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A Nonsurgical Embryo Transfer Technique for Fresh and Cultured Blastocysts in Rats

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The use of a nonsurgical embryo transfer technique in rodents eliminates the potential pain, distress, and health complications that may result from a surgical procedure and as such, represents a refinement in rodent assisted reproductive techniques. A nonsurgical technique has not been previously developed for use with rat embryos. Here we describe an efficient method to deliver either fresh or cultured blastocyst stage embryos to the uterine horn of pseudopregnant female rats using a rat nonsurgical embryo transfer (rNSET) device. The rNSET device is composed of a Teflon catheter and a hub that attaches to a 2 µL pipette. Oxytocin is used to dilate the cervix before the delivery of blastocysts, allowing passage of the rNSET catheter directly into the uterine horn for embryo delivery. The efficiency of recovery of pups after nonsurgical embryo transfer is similar to the efficiency after surgical embryo transfer. Furthermore, the technique is not stressful to the subjects, as demonstrated by the absence of a decrease in weight or increase in fecal corticosterone level in recipients of embryos delivered nonsurgically, without the use of anesthesia or analgesia.

Abbreviations: dpc, days post coitum; F344, Fisher 344; IVF, in vitro fertilization; mNSET, mouse nonsurgical embryo transfer; NP, no embryo transfer procedure; NP+A, no embryo transfer procedure with anesthesia; NSET, nonsurgical embryo transfer; rNSET, rat nonsurgical embryo transfer; rNSET+A, rat nonsurgical embryo transfer with anesthesia; SD, Sprague–Dawley; SET, surgical embryo transfer; SET+A, surgical embryo transfer with anesthesia; 3Rs, replacement, reduction, and refinement

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Targeted genetic editing in embryos is currently used for making precise alterations in mouse and rat genomes to generate specific disease and physiologic models for biomedical research. Once an embryo has been altered in vitro, it must be implanted in an appropriate recipient female to develop to term, leading to the production of founder animals. Historically, embryo transfer in rodents has been performed surgically. To minimize the potential for surgical complications, reduce the need for anesthesia and analgesia, and simplify the embryo transfer procedures, devices and methods have been developed to enable nonsurgical embryo transfer (NSET) into pseudopregnant female rodents. The first device for NSET was developed in mice (mNSET device).[15] This device effectively transfers both unmodified blastocysts[28,39,46] and blastocysts that had been genetically modified by introduction of ES cells,[3,15] pronuclear injection,[15] or lentiviral gene transfer.[1,23,25] A recent study[7] used the mNSET device to test the effect of culture conditions on post-implantation development of mouse zygotes cultured to blastocyst stage. Embryo culture, followed by mNSET, has also been used to assess toxic effects of various compounds on post-implantation embryo development and pregnancy in mice.[17,18,32] Conversely, maternal dietary requirements have been evaluated using the mNSET device to study the effects of a high fructose diet[36] and zinc deficiency[45] on transferred unmanipulated embryos.

The nonsurgical technique is an attractive alternative to surgical embryo transfer (SET) in mice as it requires no anesthesia or analgesia, reduces stress indicators,[39] and eliminates potential surgical complications.[3] NSET procedures thus meet the requirement of a refinement of technique to minimize pain and distress.[35] Modification of the techniques for the use of the mNSET device has led to a diverse expansion of its function. It has been used for transcervical sperm transfer to evaluate sperm/embryo interactions.[2] In addition, a protocol for artificial insemination in mice was developed that allows live pups to be recovered.[40] Utility of the device has expanded into areas of study such as embryo development[5,37,50] and embryonic gene expression.[20] The mNSET device was also used to deposit bacteria into uterine tissue in the development of a *Chlamydia* infection model.[8,31] This infection procedure has been used extensively to study bacterial virulence and infection,[6,12,24,33,43,52,53] the host immunologic response,[9,14,29,42,47,48] and vaccine targets.[22,38,51] While the use of the mNSET device has become routine and has expanded beyond the scope of embryo transfer in mice, the device and the technique were not immediately adaptable for use in other rodents.

