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Inducing Pseudopregnancy in Female Mice Without the Need for Vasectomized Males Prior to Non-Surgical Embryo Transfer or Artificial Insemination

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Abstract

For successfully maintaining pregnancy with embryo transfer or artificial insemination, female recipient mice must be induced into a pseudopregnant state. Female mice are traditionally paired overnight with vasectomized males, and the following morning, the presence of a copulation plug is assessed. To increase the efficiency of producing pseudopregnant females, a cervical manipulation technique has been standardized to be used in combination with non-surgical embryo transfer or artificial insemination techniques in mice. The blunt end of a small plastic rod is inserted vaginally to contact the cervix and is vibrated for 30 s by contact with a trimmer. The procedure is quick and does not require anesthesia or analgesia. This technique increases the reliability and predictability of producing pseudopregnant females and entirely eliminates the requirement for vasectomized males. For CD1 mice, the efficiency of pseudopregnancy induction using cervical manipulation was 83% for females in estrus (N = 76) but only 38% of females in estrus were plugged by vasectomized males (N = 24). Artificial insemination in CD1 mice was performed by estrus synchronization with hormones, cervical manipulation, and the uterine transfer of sperm. Artificial insemination recipients receiving cervical manipulation (N = 76) had a pregnancy rate of 72% and an average litter size of 8.3 pups. This method can also be used to produce pseudopregnant females for non-surgical embryo transfer. Therefore, inducing pseudopregnancy by cervical manipulation is a convenient and efficient alternative to mating with a vasectomized male when performing non-surgical assisted reproduction techniques. Using cervical manipulation provides 3Rs (replacement, reduction, and refinement) benefits for assisted reproduction techniques by reducing the number of animals required and eliminating the necessity for surgically altered males.

Introduction

Assisted reproduction technologies are used for the production of genetically modified mouse models, as well as the recovery of strains from cryopreservation, the rederivation of strains from a compromised health status, and strategic vivarium management, including the production of age-matched cohorts. All assisted reproduction techniques in mice require the use of pseudopregnant female recipients for embryo development. Historically, pseudopregnant recipients have been generated by mating with sterile males, which are either surgically vasectomized or genetically infertile, and the presence of a copulation plug is assessed the following morning[1]. Recently, a protocol for sonic stimulation in mice has been developed for the surgical transfer of pronuclear or two-cell mouse embryos[2]. We have also developed a cervical manipulation (CM) protocol for use with artificial insemination and the non-surgical embryo transfer of blastocysts. The rationale for the use of the procedure is to provide a 3Rs reduction in the number of animals required (no longer requiring male mice) and a refinement of the techniques used (no longer necessitating the surgical vasectomy procedure for male mice). The description of this protocol includes the associated assisted reproduction technique to aid in the integration of CM into normal workflows. The overall goal of the CM method is to replace the use of male mice in the generation of pseudopregnant females for assisted reproduction techniques, including artificial insemination and embryo transfer.

The CM protocol described here was first developed to assist with artificial insemination in mice. The artificial insemination protocol, as originally described, achieved a pregnancy rate of 50%, with an average litter size of 7 pups[3]. CD1 recipient mice were estrous synchronized with a low dose of hormones, including pregnant mare serum gonadotropin (PMSG) and human chorionic gonadotropin (hCG), at a 47 h interval prior to insemination. An advantage of estrous synchronization was that it permitted the use of the protocol during normal working hours. Females were paired with vasectomized males immediately after artificial insemination, and mating was confirmed by the presence of a copulation plug. Inconsistency in the mating rate with recipients was reported as a difficulty in the procedure. Therefore, alternatives to mating were sought for the induction of pseudopregnancy.

