos (a), oocytes (b), and abnormal oocytes (c).

Table 2 The vitrification and thawing of 2-cell stage embryos in C57BL/6Mlac mice

No. of vitrified embryos	No. of embryos discovered	No. of dead embryos	No. of surviving embryos
130	110	23	87

Table 3 The *in vitro* culture and embryo transfer in C57BL/6Mlac mice

No. of surviving 2-cell stage embryos (% survival)	Group	No. of *in vitro* culture	No. of blastocysts (% development)	No. of transferred embryos (recipients)	No. of newborns (% newborn)
87 (66.9)	1	47	29 (61.7)	NA	NA
	2	NA	NA	40 (4)	6 (15.0)

NA = No data available for this section.

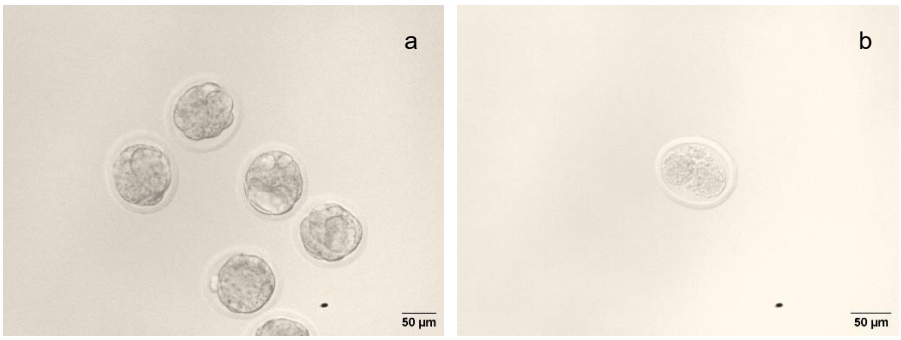

Figure 6 The blastocyst (a) and dead embryos (b) after *in vitro* culture for 72 hours.

Figure 7 The newborn of C57BL/6Mlac mice with the 1st recipient (a), the 2nd recipient (b), and the 4th recipient (c)

Discussion

In the embryo bank unit, the routine protocol for 2-cell stage embryo production involved *in vivo* embryo production. Following, 8-10 weeks old C57BL/6Mlac female mice were used for superovulation by 10 IU PMSG/hCG at a 48-hour interval, and then set natural mating with the more than 12 weeks old C57BL/6Mlac male mice (1 male per 1 female). The 2-cell stage embryos were collected from the oviducts of the donor females 43-46 hours after hCG injection. In this study, we used eleven 17-week-old C57BL/6Mlac female mice, which are typically eliminated from the colony at this age. Additionally, there was a limited number of 12-week-old C57BL/6Mlac male mice available. As it is difficult to produce 2-cell stage embryos using the standard protocol under these conditions, this study employed *in vitro* embryo production, including 10 IU PMSG/hCG for superovulation, followed by *in vitro* fertilization to collect the 2-cell stage embryos. The results show that the % superovulation was high, indicating that C57BL/6Mlac female mice at 17 weeks old can respond well to the 10 IU PMSG/hCG superovulation. In C57BL/6J mice, 10-week-old females were more responsive to superovulation with 10 IU PMSG/hCG than 5 IU PMSG/hCG (Song *et al*., 2015). The superovulation technique was used to obtain a large number of oocytes and reduce the number of laboratory animals in research. The response to superovulation is based on mouse strains, body weight, dose of hormone, and the method of embryo production. However, the number of abnormal oocytes was increased by this technique. Previous research reported that the percentage of abnormal oocytes was 54.0 and 29.0 in 46-week-old and 62-week-old C57Bl/6J female mice, respectively (Hasegawa *et al*., 2016).