Development of an NSET technique for use in rats is based in principle on the technique for mice. However, several unique technical and reproductive considerations had to be addressed for successful rNSET. As part of its development, the rNSET device had to be tested for safety, ease of use, and capacity to deliver embryos to an appropriate uterine location. Multiple prototypes were produced and tested to create a device specifically suited for embryo transfer in 2 of the most commonly used models, the Sprague–Dawley (SD) outbred stock and the inbred Fischer 344 (F344) strain. To consider the unique reproductive physiology issues encountered in the development of this method, the appropriate embryo development stage for rNSET had to be coupled with a suitably prepared female rat

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recipient. An initial hurdle was cervical dilation, as in contrast to pseudopregnant mice, the cervix of pseudopregnant recipient rats is not dilated at the optimal time of embryo transfer for blastocysts. Administration of oxytocin to dilate the cervix allowed the rNSET device to pass the cervix for placement of embryos in the uterine horn. To couple the embryo stage with a suitable recipient, morulae or blastocysts were transferred into pseudopregnant recipients at 2.5 d and 3.5 d post coitum (dpc). A rat embryo transfer procedure for use with freshly isolated blastocysts was optimized and compared with SET.

To demonstrate the potential of the technique for use in all facets of transgenic rat model generation, the transfer of in vitro cultured blastocysts was demonstrated. Embryos were cultured from single-cell stage to blastocyst and transferred to suitable recipient females. Successful recovery of pups after transfer of cultured embryos considerably expands the practicality of the technique. The goal of our study is to provide both a practical technique and animal welfare benefits in keeping with the 3Rs of animal welfare for research. Therefore, the effects of the procedure were measured using 2 noninvasive bioindicators of stress: weight loss and fecal corticosterone levels. No increase in bioindicators of stress were observed for rats. This manuscript describes the optimization of a rNSET device and procedure, and found that it is effective, can be used with fresh or in vitro cultured blastocysts, and thus, represents a technical refinement as determined by the 3Rs.[35]

Materials and Methods

Animals. All applicable international, national, and institutional guidelines for the care and use of animals were followed. All procedures involving animals were performed in accordance with the ethical standards of the Institutional Animal Care and Use Committees at ParaTechs Corporation (Lexington, KY) and the University of Missouri (Columbia, MO), where the studies were conducted. ParaTechs Corporation and the University of Missouri performed all studies involving animals under Assurances of Compliance filed with the Office of Laboratory Animal Welfare, National Institutes of Health, Public Health Service, United States Department of Health and Human Services. Animal housing and use was conducted in accordance with the Guide for the Care and Use of Laboratory Animals, 8th edition.[19] All experiments were approved by the Animal Care and Use Committee of ParaTechs Corporation. Experiments using in vitro cultured embryos for embryo transfer were performed at the Rat Resource and Research Center, Department of Veterinary Pathobiology, University of Missouri and were approved by the Animal Care and Use Committee of the University of Missouri.

Sprague–Dawley (Hsd:Sprague–Dawley SD) and Fisher 344 (F344/NHsd) SPF rats were obtained from Envigo (RMS, Indianapolis, IN). Female SD or F344 rats (>8 wk old) were used as embryo donors and recipients. Rats were acclimated for a minimum of 7 d prior to use in experiments. Male SD and F344 rats (>8 wk old) were mated with females of the same stock or strain to produce embryos for transfer. Vasectomized male SD rats (> 8 wk old) were used to induce pseudopregnancy. The vivarium at ParaTechs was maintained at 20 to 22 °C with an average relative humidity of 35% to 75% under a 12:12 h light:dark cycle (light from 0600 to 1800, EST). Rats were housed in standardized ventilated microisolation caging (Innovive, San Diego, CA). Rats had access to Teklad Global 19% protein extruded rodent diet no. 2919 (Envigo, Indianapolis, IN) and water ad libitum. The vivarium at the University of Missouri was maintained at 22 °C under a 14:10 h light:dark cycle (light from 0600 to 2000, CST) with food and water ad libitum.