The present study presents a standardized CM technique to increase the efficiency of producing pseudopregnant females. For females in estrus or proestrus, the blunt end of a small plastic rod is inserted vaginally to contact the cervix and is vibrated for 30 s by contact with a trimmer. The procedure is performed on a wire-topped cage. No anesthesia or analgesia is required. The CM technique is convenient for producing pseudopregnant females, which can produce litters after non-surgical artificial insemination without the need for mating with vasectomized males. CM can also be used for the production of pseudopregnant females as recipients of embryo transfer. Specifically, the CM technique can be paired with non-surgical embryo transfer, as described here. Non-surgical methods have been shown to be effective for the embryo transfer of blastocyst stage embryos in mice[4,5] and rats[6,7]. As this non-surgical method is an effective alternative to surgical methods, it is considered a 3Rs refinement of the technique. Based on previous research, fecal corticosterone levels, as a measure of stress, indicate that the non-surgical nature of the procedure does not increase stress levels in rodents[7,8]. The procedures are less technically challenging than surgical embryo transfer and are much faster to perform.

As the embryos are transferred to the uterus, embryos of the correct stage for uterine development must be transferred. For mice, blastocysts are transferred to 2.5 days post coitum (dpc) pseudopregnant recipients.

For the two non-surgical techniques described here, timing of the hormone administration and the CM technique differs. The timing of the CM procedure relative to estrus is important for success, as it replaces natural mating for the production of pseudopregnant recipients. By eliminating the need for vasectomized males to induce pseudopregnancy, this procedure provides 3Rs benefits by both reducing the number of animals required and eliminating the necessity for surgically altered males. The procedure itself is quick (30 s) and does not require anesthesia or analgesia. The technique greatly increases the reliability and predictability of producing pseudopregnant females for assisted reproduction.

Protocol

All applicable international, national, and institutional guidelines for the care and use of animals were followed. All procedures performed in studies involving animals were in accordance with the ethical standards of the ParaTechs Corporation Institutional Animal Care and Use Committee and conducted under the standards dictated by the Office of Laboratory Animal Welfare, National Institutes of Health, Public Health Service, United States Department of Health and Human Services. Male and female CD1(ICR) and female C57Bl/6J mice aged >8 weeks old were used for the present study. The animals were obtained from commercial sources (see **Table of Materials**).

1. Cervical manipulation procedure for use with artificial insemination in CD1 mice

1. Synchronized ovulation of female mice on day 1 and day 3
    1. Inject female mice with 2.5 IU PMSG (see **Table of Materials**) by intraperitoneal injection (IP) on day 1, 0.5 h prior to the start of the vivarium dark cycle.
    NOTE: Each male donor will provide enough sperm for 10 female recipients.
    2. Inject the females with 2.5 IU hCG (see **Table of Materials**) by IP injection on day 3, 1 h prior to the start of the vivarium dark cycle.
    NOTE: The timings of the injections, sperm transfer, and light cycle relative to each other are very important. The times specified for all the procedures in this protocol are based on a 12 h light/dark cycle.
2. Collection and capacitation of fresh sperm on day 4
    1. Prepare a 500 µL drop of sperm pre-incubation medium (PM, see **Table of Materials**) in a 60 mm tissue culture dish under paraffin oil. Prepare one dish per male donor. Equilibrate for at least 30 min at 37 °C and 5% CO_2 prior to sperm collection.
    2. At 1 h after the start of the vivarium light cycle, euthanize the male(s) according to institutional guidelines. Each male will provide enough sperm for 10 artificial inseminations.
    3. Quickly dissect the cauda epididymides from the mouse. Perform a transverse abdominal incision above the bladder using dissection scissors and toothed forceps.
        1. The testicular fat pads are located on either side of the bladder. Grasp one of the testicular fat

pads with curved forceps, and remove the testis and epididymis from the body cavity. Identify the cauda epididymis as a flat oval tubular structure just below the testis.

2. Hold the cauda epididymis with curved forceps, and resect using small, angled scissors. Remove both cauda epididymides, and transfer them to an absorbent tissue to remove fat and blood under a dissecting microscope.

4. Place two cauda epididymides into each 500 µL drop of equilibrated PM under paraffin oil at 37 ˚C. Cut the tissue by making six incisions using small scissors. If more than one male donor is used, use one epididymis from each donor for each sperm sample to reduce the variation between samples.

5. Gently swirl the dish, and allow the sperm to exit the tissue for 3 min, swirling once per minute. Remove all the tissues.

