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Impact of surgical and non-surgical embryo transfers on recipient stress level in mice

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Abstract

Embryo Transfer (ET) ist ein wichtiges Instrument in der Labortiermedizin, welches sehr häufig zum Einsatz kommt, aber für die Tiere ein Stressfaktor sein kann. Es gibt chirurgische, derzeitiger Goldstandard, und nicht chirurgische Methoden. In der Studie untersuche ich den Einfluss von einer chirurgischen (SET) und drei nicht chirurgischen (TCET, NSET/w, NSET/o) Embryotransfermethoden auf das Stresslevel der Tiere. Dies erfolgt durch die nicht invasive Messung von Kortikosteron Metaboliten im Kot über vier Tage. Alle Tiere erfuhren eine gewisse Stressbelastung, bei den Gruppen der nicht chirurgischen Methoden war diese aber wesentlich geringer. Dieses Ergebnis soll ein Anreiz sein, künftige Embryotransfers mit einer nicht chirurgischen Variante durchzuführen, um so einen Beitrag im Sinne der 3Rs zu leisten. Fragen, wie diese Methoden die Erfolgsrate der Trächtigkeit und anderweitige Parameter der Konzeption beeinflussen, werden hier nicht beantwortet.

Embryo transfer (ET) is an important tool in laboratory animal medicine, which is used very often but can be a stress factor for the animals. There are surgical, currently the gold standard, and non-surgical methods. In my study the influence of one surgical (SET) and three non-surgical (TCET, NSET/w, NSET/o) embryo transfer methods on the stress level of the animals was evaluated. This is done by non-invasive fecal corticosterone metabolite (FCM) measurements over four days. All animals experienced a certain stress load related to the procedure, but in the groups of the non-surgical methods this load was significantly lower. This result highlights the importance to perform future embryo transfers with a non-surgical method, to contribute to the 3Rs. How these methods affect pregnancy success rates and other parameters of conception is not addressed here.

1 Introduction

Embryo transfer (ET) has a long history and studies specifically in mice go back up to 80 years (1). In modern times, ETs are used in human, as well as veterinary medicine. Especially in laboratory mice, ET plays a central role in creating transgenic models, revitalizing conserved strains, rehabilitating contaminated strains, and generating many same-aged animals (2–4). To perform an ET in a surrogate mother, a pseudopregnancy must first be induced. Therefore, the female is mated with a vasectomized stimulus male. Depending on the developmental stage of the embryo, the ET can take place in the oviduct or the uterus.

Currently, the most common procedure for transferring embryos to a mouse surrogate mother is the surgical embryo transfer (SET), in which the surrogate mother is put under general anesthesia and the embryos are transferred into the oviduct or uterus after the abdominal wall has been opened. SET is the gold standard as it is efficient and inexpensive. However, it is also an invasive procedure, requiring anaesthesia, causing postoperative pain, requiring postoperative analgesia and a high level of technical expertise.

To refine and facilitate embryo transfers, a non-surgical transfer method, namely, the transcervical embryo transfer (TCET) has been developed (5). In TCET, a special device is inserted into the vagina and moved forward through the cervix into the uterus. There, the embryos are deposited and allowed to implant in the uterine wall. In contrast to SET, anesthesia of the surrogate mother is not necessary, but is recommended to facilitate the procedure. An advantage of TCET is that it requires less technical expertise, it is faster to perform than SET and causes less physical trauma. Unfortunately, with TCET and other non-surgical transfer methods, only embryos at the morulae or blastocyst stage can be transferred, because the embryos are placed directly into the uterus. In 2014, Cui et al. (6) compared the success rates of SET and TCET and reported that the number of live pups born per transferred embryo was comparable between both methods, indicating that TCET can be equally successful than the established SET.

Similarly, to TCET, other non-surgical procedures emerged. In 2009, ParaTechs (Lexington, USA) developed the so-called “non-surgical embryo transfer” (NSET) to simplify and expand the application of ETs in mice. The advantages of TCET also apply to NSET. The devices are different, but the two procedures are identical. Animals recover quickly from the manipulation,

no anesthesia and analgesia are required, and the success rates are comparable to SET in terms of pregnancy rates and litter sizes (7). In addition, others reported that pregnancy and birth rates can further increase with routine practice (8).