Embryo donor preparation, embryo collection, and embryo culture. Four d prior to mating with a stud male, female embryo donors underwent estrous cycle synchronization by intraperitoneal injection of 40 µg luteinizing hormone-releasing hormone antagonist (Sigma–Aldrich, St Louis, MO) in injectable phosphate buffered saline (Covetrus, Dublin, OH). The mating status of females was confirmed by the presence of a copulation plug using an otoscope (Riester, Jungingen, Germany).[41] Morula or blastocyst stage embryos were flushed from the uterine horn with M2 medium (Sigma–Aldrich) at 3.5 or 4.5dpc, respectively. For isolation of single-cell embryos prior to in vitro culture, presumptive zygotes were collected at 0.5dpc from copulatory plug-positive SD females using mR1ECM-Hepes[27,30] and denuded by brief exposure to 1 mg/mL hyaluronidase (Sigma–Aldrich) in mR1ECM-Hepes. Embryos were rinsed and cultured to blastocyst stage in KSOM-R[27] with 1mM glutamine supplementation (Sigma–Aldrich). KSOM-R + 1 mg/mL bovine serum albumin (Sigma–Aldrich) was used for early stage embryos and KSOM-R + 10% fetal bovine serum (Gibco, Thermo Fisher Scientific, Waltham, MA) was used for embryos at 16-cell stage or later.

Pseudopregnant female rat recipients for rNSET. Pseudopregnant embryo recipients were estrous cycle synchronized by intraperitoneal injection of 40µg of luteinizing hormone-releasing hormone antagonist 4 d prior to mating with a vasectomized male. Mating status was confirmed by the presence of a copulatory plug.

rNSET protocol for use with the rNSET device. Experimental groups for rNSET were 20 recipients ($n = 20$) for freshly isolated blastocysts and 12 recipients ($n = 12$) for cultured blastocysts. Pseudopregnant females were used for embryo transfer at 2.5 or 3.5dpc, as indicated. Female pseudopregnant recipients at 3.5dpc received an intraperitoneal injection with 2IU oxytocin (Oxoject, Covetrus), 2 h prior to embryo transfer. Embryos (11 to 19) were prepared for transfer in a 70 µL drop of M2 medium. The rNSET device (ParaTechs, Lexington, KY) was fitted onto a Rainin PR-2 Pipette (Mettler-Toledo Rainin, Oakland, CA) set to 1.8 µL . Embryos were loaded into the rNSET device, and the pipette set to 2.0 µL to create a small air bubble at the tip of the catheter. The rat was held loosely in position on the benchtop between the technician's arm and torso facing away from the procedure. The base of the tail was held gently and angled slightly upward. A speculum was inserted into the vagina to access the cervix. The rNSET device catheter was then inserted through the speculum, past the cervix, and into the uterine horn. Embryos were delivered to one uterine horn only. No monitoring or maintenance was required after the procedure.

SET. Experimental groups for SET were $n = 20$ for freshly isolated blastocysts and $n = 9$ for cultured blastocysts. SETs were performed on 3.5dpc pseudopregnant recipients given 2.5% to 3% inhaled isoflurane delivered with O_2 using a VetEquip inhalation anesthesia system (Pleasanton, CA). The following analgesics were administered subcutaneously; buprenorphine (Covetrus) 0.02 mg/kg and meloxicam (Covetrus) 2.0 mg/kg. Under aseptic conditions, a dorsal incision was made and the ovary, oviduct, and upper uterine horn were exteriorized. Embryos were delivered by pipette to the uterine horn through a small incision made with a 26G needle. After the procedure was finished, the dorsal incision was closed with internal sutures and wound clips. Postsurgical monitoring and thermal maintenance were performed for at least 30 min and until the embryo recipient had resumed normal behavior.

Body weight and fecal corticosterone responses to SET and rNSET. Female rats were divided into 5 groups with 26 rats

per group; no procedure (NP), no procedure with anesthesia (NP+A), surgery with anesthesia (SET+A), rNSET with anesthesia (rNSET+A), and rNSET without anesthesia (rNSET). Females for embryo transfer groups (SET+A, rNSET+A, rNSET) were pseudopregnant and the procedures performed as described at 2.5dpc. Females for the groups that did not receive embryos (NP, NP+A) were not pseudopregnant. For animals receiving anesthesia, inhaled isoflurane (Covetrus) was administered at 2.5% to 3% for 15 min with the following analgesics administered subcutaneously; buprenorphine 0.02 mg/kg and meloxicam 2.0 mg/kg.