6. Incubate the sperm sample for 45 min to 1 h at 37 °C and 5% CO_2 to capacitate.

7. Optional: Measure the sperm count, and assess the motility under a microscope using a hemocytometer. For counting, dilute the sperm in PM as needed.

3. Confirmation of estrous cycle synchronization by cytological evaluation

1. Using a small, pre-moistened swab (see **Table of Materials**), gently collect vaginal cells from the vaginal wall by rolling the swab against the tissue, smear them into a 20 µL drop of sterile water on a microscope slide, and air-dry.

2. Under a microscope using 100x magnification with bright-field illumination, evaluate for the presence of cornified epithelial cells. Females in estrus or late proestrus will have cornified epithelial cells present and should be selected for cervical manipulation.

3. Optional: Record the weight of the female recipients prior to insemination.

4. Performing the cervical manipulation (CM)

1. Approximately 0.5 h prior to insemination, place a recipient female on the top of a cage with a wire rack, allowing the mouse to "grab" the cage bar surface. Grasp near the base of the tail using the thumb and forefinger and angle the tail upward while stabilizing the animal (**Figure 1**).

NOTE: The mouse will hold still for the duration of the procedure when the handling technique is performed correctly. If the mouse moves out of position, gently reposition and continue. If an aggressive animal is chosen as a recipient, allowing the animal to step into an enrichment tunnel during the procedure can be calming. Using the tunnel is optional, but it can assist researchers in handling the animals by providing a distraction during this procedure.

2. Insert the blunt end of a small plastic rod (see **Table of Materials**) vaginally to contact with the cervix, and vibrate for 30 s by contact with a trimmer (see **Table of Materials**).

NOTE: Place the rod in position before starting the trimmer. Both are held in one hand during the procedure. No anesthesia or analgesia is required.

5. Non-surgical sperm transfer for artificial insemination

1. Place the insemination device (see **Table of Materials**) on a P200 pipette set to 40 µL, and remove the protective cover.

2. Remove an aliquot of the capacitated sperm sample from the dish at 37 °C and 5% CO_2, and transfer it to a 35 mm tissue culture dish without oil at 37 °C. The sperm sample will be used immediately for transfer.
   NOTE: The sperm will lose motility rapidly outside the CO_2 incubator. Keep the capacitated sperm sample at 37 °C and 5% CO_2 as much as possible.

3. Press the pipette plunger to the first stop, lower the catheter tip into the sperm sample at 37 °C, and slowly load the sperm into the transfer device. Avoid any clumps. Remove residual oil from the exterior of the insemination device using an absorbent tissue. Set aside the pipette.
   NOTE: The transfer of paraffin oil to the uterine horn must be avoided. Remove residual oil from the exterior of the insemination device using an absorbent tissue.

4. Place the recipient female on the wire rack cage top. Hold the mouse in position using the same handling technique used for the CM; grasp near the base of the tail using the thumb and forefinger, and angle the tail upward while stabilizing the animal.

5. Place the small speculum (see **Table of Materials**) into the vagina.

6. Insert the insemination device catheter into the speculum, through the cervix, and into the uterus. Once the device hub contacts the speculum, dispense the sperm by pressing the pipette plunger to the first stop. Avoid the transfer of extra air into the uterine horn.
   NOTE: If the catheter is not positioned correctly, it will hit the tissue surrounding the cervix, causing it to flex and eventually bend. If the catheter does not glide through the cervix on the first attempt, gently back the catheter out, and reattempt until successful. Repositioning of the speculum may be necessary.

7. Remove the device and speculum. No post-procedure monitoring is required.

6. Optional: Pregnancy check on days 10-12

   1. Use weight gain to determine the pregnancy status. Weight gain will be strain dependent, but an increase of at least 1-2 g correlates with pregnancy.