Common for all non-surgical ET methods is that they are less invasive and therefore should be less stressful for the animals. But to date, no study has been performed that simultaneously compares the stress levels of animals that experience SET, TCET, and NSET. There are already some studies that have looked at specific transfer methods and their respective stress impact on mice. Steele et al. (9), for example, demonstrated that stress levels were higher after SET than after NSET, independent of whether NSET was performed with or without anesthesia. In addition, animals experiencing anesthesia and SET showed more fluctuation in their cardiac rhythm, which might indicate that animals face higher stress levels with surgery. However, it should be mentioned that no embryos were transferred in this study, as only sham transfers were performed.

In this experiment we performed SET, TCET (with anesthesia), and NSET (with and without anesthesia) and investigated how the respective transfer methods affect the stress levels of the mice. We determined animal stress levels by measuring fecal corticosterone metabolites (FCM). This non-invasive stress evaluation has been successfully established by Palme et al. (10) and validated for mice by Touma et al. (11,12). We assume that non-surgical ETs are less stressful for surrogate mothers than surgical ETs, and we have no clear predictions regarding the stress levels between the non-surgical methods. If our assumption is correct, the replacement of SET by a non-surgical alternative would affect the life of uncountable mice in laboratories worldwide and could contribute to the 3Rs by refining this procedure for surrogate mothers.

2 Materials and Methods

2.1 Experimental Animals

In this experiment, we used donor mice (n=80) and recipient mice (n=93) for the ETs. As donor strain, we used female C57BL/6N, which were purchased at an age of 3–4 weeks at Janvier Labs, France and male C57BL/6N as sperm donors, which were at least 8 weeks old. Recipients were hybrid females (crossing between C57BL/6N and DBA/2) that were homebred and used at an age of 8–16 weeks. All mice were kept under standardized housing conditions (21 ± 1 °C room temperature, 50 ± 10 % humidity, 12:12 h light-dark cycle with lights on at 6:00 a. m.) in the experimental animal facilities of the University of Veterinary Medicine, Vienna. Commercial mouse diet (V1534, M/R-H irradiated, Ssniff GmbH) and water (chlorinated tap water) were offered *ad libitum*. Mice were kept in same-sex groups in Macrolon cages equipped with wood bedding (Lignocel Select, J. Rettenmaier und Söhne GmbH + Co-KG), nesting material (Pur-Zellin® tissue swabs, Paul Hartmann GmbH) and houses or tubes (SDS Deutschland c/o Jung GmbH). There was a daily visual inspection of the animals to evaluate their health and welfare.

2.2 Study Design

We investigated the impact of different ET methods on female recipients in assessing their fecal stress hormone metabolite levels. Recipient females experienced either a surgical embryo transfer (SET), transcervical embryo transfer (TCET) or a non-surgical embryo transfer with (NSET/w) or without anesthesia (NSET/o). We used 93 hybrid surrogate mothers, 24 for the surgical transfer and 23 for each of the non-surgical ETs.

We ran the experiment in two blocks. In the first block, we performed 59 ETs on 7 consecutive days, in the second block, we performed 34 ETs on 5 consecutive days. On each day, we performed all four ET methods, and the number of ETs was balanced for transfer method. The order in which we performed the ETs was randomized between days. We always transferred 10 C57BL/6N blastocysts per ET, which were generated by IVF.

2.3 Embryo Transfer Methods

2.3.1 Surgical Embryo Transfer (SET)

The surrogate mothers received an injection anesthesia i. p. of ketamine and xylazine (Ketasol®, Xylasol®, both Graeub, Switzerland; 10 mg/100 g bw ketamine; 0.4 mg/100 g bw xylazine; 0.07 ml per 10 g bw). Once the necessary anesthetic depth was reached, the eyes were covered with eye ointment (Oleovit®, Fresenius Kabi, Austria). For analgesia, 0.05 mg/100 g meloxicam (Metacam®; Boehringer Ingelheim, Germany) was given s. c. once before surgery. The mice were placed in lateral position and a 4–6 mm skin incision was made either in the right or left flank (randomly chosen) followed by a slightly smaller incision in the abdominal skin. We always performed one sided transfer and the side of transfer was randomized between females. The ovary was pulled out of the abdominal cavity together with the oviduct and the tip of the uterine horn. A 26G cannula was used to pierce a small hole in the uterine wall near the uterotubal junction through which blastocysts were transferred with a glass pipette. After repositioning the ovary, oviduct and uterus into the abdominal cavity, the peritoneum was sutured with resorbable suture material, and the skin was closed with wound clips (Michel clips). The mice were kept on a warming plate until they recovered from anesthesia before being returned to their home cage.