Animals were single housed at the start of data collection. Animal handling was minimized to just those tasks necessary for vivarium maintenance and data collection, both prior to and during the experiments. Rat were weighed and fresh fecal samples were collected 24 h prior to a scheduled procedure. Body weight was measured daily and fecal samples were collected at the following time points after procedure: 7.5, 24, 31.5, 55.5, and 79.5 h. Fecal samples were stored at −80 °C until processing. Fecal corticosterone levels were measured in triplicate by ELISA assay (Cayman Chemical, Ann Arbor, MI) according to the manufacturer's directions. Standard curves for concentration determination were prepared for each 96 well plate, as supplied by the manufacturer. Concentration in ng/g fecal sample was determined by comparison to the standard curve and by using the Cayman data analysis tool.

Statistical analysis. Statistical analyses were performed using GraphPad Prism version 8.0 for Windows, GraphPad Software, San Diego, CA, USA. Weight and stress data were analyzed using 2-way repeated measures ANOVA followed by the Tukey multiple comparisons test. The embryo transfer birth rates were compared by one-way ANOVA followed by the Bartlett test. Multiple comparisons using the one-way ANOVA data were followed by the Tukey multiple comparisons test. The statistical significance threshold was set at $P < 0.05$.

Results

Development of the rNSET device and transfer technique. The rNSET technique was dependent on development and testing of a suitable rNSET device. Prior to use for embryo transfer, the rNSET device (Figure 1) was selected from several prototypes after evaluation of catheter length, depth of uterine insertion, and safety. The catheter is made from a flexible Teflon material with a tapered tip for ease of passage through the cervix. A 19 mm polyethylene vaginal speculum was designed for use based upon the average length sufficient for passage of the catheter through the vaginal canal of female SD and F344 rats aged 2 to 7 mo and optimal catheter tip location in the uterine horn. Repeated insertions were performed to test the safety of passage of the catheter through the cervix and into the uterine horn. No overt uterine horn damage was detected after multiple insertions of any of the prototypes ($n = 48$), with rats showing no evidence of bleeding or discomfort after the procedure and no evidence of swelling, bleeding, or tissue damage upon visual inspection of the reproductive tract within 1 h after the procedure. The rNSET device was chosen from among the prototypes based on ease of use and proper placement within the uterine horn of both SD and F344 female rats. The device has a catheter length of 37 mm and a diameter of 0.71 mm.

Passage of the catheter through the cervix of pseudopregnant rats was determined at various time points measured as dpc. Passage of the rNSET device through the cervix was often blocked by the presence of a postcoital plug at 0.5 to 1.5dpc. The rNSET device was able to consistently pass the cervix at 2.5dpc, but not 3.5dpc. Embryo development in rats indicates that morulae would be appropriate to transfer into 2.5dpc pseudopregnant females and blastocysts would be transferred to 3.5dpc recipients.[41] Because the cervix was dilated at 2.5dpc, rNSET of morula and blastocyst stage embryos were attempted at 2.5dpc. Transfer of blastocysts into the uterine horn of 2.5dpc recipients resulted in recovery of live pups both surgically and nonsurgically, but the birth rate was low (8 to 9% of blastocysts), and litter size was small (1 to 3 pups). SET of morulae to the uterine horn at 2.5dpc did not result in pregnancies, and transfer with the rNSET device resulted in a low birth rate (13%) and small litter size (1 to 3 pups). SET of blastocysts to 3.5dpc pseudopregnant female rats was much more efficient (Table 1). SET of 11 to 19 embryos to SD or F344 females resulted in birth rates of 42% and 34%, respectively. Because SET of blastocysts to the uterine horn was more efficient at 3.5dpc as compared with 2.5dpc, development of a protocol for rNSET to 3.5dpc pseudopregnant females was pursued.