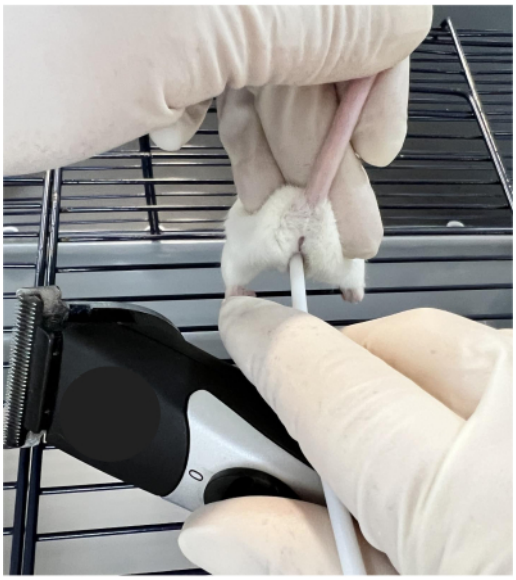

Figure 1: The mouse-holding technique for cervical manipulation. The mouse rests on a wire cage top and is stabilized at the tail and on both sides at the front of the hind legs. The blunt end of a small plastic rod is inserted vaginally to contact the cervix and is vibrated by contact with a trimmer. Please click here to view a larger version of this figure.

2. Non-surgical embryo transfer procedure for use with cervical manipulation in CD1 mice

1. Synchronized ovulation of the female mice on day 1 and day 3
    1. Inject female mice with 2.5 IU PMSG by IP injection on day 1, approximately 6 h after the start of the vivarium light cycle.
    2. Inject the females with 2.5 IU hCG by IP injection on day 3, at a 47 h interval after the PMSG injection or approximately 5 h after the start of the vivarium light cycle.
2. Confirming estrous cycle synchronization by cytological evaluation
    1. On day 3, about 1 h prior to the CM, perform cytology on the potential recipients. Using a small, pre-moistened swab, gently collect vaginal cells from the vaginal wall by rolling the swab against the tissue, smear them into a 20 µL drop of sterile water on a microscope slide, and air-dry.
    2. Under a microscope using 100x magnification with bright-field illumination, evaluate for the presence of cornified epithelial cells. Females in estrus or late proestrus will have cornified epithelial cells present and should be selected for cervical manipulation.
    3. Optional: Record the weight of the potential female embryo recipients.
3. Performing the CM

1. On day 3, 1 h prior to the start of the vivarium dark cycle, place a recipient female on the top of a cage with a wire rack, allowing the animal to "grab" the cage bar surface. Grasp near the base of the tail using the forefinger and the thumb, and then angle the tail upward while stabilizing the animal (**Figure 1**).

   NOTE: The mouse will hold still for the duration of the procedure when the handling technique is performed correctly. If the mouse moves out of position, gently reposition, and continue. If an aggressive animal is chosen as a recipient, allowing the animal to step into an enrichment tunnel during the procedure can be calming.

2. Insert the blunt end of a small plastic rod vaginally to contact the cervix, and vibrate it for 30 s by contact with a trimmer.

   NOTE: Place the rod in position before starting the trimmer. Both are held in one hand during the procedure. No anesthesia or analgesia is required.

4. Non-surgical embryo transfer on day 6

   1. Place a 20 µL drop of M2 medium (see **Table of Materials**) onto the lid of a tissue culture dish.

      NOTE: The lid is chosen as it has a shorter rim and, thus, is convenient for accessing the embryos in the drop. Do not at any time layer paraffin oil over the drop of M2, as introducing oil into the uterine horn appears to negatively affect embryo transfer.

   2. Under a microscope and using reflective lighting, load 10-20 blastocysts into the medium drop using a standard embryo handling pipette (see **Table of Materials**).

      NOTE: The optimal number of embryos to transfer depends on the mouse strain and any manipulations the embryos have undergone. For healthy unmanipulated embryos, transferring 10-15 embryos should be sufficient. For rederivation or genetically modified embryos, the transfer of more embryos is appropriate.

   3. Secure the non-surgical embryo transfer device (see **Table of Materials**) onto a P2 pipette set to 1.8 µL. Remove the protective catheter cover.

   4. Press the plunger of the pipette to the first stop, lower the tip of the catheter into the medium, and slowly pull the embryos into the catheter tip of the device. Remove the tip from the medium.