2.3.2 Transcervical Embryo Transfer (TCET)

The surrogate mothers received an inhalation anesthesia with sevoflurane and oxygen as carrier gas (induction 8%, maintenance 2–3% sevoflurane; Harvard Apparatus, Hugo Sachs Elektronik, Germany). An apparatus positioned the animals in a 45° angle head downward (Fig. 1). Once the necessary anesthetic depth was reached, the ETs were performed with the TCET device (Fig. 2). Therefore, females were fixed by the root of the tail with one hand while a provided speculum (moistened with physiological saline) was carefully inserted into the vagina. Then the device was inserted through the speculum and the cervix to one of the uterine horns. Subsequently, the blastocysts were released in pulling a part of the device to its base. Afterwards the speculum was removed, and the mice were placed into their home cage.

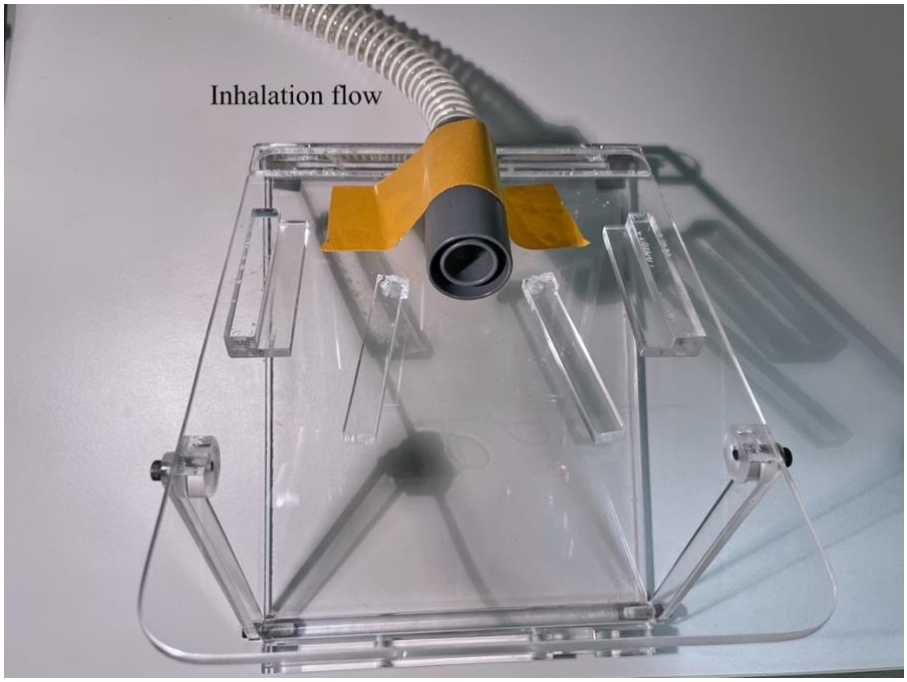

Fig. 1. Apparatus to fix the mouse for TCET and NSET/w lying on the abdomen in a 45° angle head facing downwards to the inhalation flow.

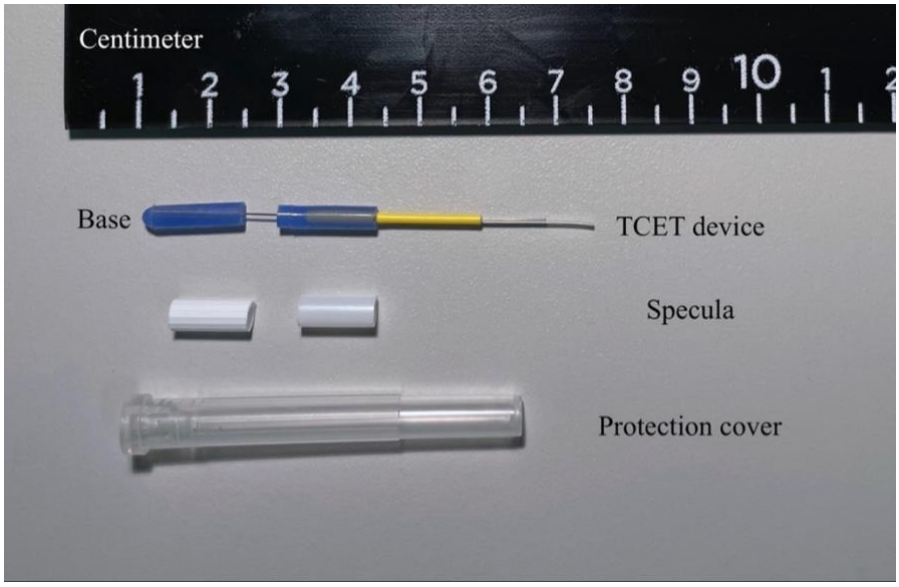

Fig. 2. TCET device with two different specula and protection cover.