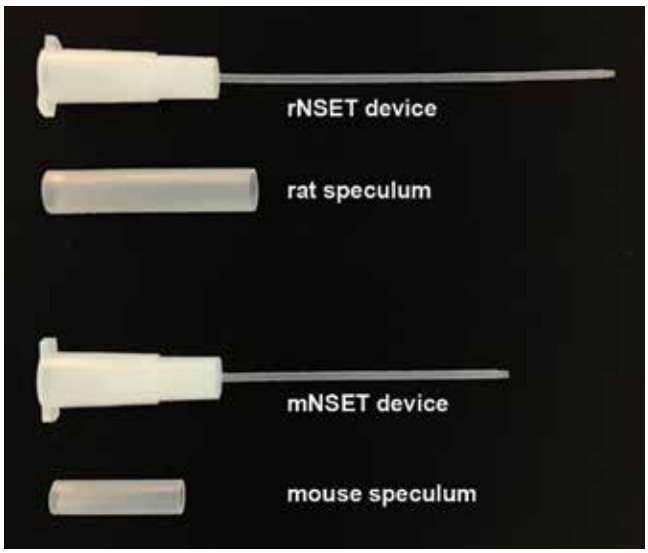

Figure 1. Manufactured rNSET device and speculum. The rNSET catheter is made from Teflon and the hub is molded from polyethylene. The hub is designed to fit securely on the end of a PR-2 pipette. The tip is tapered to facilitate passage through the cervix, with the length of the catheter (37 mm) optimized for embryo delivery through the cervix to the uterine horn. The catheter is flexible so that it readily passes through the cervix without damage to the uterine wall. The rat speculum is made from polyethylene and the length (19 mm) is optimized for use in SD and F344 rats. The mNSET and rNSET devices and specula are shown for comparison.

To dilate the cervix of 3.5dpc pseudopregnant rat recipients, 2IU of oxytocin was administered by intraperitoneal injection. Of injected female rats, 73% of 28 SD rats and 83% of 24 F344 rats became dilated, allowing the rNSET device catheter to be passed through the cervix by 2 h after injection. With administration of oxytocin, blastocysts were transferred to SD and F344 rats and the rNSET technique was compared to the SET technique (Table 1). rNSET in SD rats resulted in an 80% pregnancy rate and 39% birth rate compared to the SET pregnancy rate of 65% and 42% birth rate. In F344 rats, the rNSET recipients had a pregnancy rate of 90% and a birth rate of 36%, as compared with a SET pregnancy rate of 80% and birth rate of 34%. Data analysis of birth rates by one-way ANOVA indicates that the differences seen between the 4 groups in Table 1 are not significant. The administration of oxytocin did not appear to have deleterious effects on pregnancy after rNSET. These data provide evidence that use of the rNSET device for rNSET of blastocysts permits recovery of live offspring from SD and F344 rats.

Table 1. Blastocyst transfer to 3.5dpc pseudopregnant Sprague–Dawley and Fischer 344 rats.

			Embryos per transfer		Litter size			
Procedure	Strain	Recipients	Range	Average	Range	Average	Pregnancy rate	Birth rate
rNSET	SD	20	11–19	14.5	1–10	5.4	80%	39% ± 4.9
SET	SD	20	11–19	14.4	2–11	5.9	65%	42% ± 6.2
rNSET	F344	20	11–19	14.2	1–10	5.1	90%	36% ± 4.2
SET	F344	20	11–19	14.9	2–8	4.9	80%	34% ± 3.6

Birth rates were compared by one-way ANOVA. Birth rate is given as the mean ± SEM for pups born to pregnant females per number of embryos transferred. No statistical significance was seen between SET and rNSET birth rates for any of the groups.

Transfer of in vitro cultured blastocysts. To expand the utility of rNSET, we explored the use of SD embryos developed during in vitro culture. Embryos isolated at single cell stage were cultured to blastocyst stage and transferred by either SET or rNSET. The birth rate for cultured embryos transferred by SET was 58% and those transferred by rNSET was 31% (Table 2). Statistical analysis of the birth rates for the cultured embryos indicates a significant difference between the groups receiving cultured embryos by SET and rNSET. However, comparison of the birth rates between fresh (Table 1) and cultured (Table 2) SD embryos transferred either by SET or rNSET found no significant difference between groups.

Evaluation of body weight and fecal corticosterone after SET and rNSET. The use of a nonsurgical technique as a replacement for a surgical procedure provides clear advantages to the welfare of animals in research. However, to prove that the rNSET device is safe and does not cause undue stress to embryo recipients, a direct comparison of stress biomarkers in rats undergoing either SET or rNSET was performed. Two noninvasive biomarkers used for stress analysis were measured; weight loss and fecal corticosterone levels. Five study groups were compared ($n = 26$). Two groups of female SD rats did not undergo an embryo transfer procedure; one of these was administered anesthesia/analgesia (NP+A) and the other was not (NP). Three embryo recipient groups were compared using 2.5dpc pseudopregnant females. For one group, uterine surgery was performed with anesthesia and appropriate analgesia (SET+A). Two groups had embryos transferred using a rNSET device, either with (rNSET+A) or without anesthesia/analgesia (rNSET).