      NOTE: Visualizing the embryos under low magnification will allow for easier loading of the embryos into the device. If the embryos are scattered in the drop, gently shake the dish from side to side to concentrate the embryos in the center of the drop, or reposition them using an embryo handling pipette.

   5. Set the pipette volume to 2.0 µL to generate a small air bubble at the tip of the catheter. Gently lay the pipette with the embryos loaded near the mouse cage.

   6. Place the recipient female on the wire rack cage top. Hold the mouse in position using the same handling technique used for CM; grasp near the base of the tail using the forefinger and the thumb, and then angle the tail upward while stabilizing the animal.

   7. Place a small speculum (see **Table of Materials**) into the vagina.

8. Insert the transfer device catheter into the speculum, through the cervix, and into the uterus. Once the device hub contacts the speculum, dispense the embryos by pressing the pipette plunger to the first stop. Avoid the transfer of extra air into the uterine horn.

9. Without releasing the pipette plunger, remove the device and speculum. No post-procedure monitoring is required.

Representative Results

As electrical[9] and sonic[10] cervical stimulation have been used to induce pseudopregnancy in rats, this work presents a standardized mechanical procedure that can be used in mice. Vaginal cytology can aid in the identification of females in various stages of estrous. To confirm pseudopregnancy in females, this same method was employed. First, cytology profiles for females were compared for CD1 and C57Bl/6 mice throughout estrous cycles, during pregnancy, and during pseudopregnancy induced by mating or CM. Vaginal swabs were taken from the females and stained using a Papanicolaou staining system (see **Table of Materials**). The cells were observed under 100x magnification with bright-field illumination. The cells observed included leukocytes, nucleated epithelial cells, and anucleate cornified epithelial cells. The determination of the estrous cycle stage was based on the relative percentage of each cell type[11,12]. Estrus is characterized by the predominance of cornified epithelial cells. As estrus ends, metestrus begins, and leukocytes begin to appear, while cornified epithelial cells become less evident. Diestrus has moderate to low cellularity, with leukocytes predominating and nucleated epithelial cells beginning to appear. Proestrus is distinguished by the loss of leukocytes, an increase in nucleated epithelial cells, and the appearance of cornified epithelial cells. After proestrus, estrus begins, and the cycle continues.

To develop a baseline profile, vaginal cytology was recorded for at least two full estrous cycles for each female (N = 20 for CD1, N = 20 for C57Bl/6) prior to mating. The cycle lengths and individual profiles varied among the mice; however, the expected general trends were observed. The average length of an estrous cycle for the CD1 and C57Bl/6 mice was 3.8 days, with a range of 3-5 days. The day before natural mating occurred, all the female mice were in proestrus. After mating, cytology was performed again 1.5 days post coitum (dpc) until estrous cycling resumed. **Figure 2** shows the cytological profile for pseudopregnant CD1 and C57Bl/6 females mated with vasectomized males.