2.3.3 Non-surgical Embryo Transfer with Anesthesia (NSET/w)

The surrogate mothers received an inhalation anesthesia with sevoflurane and oxygen as carrier gas (induction 8%, maintenance 2–3% sevoflurane; Harvard Apparatus, Hugo Sachs Elektronik, Germany). An apparatus positioned the animals in a 45° angle head downward (Fig. 1). Once the necessary anesthetic depth was reached, ETs were performed with a NSET device (Fig. 3). Therefore, females were fixed by the root of the tail with one hand while a provided speculum (moistened with physiological saline) was carefully inserted into the vagina. Once the speculum was positioned, the NSET device, which was placed on a 0.5–2 µl pipette, was introduced through the cervix to one of the uterine horns and the blastocysts were released by pressing the plunger to the first stop. After waiting for three seconds the pipette was retrieved while pressing the plunger and the mice were placed back into their home cage.

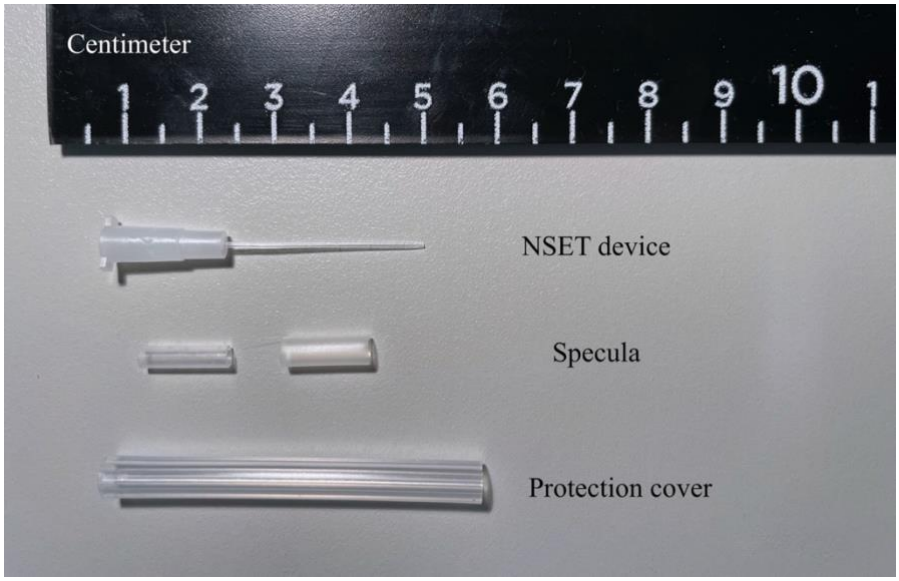

Fig. 3. NSET device with two different specula and protection cover.

2.3.4 Non-surgical Embryo Transfer without Anesthesia (NSET/o)

ET was performed with an NSET device without anesthesia (Fig. 3). Therefore, surrogate mothers were placed on the feeding grid of a grid cover where they could hold on. The root of the tail of the animals was lifted to position the animals with a 45° angle head downward. The provided speculum (moistened with physiological saline) was carefully inserted into the vagina. Then the NSET device, which was placed on a 0.5–2 µl pipette, was introduced through the

cervix to one of the uterine horns and the blastocysts were released as described above (Fig. 4). Subsequently, the NSET device and the speculum were removed, and the mice were placed into their home cage.

Fig. 4. NSET device placed on a 0.5–2 µl pipette is introduced into the speculum and through the cervix to one of the uterine horns. The mouse is unanesthetized.

2.4 Fecal Sample Collection

To evaluate the impact of the ET methods on the stress level of surrogate mothers, we collected the feces from all mice at four consecutive days and measured FCM concentrations. To determine baseline levels, we collected feces one day before ET. To determine the stress levels of the transfers, we collected feces on the day of ET and then one and two days later to estimate

how long a potential stress effect would last. Feces collection always occurred six to eight hours after ET. Peak excretion rates are expected around ten hours after a procedure (11). For collection each mouse was transferred to a small clean plastic box, in which it remained for 30 minutes or shorter if it had dropped three to four pellets (Fig. 5). Individuals were always sampled at the same day time across the four days, to control for daytime specific variations in FCM levels. After collection, feces were stored in Eppendorf Tubes, which were labeled with the consecutive sample number and stored in the freezer (-20 °C) until further processing.