For the weight analysis, animals were single housed and weighed 1 d before the procedure. Weights were taken daily for 5 d post procedure. The analyses compared effects of surgery, sedation, and the rNSET procedure on the mean % weight change for each group over time (Figure 2). Rats that did not undergo embryo transfers (NP and NP+A) experienced a slight transient weight loss at the start of the experiment. Analysis showed no statistical significance between the NP and NP+A groups. These results indicate that anesthesia by itself had no effect on weight. There was also no difference between the rNSET+A and SET+A groups, which both showed a lack of weight gain for 2 days after anesthesia. However, a significant difference ($P < 0.05$) was detected between the groups receiving both anesthesia and embryo transfer (SET+A and rNSET+A) as compared with groups that did not receive embryos (NP and NP+A). One difference between these groups is that the SET+A and rNSET+A were 2.5dpc pseudopregnant while the NP and NP+A females were not. The greater weight change of the rNSET group was significant ($P < 0.05$) as compared with all other groups. While the rNSET group consistently gained weight throughout the study period, a slight decrease in weight or decrease in weight gain was observed for all other groups. The effects of the rNSET procedure on weight were independent of pregnancy status.

In addition to the weight analysis, fecal corticosterone levels were analyzed for all study groups (Figure 3). Feces were collected from each animal 24 h before the procedure and at 7.5, 24, 31.5, 55.5, and 79.5 h after the procedure. Corticosterone levels were measured by ELISA assay and compared between groups as the mean % change in fecal corticosterone levels over time starting at 24 h prior to the procedure. No significant difference was seen between any of the groups.

Discussion

This study describes the development of a rNSET device and a protocol for its use in SD and F344 rats. Recipient females are prepared for embryo transfer by estrus synchronization and establishment of pseudopregnancy. On the day of embryo transfer, 3.5dpc recipients are injected with oxytocin to dilate the cervix, and a speculum is inserted into the vagina and used to guide the rNSET catheter through the cervix and into the uterine horn. The blastocysts are then deposited, and the device and speculum are removed. This procedure results in the birth of live pups and is an effective alternative to SET of blastocyst stage embryos. Transfer of cultured embryos also resulted in live pups. While the birth rate for rNSET was lower than that of SET using cultured embryos, the ability to recover pups by a nonsurgical method makes this protocol useful in conjunction with other assisted reproduction techniques such as in vitro fertilization (IVF), pronuclear injection, intracytoplasmic sperm injection (ICSI), embryonic stem (ES) cell injection, cryopreservation, and targeted gene editing. These methods are important for vivarium management, disaster recovery for cryopreserved models, rederivation from a pathogen compromised facility, and experimental model production using gene editing procedures.

Prior to the rNSET procedure, oxytocin was administered by injection to 3.5dpc pseudopregnant rat recipients. Oxytocin has known pleotropic effects on physiology, reproduction, and behavior in mammals, is found in the brain and peripheral systems, and upon binding to its receptor, activates several signaling cascades.[21] As an example, oxytocin levels in the blood increase during parturition and lead to uterine contractions. Oxytocin has also recently been used to both dilate the cervix in sheep prior to artificial insemination[26] and for nonsurgical collection of embryos from the uterus.[13] The use of oxytocin prior to the rNSET procedure was able to efficiently dilate the cervix and did not appear to have deleterious effects on pregnancy or birth rate in SD and F344 rats.

Replacement of a surgical procedure with a nonsurgical alternative is a refinement in the use of animals in research. As reported here, the rNSET procedure can effectively replace SET. To ensure the procedure does not cause undue stress to embryo recipients, we directly compared 2 markers of wellbeing in rats undergoing either SET or rNSET. Surgery can have a measurable effect on corticosterone levels in rats[34] and fecal corticosterone levels are an appropriate noninvasive sampling method for the stress response in rats.[44] We have previously shown in mice that

Table 2. Transfer of in vitro cultured blastocysts to 3.5dpc pseudopregnant Sprague–Dawley rats.