In this study, COCs were collected from the oviducts and incubated overnight with sperm in HTF medium. After *in vitro* fertilization, the % *in vitro* fertilization was 42.6. Previous research reported that the % *in vitro* fertilization was 46.0 in 62-week-old C57BL/6J female mice, in which oocytes and frozen sperm were incubated in HTF medium for 3-4 hours (Hasegawa *et al*., 2016). In 9-week-old C57BL/6N female mice, COCs and fresh sperm were cultured in HTF medium for 3-4 hours. The % *in vitro* fertilization was more than 50.0 in C57BL/6NKorl, C57BL/6NA, and C57BL/6NB female mice (Yun *et al*., 2017). The % *in vitro* fertilization was greater than 65.0 in 8-week-old C57BL/6NCrl, C57Bl/6J and C57BL/6NTac female mice. COCs and fresh sperm were cultured in HTF medium for 4 hours (Liu *et al*., 2009). In 5-week-old C57BL/6J female mice, COCs and fresh sperm were cultured in HTF medium for 3 hours. The % *in vitro* fertilization was 78.3 (Zhao *et al*., 2021). The % *in vitro* fertilization was 70.0 in 3-week-old C57BL/6J female mice. COCs were placed in a drop of HTF medium with fresh sperm for 5 hours. After that time, oocytes were washed to eliminate excess sperm and then cultured overnight in HTF medium (Sztein *et al*., 2000). Other studies found that the % *in vitro* fertilization was 62.5 in 8-week-old C57B/6J female mice, in which oocytes and sperm were incubated in TYH medium for 4 hours (Pornwiroon *et al*., 2006). However, COCs were

used in this study. These COCs have consisted of both normal oocytes and abnormal oocytes; it is unclear whether the abnormal oocytes were caused by superovulation or the *in vitro* fertilization protocol. In the next research, the dose of hormone will be studied for superovulation in adult C57BL/6Mlac mice. it is expected that reducing the number of abnormal embryos will increase the % *in vitro* fertilization. Although younger mice yield better *in vitro* fertilization outcomes, the use of aged mice remains effective for producing 2-cell stage embryos, which is crucial for preventing the long-term loss of laboratory animal strains.

The % survival of 2-cell stage embryos was high in this study. Vitrification and thawing followed the routine protocol in our laboratory. The cryoprotectant reagent in the vitrification solution, ethylene glycol, has properties that help prevent ice crystal formation inside the cells, with minimal toxicity to the cells. Ethylene glycol has been used in vitrification solutions for various stages of mouse embryos (Kohaya *et al*., 2013). The % survival of *in vitro* 2-cell stage embryos was 97.0 in C57BL/6J and C57BL/6N female mice (Mochida *et al*., 2013). For *in vivo* 2-cell stage embryos, the % survival was 69.0 in C57BL/6J female mice (Qiu *et al*., 2021).

The embryo transfer techniques are beneficial in the production of laboratory animals. The success of embryo transfer in mice depends on several factors, such as the stage of embryos, the quality of embryos, the recipients, and the operator's skills. In this study, the quality of 2-cell stage embryos after vitrification and thawing was tested using the protocol of *in vitro* culture and embryo transfer. It was found that the % development after *in vitro* culture was high, which ensured that the 2-cell stage embryos were a good quality. The recipients were on day 1 of pseudopregnancy. The surviving 2-cell stage embryos were transferred to the ampulla, and the surgery was successful. However, the % newborn was low. The previous research reported that the % newborn was 82.0 in C57BL/6J female mice, 53.0 in C57BL/6N female mice, 50.0 in the thalassemia transgenic female mice, and 33.2 in C57BL/6NTac female mice (Sa-ardrit *et al*., 2006; Liu *et al*., 2009; Mochida *et al*., 2013). Therefore, the operator should continuously practice embryo transfer skills to increase the % newborn.

Conclusions

C57BL/6Mlac female mice with adult age of 17-week-old can be used to produce the 2-cell stage embryos through *in vitro* embryo production, which involves 10 IU PMSG/hCG superovulation and *in vitro* fertilization. These 2-cell stage embryos exhibit resistance to cryopreservation, making them suitable for use in the embryo bank unit. Although the % *in vitro* fertilization and the % newborn were low, there are sufficient numbers of newborns to establish a new colony in C57BL/6Mlac mice. This approach also enhances the effective use of laboratory animals at the National Laboratory Animal Center.

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