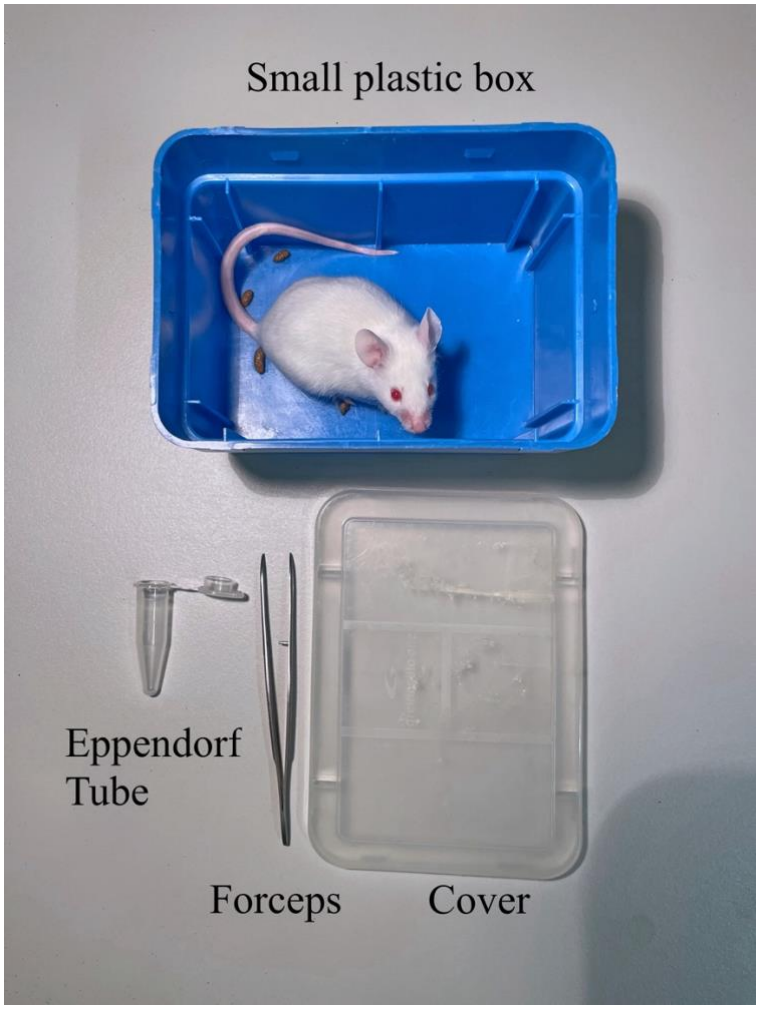

Fig. 5. Feces collection equipment: a small plastic box, a cover so that the mice can hide (not airtight), and a forceps to transfer the feces into an Eppendorf tube.

2.5 Fecal Corticosterone Metabolite (FCM) Measurement

Fecal samples were subjected to a standard extraction procedure. This method was established by Palme et al. (10) and validated for mice by Touma et al. (11,12). In short, after thawing, each sample was dried in the opened tube overnight in an incubator at approximately 35 °C and homogenized the next day using FastPrep-24^{TM} 5G. An aliquot of 0.05 g was vortexed with 1 ml of 80% methanol for 30 minutes. If less than 0.05 g were available, the amount of methanol was reduced and adapted accordingly. After a 15 min centrifugation at 2500 g, a 100 µl aliquot of the supernatant was pipetted off and transferred to a counter plate. For the analysis, we used an established 5α-pregnane-3β,11β,21-triol-20-one enzyme immunoassay to determine FCMs.

2.6 Statistical Analysis

All statistical analysis were performed with IMB SPSS Statistics (Version 28). To test how the different transfer methods affected the stress levels of the surrogate mothers over time, we ran a repeated measures ANOVA. To test whether the ET method had a differential effect on individual rises in stress levels, we ran an ANOVA and applied Tukey as post-hoc test.

3 Results

We found significant variations in FCM levels over the four sampling time points (day -1 to day 2)($F=15.6$, $p<0.001$, Fig. 6). The respective means and standard deviations (SD) of the four ET methods on each day are reported in Table 1. In all four methods, there was an increase in FCM levels on the day of ET (day 0) and a decrease to baseline levels one day after the transfer (day 1)(Fig. 6). The individual changes in FCM levels from baseline (day -1) to the day of ET (day 0) differed significantly between transfer methods ($F=3.4$, $p=0.03$, Fig. 7): Mice experiencing SET doubled on average their FCM levels and showed significantly stronger increases in FCM levels than mice from NSET/w ($p=0.018$) and NSET/o ($p=0.022$) group. The increase in individual FCM levels in the TCET group was marginally non-significant compared to the SET group ($p=0.058$). There was no difference in individual FCM level changes between the non-surgical methods (all $p>0.98$).