Procedure	Strain	Recipients	Embryos per transfer	Pregnancy rate	Birth rate
rNSET	SD	12	12	100%	31% ± 0.3
SET	SD	9	12	100%	58%* ± 5.4

Birth rates were compared by one-way ANOVA. Birth rate is given as the mean ± SEM for pups born to pregnant females per number of embryos transferred. A statistical significance was seen between SET and rNSET birth rates for in vitro cultured blastocysts (* $P < 0.05$).

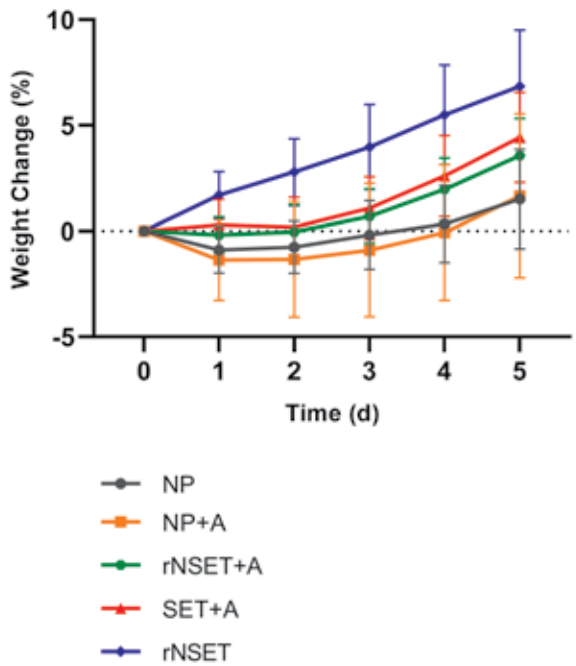

Figure 2. Weight analysis. Rats were weighed daily and the % weight change was calculated from a baseline average of weights taken 24 h prior to the procedure and just before the procedure (n = 26 per group). Two-way repeated measures ANOVA followed by Tukey multiple comparisons test were performed for statistical analysis. Data are presented as the average % weight change ± SD. The average starting weights of each group were compared by one-way ANOVA and statistical differences were found between treatment groups ($P < 0.05$). The following are the average weight ± SD at day 0 for each group; NP, 242.3g ± 31.6; NP+A, 205.9g ± 15.1; rNSET+A, 240.2g ± 16.13; SET+A, 221.9g ± 16.7; rNSET, 220.6g ± 17.1.

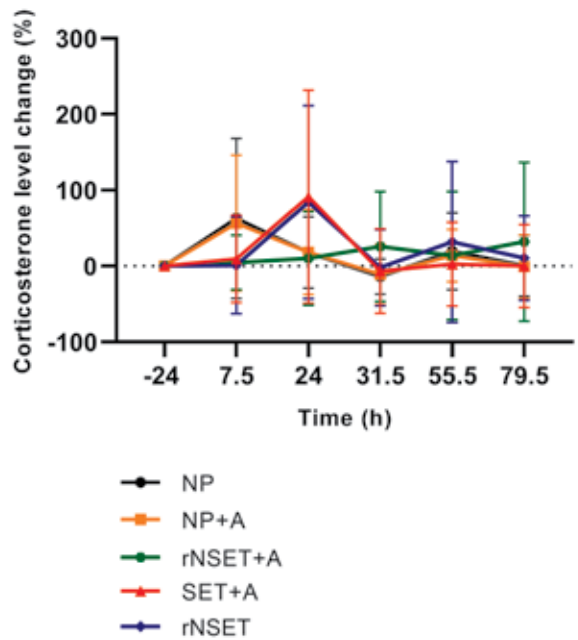

Figure 3. Fecal corticosterone analysis. Rat feces were collected at several timepoints before and after the procedure. Fecal corticosterone levels were measured as ng/g fecal matter collected. The % change in corticosterone level was calculated for each animal (n = 26 per group) at each time point relative to the baseline level measured at 24 h prior to the procedure. Two-way repeated measures ANOVA followed by Tukey multiple comparisons test were performed for statistical analysis. Data are presented as the average % change ± SD. The average baseline fecal corticosterone level for each group were compared by one-way ANOVA and statistical differences were found between treatment groups ($P < 0.05$). Initial average fecal corticosterone levels ranged from 248 to 418 ng/g feces between groups.