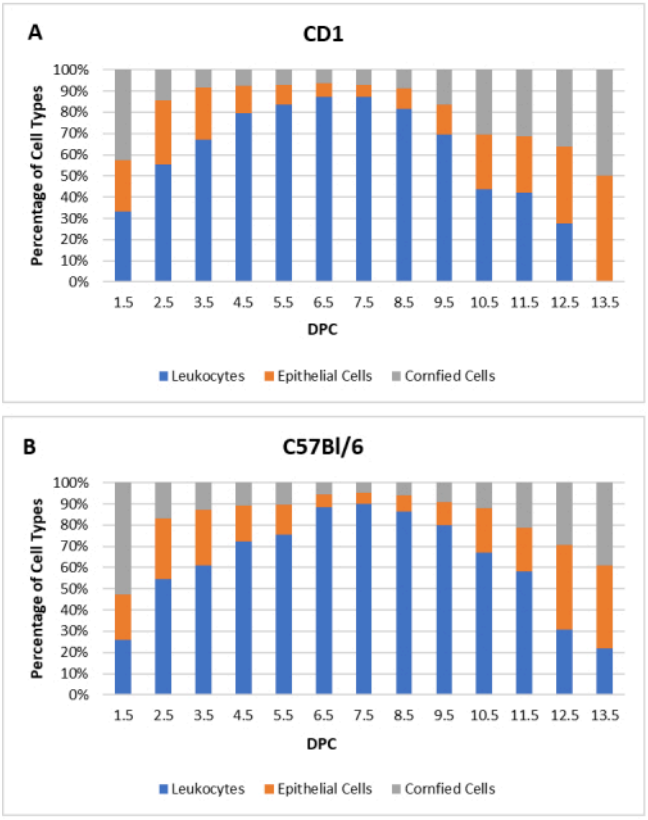

Figure 2: Cytological profile for pseudopregnant female mice. The average percentages of each cell type for leukocytes, nucleated epithelial cells, and cornified epithelial cells are shown as a function of days post coitum (DPC) for (**A**) CD1 (N = 20) and (**B**) C57Bl/6 (N = 20) mice. Please click here to view a larger version of this figure.

In this work, a CM technique has been standardized to increase the efficiency of producing pseudopregnant females. For females in estrus or proestrus, the blunt end of a small plastic rod is inserted vaginally to contact the cervix and is vibrated for 30 s by contact with a trimmer. The procedure is performed on a wire-topped cage. No anesthesia or analgesia is required. To determine the effectiveness of the CM procedure, vaginal cytology was compared for female CD1 (N = 20) and C57Bl/6 (N = 20) mice after mating with vasectomized males and after CM (total N = 40). The cytological profile of pseudopregnancy induced by CM was similar to the profile of pseudopregnancy induced by mating, as shown in **Figure 3**.

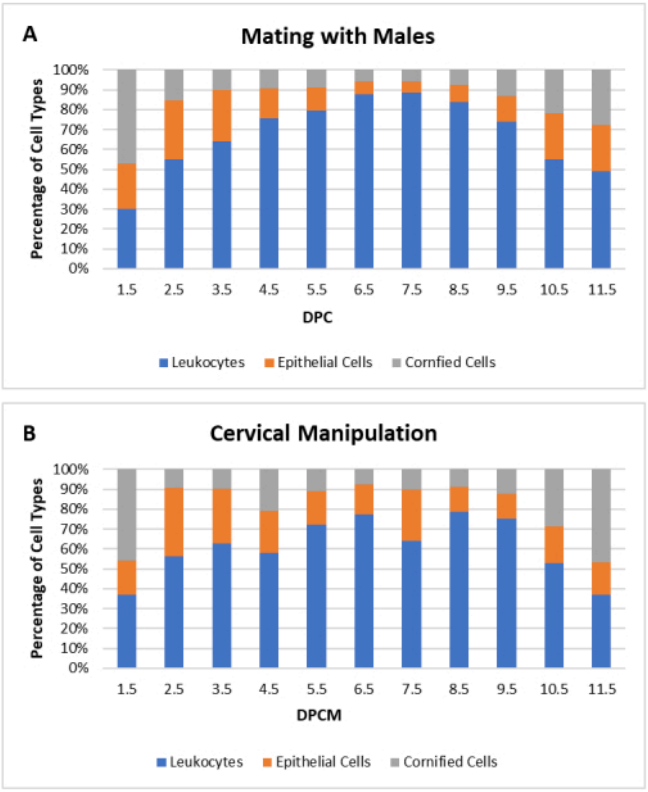

Figure 3: Cytological profile for pseudopregnant female mice after cervical manipulation (CM). The percentage of each cell type for leukocytes, nucleated epithelial cells, and cornified epithelial cells are shown as a function of days post coitum (DPC) or days post cervical manipulation (DPCM). The average cell type percentages are shown for the CD1 and C57Bl/6 mice (N = 40) (**A**) mated with vasectomized males or (**B**) after CM. Please click here to view a larger version of this figure.