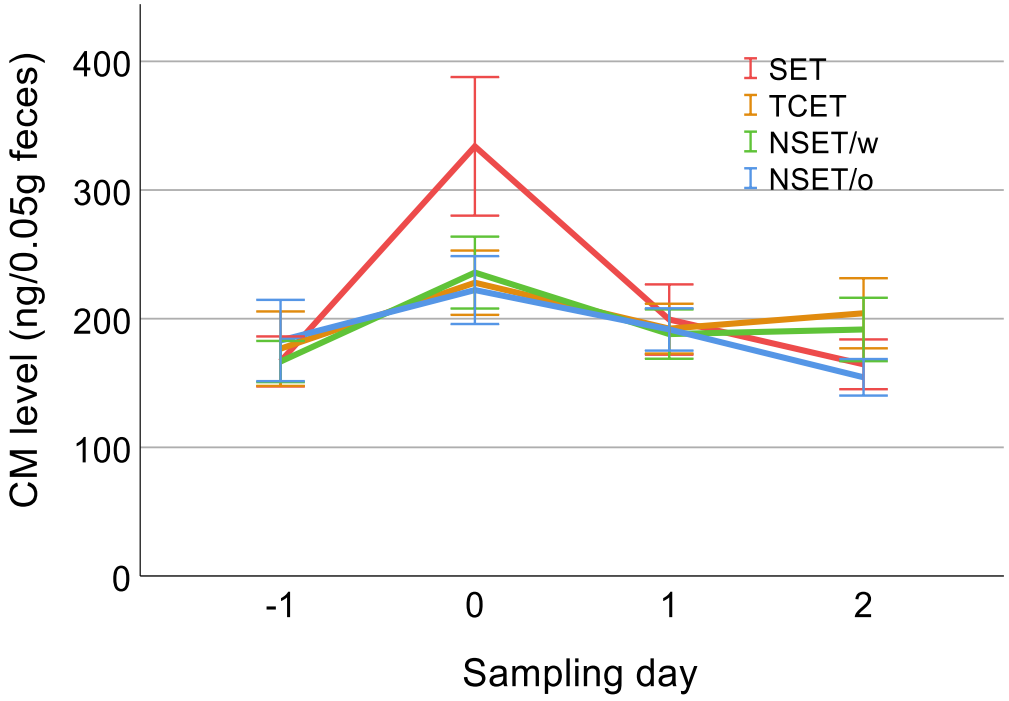

Fig. 6. Mean (± 1 SE) corticosterone metabolites (CM) levels in mice one day before embryo transfer (day -1), on the day of embryo transfer (day 0) and one (day 1) and two days (day 2) after the transfer depending on the transfer method. SET: surgical embryo transfer (red), TCET:

transcervical embryo transfer (orange), NSET/w: non-surgical embryo transfer with anesthesia (green), NSET/o: non-surgical embryo transfer without anesthesia (blue).