fecal corticosterone levels are increased in response to surgery but not in response to anesthesia or mNSET.[39]

A correlation between a decrease in body weight (or a decrease in weight gain) and postoperative pain has also been found in rats.[4,16] Weight loss and fecal corticosterone levels were measured to determine if the procedures caused an acute stress response (an increase in fecal corticosterone levels near the time of the procedure) or a more subtle stress response (reduction of weight gain or weight loss over several days). The rNSET procedure itself did not induce a change in either of these 2 measures. Weight loss and fecal corticosterone levels of the SET recipients, which received anesthesia (isoflurane) and an analgesic (buprenorphine), were not increased over controls. None of the groups tested showed significant changes in fecal corticosterone levels as compared with the unmanipulated group. However, we did detect a significant difference between the steady weight gain of the rNSET recipients and a short pause in weight gain or slight weight loss for all other groups tested. Comparison of the weight gain profiles of the study groups indicates that the groups not undergoing embryo transfer were statistically equivalent, as were groups receiving anesthesia and undergoing embryo transfer procedures. Two experimental variables in this study with potential to alter weight gain are the anesthetic buprenorphine[4] and oxytocin.[21] The lack of weight gain seen in all study groups other than the rNSET group cannot be attributed to the use of anesthesia alone as the NP control group also showed this effect.

Because oxytocin has been shown to affect both weight and corticosterone levels,[21] none of the females used for stress evaluation in this study received oxytocin prior to embryo transfer. Given that all embryo transfer groups were pseudopregnant whereas the NP groups were not, the difference in weight gain relative to these controls may be due to the pseudopregnant state of the embryo recipients. The difference between the rNSET group and other embryo transfer groups (rNSET+A and SET+A) is the use of anesthesia. Anesthesia use, in combination with the pseudopregnant state, may have impacted weight gain for these groups. An additional difference between these groups is that the rNSET recipients were handled without anesthesia for the duration of the embryo transfer procedure. The extended exposure to handling may have allowed a faster acclimation to the daily schedule of weighing and fecal sample collection. Regardless, the steady increase in weight by the rNSET group suggests a lack of stress during the study period and verifies

that the procedure is appropriate for use without anesthesia or analgesics. In addition, rNSET eliminates the complications associated with surgery and postoperative care. The rNSET procedure described here therefore provides a refinement for laboratory animal research, as defined by the 3Rs.

One of the concerns about the use of assisted reproduction in mammals is that the techniques used could damage the developing embryo. Epigenetic errors have been introduced on imprinted genes during culture of mouse embryos derived by IVF.[10] The study of embryo development and differential gene expression has been facilitated by the use of the mNSET device in mice. In a study to determine potential sources of epigenetic errors in mouse assisted reproduction procedures, the mNSET procedure did not change epigenetic profiles or reduce fetal weight.[11] No long-term physiologic effects were attributed to embryo transfer in mice.[11] However, all of the assisted reproduction techniques tested (embryo transfer, superovulation, and IVF) did increase placental weight.[11] Currently, the features of assisted reproduction that may cause epigenetic errors have not been identified. Some possibilities include deleterious effects of the method of euthanasia of the donor animal,[49] exposure to embryo culture media, increased atmospheric O_2 concentrations in the air relative to the uterine environment, temperature fluctuations, or potential physiologic differences between pseudopregnant embryo recipients as compared with naturally pregnant females.

Future research in the development of assisted reproduction techniques for use in mice and rats will no doubt find ways to reduce the impact of in vitro techniques on embryo development. In the meantime, assisted reproduction is crucial to the development of animal models for biomedical applications. For example, cryopreservation of germplasm provides the flexibility necessary for strategic vivarium maintenance. Cryopreserved sperm and embryos are used for storage of models that are not frequently needed, significantly reducing the number of animals bred for research and reducing costs. Cryopreservation also provides a source of animals for disaster recovery, can be used to mitigate genetic drift, and can aid in producing age-matched cohorts or rapid colony expansion when needed. Along with cryopreservation, IVF, artificial insemination, and embryo culture, embryo transfer is vital for various aspects of rodent model maintenance, recovery, and generation. The novel rNSET technique described here reduces the expertise necessary to produce rats by embryo transfer and adds a new methodology to the repertoire of assisted reproduction technologies available for rats used in biomedical research.

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