To determine if the CM technique is sufficient to establish pregnancy for assisted reproduction, CM was performed as part of a non-surgical artificial insemination (NSAI) protocol in CD1 females. The artificial insemination protocol included estrus synchronization of the females with a low dose of the hormones PMSG and hCG prior to sperm transfer. CM was performed just prior to the sperm transfer. For CD1 mice, the efficiency of pseudopregnancy induction using CM for females in estrus (N = 76) with this technique was 83%. In a control experiment, only 38% of females in estrus were plugged by vasectomized males (N = 24). The artificial insemination recipients receiving cervical manipulation (N = 76) had a 72% pregnancy rate and an average litter size of 8.3 pups. Thus, induction of pseudopregnancy by CM is a convenient and efficient replacement to mating with a vasectomized male when performing NSAI. Using CM to prepare CD1 recipients (N = 4) in estrus for the non-surgical embryo transfer of fresh CD1 blastocysts resulted in a 100% pregnancy rate. The transfer of 9-15 embryos yielded three healthy litters with a 45% birth rate and an average litter size of

6 pups. In comparison, the CD1 recipients (N = 20) that were mated with vasectomized males to induce pseudopregnancy had an 80% pregnancy rate and a 46% birth rate after the transfer of 20 fresh B6C3F2 blastocysts.

Results for assisted reproduction techniques will likely be strain-specific, as variables such as the dose and timing of hormone administration for superovulation have been found to be strain-dependent[13]. In addition, factors such as recipient age and weight can affect reaction to hormones[14]. In this work, when performing the CM procedure for artificial insemination, only females in late proestrus or estrus were found to be responsive. In general, to increase the number of available recipients in a population, the females are first estrus-synchronized with a low dose of hormones[3]. Estrus synchronization with 2.5 IU of PMSG and hCG was 78% effective for 11-14 week old CD1 females (N = 27) and 60% effective for 18-32 week old C57Bl/6 females (N = 22) in this work. The efficiency of pseudopregnancy induction using cervical manipulation on CD1 females was 83% for females in estrus (N = 76) and 82% (N = 100) for C57Bl/6 females in estrus with this technique.

Discussion

The 3Rs is an ethical framework for animal use in research, as described in 1959 by Russel and Burch in "The Principles of Humane Experimental Technique"[15]. The 3Rs represent replacement, reduction, and refinement in animal use. The protocols highlighted here are in alignment with the 3Rs. The cervical manipulation technique reduces the number of animals needed by no longer requiring the use of males to produce pseudopregnant females. The technique also eliminates the need to perform vasectomies on the males, thus providing refinement by reducing pain and distress. The assisted reproduction techniques described here (artificial insemination and embryo transfer) are non-surgical, and thus, both provide a 3Rs refinement by reducing the pain and distress[8] caused by their surgical alternatives.

The use of pseudopregnant females is necessary for the recovery of pups when performing assisted reproduction in mice[1]. The CM procedure is an effective method for producing pseudopregnant females, but the synchronization of the phase of the estrous cycle of the recipient females is a critical first step in the process. Estrous synchronization can drastically reduce the number of females needed in the colony to prepare potential recipients and aids in producing timed pseudopregnant females on demand. Using a low dose of hormones does not seem to cause any deleterious effects on the recovery of live litters in CD1 mice. Care must be taken with other strains to find the hormone and concentration combination that produces the best-quality recipient females for the embryos or sperm transferred. Synchronization can be achieved using PMSG and hCG[16], but doses that produce superovulated females may not be appropriate for a sustained pregnancy[17].

To determine if a female is in estrus, a cytological evaluation was performed in this work. The estrous phase can also be evaluated by the observation of the vaginal opening[11,18]. While this method is extremely helpful and can be used by itself or as confirmation, it is more subjective than the use of cytology. Vaginal cytology without staining is both rapid and effective for choosing females in estrus because cornified epithelial cells can be easily identified. In this protocol, the cytological evaluation is performed prior to CM to determine potential recipients. It is important to perform cytology prior to CM, as the procedure tends to fragment the cells sloughed from the vaginal area, thus making identification difficult. Cytological evaluation for pseudopregnancy or pregnancy

can be performed at 3.5-11.5 days post CM (dpcm) for 3 consecutive days. The profile of an estrous cycling female should have at least 1 day with considerable infiltration of cornified epithelial cells. Pseudopregnant/pregnant females should display a diestrus profile (mostly leukocytes with potentially low cell numbers) for 3 consecutive days.