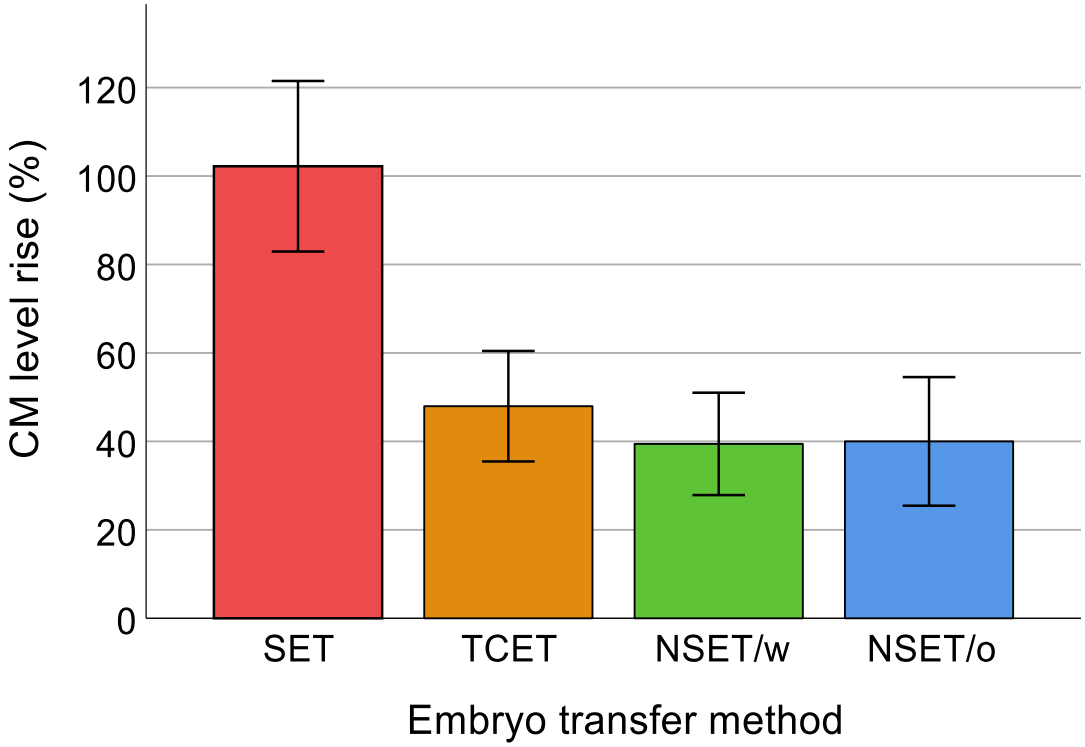

Fig. 7. Mean (± 1 SE) rise in individual corticosterone metabolites (CM) levels (%) from one day before the embryo transfer to the day of embryo transfer depending on the transfer method. SET: surgical embryo transfer, TCET: transcervical embryo transfer, NSET/w: non-surgical embryo transfer with anesthesia, NSET/o: non- surgical embryo transfer without anesthesia.

Table 1. Mean and standard deviation (SD) of fecal corticosterone metabolite levels (ng/0.05g feces) measured the day before embryo transfer (day -1), on the day of embryo transfer (day 0), one (day 1) and two days (day 2) after embryo transfer depending on the embryo transfer method.

	SET		**TCET**		**NSET/w**		**NSET/o**	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Day -1	166.8	93.2	176.7	129.4	166.7	75.0	183.1	144.7
Day 0	333.9	258.3	228.0	117.2	235.8	128,1	222.2	120.9
Day 1	199.3	128.1	192.3	90.7	188.0	89,9	191.6	77.0
Day 2	164.5	92.9	204.2	127.8	191.6	112.9	154.4	65.0

SET: surgical embryo transfer, TCET: transcervical embryo transfer, NSET/w: non-surgical embryo transfer with anesthesia, NSET/o: non- surgical embryo transfer without anesthesia

4 Discussion

Embryo transfer in laboratory mice is an important method in assisted reproduction. The most common method is the surgical ET. This method is invasive, relatively time consuming and demands a certain amount of technical expertise and postoperative care. In this study, I aimed to experimentally test the influence of this and three other non-surgical ET methods (TCET, NSET/w, NSET/o) on the stress level of recipient mice. By performing these methods while measuring FCMs of surrogate mothers. All mice had an approximately equal baseline CM level on the day before ET (Table 1). On the day of ET, FCM levels increased, and all animals returned to their baseline levels within a day after ET. Mice that experienced SET showed individual rises in FCM levels of more than 100%. Mice from the non-surgical groups, also showed increases in FCM levels, though, their rises were only half as much. Thus, all animals experienced stress related to the procedure, but the stress impact was highest for animals from the SET group. This is probably due to the severe physical trauma. The cutting of the skin, the opening of the abdominal cavity and the closure with metal clamps cause stress to the body. Due to the physical injury, the body releases more cortisol, which suppresses the immune system and acts anti-inflammatory. But trauma can also lead to a reduced ability to fight infections or a destructive inflammatory response (13). The body needed a short time to adapt to the new physical conditions, after which the stress hormones decreased again. Interestingly, stress levels in the SET group varied stronger than in the non-surgical groups. This suggests that the stress impact of SET is less predictable, as it might vary with the condition of the surrogate mother, as well as the duration of the procedure, eventually occurring complications during the procedure or during the recovery phase. To determine the impact of the injection anesthesia *per se*, a control group with injection anesthesia without ET needs to be included in future studies.