Through the development of the CM technique, some mice were found to be more receptive to the procedure than others. CD1 female mice are excellent candidates because of their calm nature and excellent nurturing instincts. This strain is easy to handle and performs well during the CM and non-surgical assisted reproduction techniques. C57Bl/6 mice tend to be more aggressive and less nurturing. While this protocol effectively produced pseudopregnant C57Bl/6 females using CM, they were less likely to be consistently permissive of the procedure. This seemed to correlate somewhat with the estrous phase during CM. Females in estrus or proestrus were more receptive. The use of an enrichment tube for the animal to enter allowed access to the vagina for the procedure and helped calm the female. The procedure itself does not fully restrain the female, so the animal can pull away at any time. If this occurs, the animal can be repositioned, and the procedure can then be continued. The timing of the procedure stops if the female walks away and resumes when the procedure is resumed. Critical to the success of the procedure are the phase of the estrous cycle (late proestrus and estrus) and the contact of the rod with the cervix. The vibration of the trimmer provides standardized CM. To ensure contact with the cervix, gentle pressure is applied to the rod, and the positioning of the rod against the cervix is assured with small back-and-forth movements of the rod.

The use of CM has improved the NSAI protocol, as females in the correct phase of the estrous cycle can be chosen prior to sperm transfer, and the protocol is no longer contingent upon mating with vasectomized males. The artificial insemination estrous cycle synchronization is timed such that oocyte maturation corresponds to sperm transfer on the morning of day 4. Critical to the success of the protocol is the adaptation of the timing of ovulation such that fertilization can occur. Care must be taken to administer hCG 15-17 h before expected sperm transfer, as is suggested for the timings used for *in vitro* fertilization[1]. The quality of the sperm sample will directly affect the outcome of artificial insemination. Fresh sperm that have been capacitated will perform best. Cryopreserved sperm of good quality can produce fertilized embryos *in vivo*. However, care should be taken with the direct transfer of thawed sperm, as residual cryoprotectants transferred to the uterine horn may inhibit implantation (unpublished observations).

The use of CM in conjunction with embryo transfer is conceptually an easy adaptation. Estrous cycle synchronization reduces the number of females necessary for producing the recipient pool. Determining the estrous stage prior to CM increases the likelihood of obtaining pseudopregnant recipients. One drawback of the method is that the cytology of the recipients at the time of embryo transfer is in a stage of flux. All cell types are present if the female is transitioning from estrus to the pseudopregnancy profile, and pseudopregnancy becomes obvious only if the cytology is tracked for several days. Based on the success (>80%) of the transition from estrus to pseudopregnancy for CD1 and C57Bl/6 mice, this method is expected to be suitable for embryo transfer recipients. The preliminary results show good success with limited non-surgical embryo transfer. In general, the efficiency of non-surgical embryo transfer is comparable to that of the surgical technique[4,5], and non-surgical transfer can replace surgical embryo transfers at the

blastocyst stage. For earlier-stage embryos, embryo culture to the blastocyst stage is required. However, if a surgical transfer is preferred, it is possible to adapt the CM technique to the correct timing needed for appropriate pseudopregnant recipients[2]. In general, the embryo recipients are 1 day less advanced than the embryo. For example, blastocysts are harvested at 3.5 dpc from donors and transferred to 2.5 dpc recipients. Therefore, CM will need to be performed such that the recipient is in a less developed pseudopregnant state than the embryos.

In conclusion, the CM technique outlined here shows excellent promise for integration with other assisted reproduction techniques for mice. We have provided successful protocols for artificial insemination and embryo transfer using non-surgical techniques. In combination, the CM technique provides 3Rs advantages, including (1) a reduction in the number of animals by eliminating the need for vasectomized males and (2) a refinement of techniques by replacing surgical techniques with non-surgical alternatives.

Disclosures

Barbara Stone and Sarah Srodulski are both employed at ParaTechs Corporation, Lexington, KY, USA. ParaTechs holds exclusive licensing rights to manufacture the mNSET device for mice. ParaTechs Corp has developed and sells the mNSET and mC&I devices, which can be used for non-surgical embryo transfer and non-surgical artificial insemination, respectively.

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