The stress levels of the three non-surgical groups on the day of ET showed no significant differences between each other, suggesting that the experienced stress levels for the animals were comparable. Thus, anesthesia has no detectable effect on the stress level, as there were no differences between the two NSET groups, or between the NSET/o and the TCET group. We assume that the advantage of the anesthesia might be overwritten by the required handling and manipulation of the mice to induce the anesthesia. Therefore, the effort, cost, and risk of the

anesthesia (e.g., more fluctuation in cardiac rhythm) (9) can be spared, since it has no measurable benefit in terms of stress reduction measured by FCM.

A question which came up during the discussion about the results is whether the anesthesia or possible gastrointestinal diseases could affect bowel emptying and thus excretion of FCMs. There is literature that says that bowel emptying should be unaffected after a short anesthetic, but prolonged anesthesia and the presence of diseases may delay it. As well as the use of opioids, which are the most common reason for delay after surgery (14). We assume that our anesthesia duration was so short that it had no effect on the emptying rate and thus delayed excretion of CMs. Also, there was no evidence of gastrointestinal diseases in our mice.

Mean baseline FCM levels ranged between 168 and 183 ng/0.05 g feces between transfer groups and were comparatively high in our study. Another study, which has been conducted at our institute found that young virgin ICR outbred females in the same age class had baseline levels of approx. 48–58 ng/0.05 g feces and 62–78 ng/0.05 g feces on the day of ET (15). Others also report baseline levels of approx. 28–70 ng/ 0.05 g feces in 10–13 weeks old C57BL/6 females (12). The collection time in the first study was from 5 to 9 p.m., in the second from 5 to 7 p.m. and in this study from 4:15 to 6:15 p.m. The baseline levels of mice in this study were higher (Table 1), even though we collected samples prior to the expected peak FCM excretion. FCM excretion levels can vary depending on the age and strain of the mice. C57BL/6 females have a constant excretion level in the range of 60–250 ng/0.05g feces when collected over one year, whereas Crl:CD1 mice increase FCM levels continuously from 80–200 to 130–500 ng/0.05 g feces over the same time course (16). In our study, animals were between 8 and 16 weeks old during data collection, thus we can exclude large time effects. However, strain specific differences in baseline FCM levels are known. C57BL/6 mice, for example, excrete higher amounts of FCM than BALB/c mice (17). We used hybrid mice (crossing between C57BL/6 and DBA/2), and these genetic differences might likely explain the observed differences in FCM levels.

Hormone secretions are affected by diurnal fluctuations. In addition, one must consider gut passage times when measuring FCM levels. Depending on the activity pattern of the animal, gut passage time will vary: During the light phase, i.e., during the resting phase of the animal,

peak excretion of FCM is expected to occur about ten hours after a stressful event, compared to only four hours when the event occurred during the dark phase, i.e., during the active period of the animal (11). In this study, ETs were performed during the resting phase of the mice and feces were collected approximately eight hours later at the onset of their active period. Thus, we assessed FCM levels slightly before peak excretions were expected and thus might even have underestimated actual stress levels.

We can conclude that NSET with and without anesthesia, as well as TCET have little difference in the influence on the stress level of the recipient animals. The results are not surprising, as they support our hypothesis that a less invasive ET exposes the animal to less pain and stress. Stress in mice, especially in the first days of pregnancy, can have fatal consequences on pregnancy rates and litter sizes, reducing them by up to half. Additional effects of stress on reproductive characteristics of pregnant mice include more animals with abnormal corpora lutea, fewer implantation sites per animal, and lower serum progesterone levels (ng/ml) than animals in the control group (18). Therefore, stress in a pregnant female should be avoided.

We do not report information on how the different ET methods affected pregnancy rates, litter sizes, and juvenile survival. However, the impact of these methods on reproduction is important when choosing a method. There have already been some studies performed on how specific embryo transfer methods effects reproductive outcome (6–8), which reveal that non-surgical methods are as effective as SET. The above studies uniformly argue why neither NSET nor TCET have replaced the surgical method. Potential explanations are the lack of experience in personnel with non-surgical ET devices, which could have had a negative impact on the success rates. However, success rates can evidently improve, as the devices itself have shown improvement. As mentioned in the introduction, non-surgical devices only allow the transfer of morulae or blastocyst stage embryos, thereby limiting its area of use.

In summary, we showed that the use of non-surgical ET methods is less stressful for recipient females and is thus consistent with the concept of 3Rs (Replacement, Reduction, Refinement). Using non-surgical methods can refine ET without lowering success rates. Since the three non-surgical ET methods are also generally faster, and easier to learn and perform, many laboratories could establish non-surgical ETs and positively impact the life of many surrogate mothers.

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