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Repeated Use of Surrogate Mothers for Embryo Transfer in the Mouse

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ABSTRACT

Embryo transfer in mice is a crucial technique for generation of transgenic animals, rederivation of contaminated lines, and revitalization of cryopreserved strains, and it is a key component of assisted reproduction techniques. It is common practice to use females only once as surrogate mothers. However, their reuse for a second embryo transfer could provide hygienic and economic advantages and conform to the concept of the 3Rs (replace, reduce, refine). This investigation evaluated the potential for a second embryo transfer in terms of feasibility, reproductive results, and experimental burden for the animal. Virgin female ICR mice (age 8–16 wk) were used as recipients for the first embryo transfer. Immediately after weaning of the first litter, a second surgical embryo transfer was performed into the same oviduct. Virgin females of comparable age to the reused mothers served as controls and underwent the same procedure. The first surgery did not affect the success of the second embryo transfer. Histological sections showed excellent wound healing without relevant impairment of involved tissues. We observed no differences in pregnancy rates or litter sizes between the transfer groups. Most importantly, we found no change in behavior indicating reduced well-being and no increase of corticosterone metabolites in the feces of surrogate mothers reused for a second embryo transfer. We conclude that a second embryo transfer in mice is feasible with regard to reproductive and animal welfare aspects.

embryo transfer, mouse, repeated use, surrogate mother

INTRODUCTION

Since the initial reports by Beatty [1] and by McLaren and Michie [2], embryo transfer in laboratory mice has been an indispensible technique with multiple applications in biomedical research. It is pivotal for the generation of transgenic mice [3, 4] and is an important part of assisted reproduction techniques to overcome fertility problems in mutants. Rederivation of contaminated mouse strains by sterile embryo transfer is crucial to get rid of pathogens [5]. The cryopreservation of gametes and embryos is only useful with embryo transfer to revitalize the archived strains [6]. Cryopreservation also allows shipment of embryos instead of live mice, thus addressing animal welfare concerns. Additionally, embryo transfer is used to accelerate the production of congenic strains by superovulation of juvenile females or use of male first-wave germ cells [7, 8].

Embryo transfer is feasible with all preimplantation stages of embryos and is usually conducted as a surgical procedure. In addition to the well-known protocols for oviduct and uterus transfer published by Nagy et al. [9], some variations have been described, such as uterine transfer via the uterotubal junction [10] or puncture of the oviductal wall [11, 12]. In all these protocols, recipients were used only once. However, reusing a surrogate mother for a second embryo transfer has many potential advantages. Obviously, it could reduce the number of animals required. Also, because experimental facilities do usually not breed their own recipient colony and depend on external supply, a risk of pathogen introduction exists even if the animals come from a reliable source. Reusing surrogate mothers reduces this risk by lessening animal imports. Reuse will also save space in the animal facility by reducing the number of cages for adaptation and storage of newly received females, thereby saving costs in terms of both animals and animal housing. Furthermore, better reproductive performance could be expected from reused surrogate mothers that have already successfully raised a litter.

The recently revised European Directive on the protection of animals used for scientific purposes states in Article 16 that animals can be reused in a new procedure provided (among other conditions) that the first and second procedures are of only mild or moderate severity [13]. The severity of surgical embryo transfer is classified as moderate, and repeated use of surrogate mothers is thus de jure allowed. However, unknown impairments and increased suffering may arise from repeating the same procedure at the same site. These possibilities need to be systematically addressed to make ethical decisions and to evaluate the reuse of surrogate mother in terms of the 3Rs [14]. The aim of the present study was therefore to evaluate the practical feasibility, pregnancy rate and litter size, and potential for involved females to experience pain, suffering, or distress with a second embryo transfer.

MATERIALS AND METHODS

Female ICR mice of SOPF (specific and opportunistic pathogen free) quality were bred in our facility according to a Robertson rotation system. Animals were housed in Macrolon cages under standard laboratory conditions (room temperature, 21 ± 1°C [mean ± SEM]; relative humidity, 40%–55%; photoperiod, 12L:12D) and supplied with a standard breeding diet (V1126; Ssniff Spezialitäten GmbH) and tap water ad libitum. The present study was discussed and approved by the institutional ethics committee of the University of Veterinary Medicine, Vienna, and animal experiment license was granted under BMWF-68.205/0084-II/10b/2008 (Austrian Federal Ministry of Science and Research). B6D2F1 hybrid mice were purchased from Charles River Laboratories and used as embryo donors. Hybrid females were superovulated at 8 wk of age by i.p. injection with 5.0 IU of equine chorionic gonadotropin (Folligon; Intervet) and, 48 h later, with 5.0 IU of human chorionic gonadotropin (Chorulon; Intervet) and then mated with B6D2F1 males. At

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TABLE 1. Experimental groups and results of reproductive performance.

Experimental group	Females used (n)	Age (wk)	Pregnancy rate (%)	Litter size (pups)	Mating days until vaginal plug detected
1A	22	8–16	95.7	9.1 ± 2.96	–
1B	16	16–21	95.5	8.3 ± 3.47	–
2	22	16–21	87.5	7.5 ± 2.77	–
3	20	8–16	–	–	2.4 ± 0.8
4	18	16–21	–	–	6.7 ± 2.2*

* $P < 0.001$.

1.5 days postcoitus (dpc), donors were killed by cervical dislocation to isolate both oviducts. Two-cell embryos were flushed and stored on a warming plate using M2 medium.

Embryo Transfer

Pseudopregnant ICR recipients were produced by mating with vasectomized ICR males and identified by vaginal plug control (group 1A) (Table 1). For the second embryo transfer, group 1A females were mated to sterile males immediately after weaning of their first litter (group 1B). To evaluate the effect of the advanced age of reused surrogate mothers, we accomplished in parallel embryo transfer in age-matched virgin females (group 2).

Embryo transfer has been described earlier in detail [15, 16]. Briefly, anesthesia in 0.5-dpc pseudopregnant females was applied by i.p. injection of ketamine/xylazine (10 mg/100 g body wt of ketamine [Ketasol; Graeub Veterinary Products] and 0.4 mg/100 g body wt of xylazine [Rompun; Graeub Veterinary Products]). Eyes were covered with eye ointment (Oleovit; Fresenius Kabi) to prevent them from drying. An incision of the unshaved skin and the peritoneum was made on the right side near the ovary to pull out the reproductive tract. The ovarian bursa, a transparent tissue membrane that covers the ovary, was ruptured, and twelve 2-cell-stage hybrid embryos were transferred via the ovarian infundibulum into the ipsilateral ampulla of the uterine tube. Then, the reproductive tract was gently placed back into the abdominal cavity, the peritoneum sutured, and the skin closed with a Michel clamp. The whole procedure was conducted on a warmed table of a laminar flow hood, and the recipient females were placed in their cage after awakening. Analgesia, routinely implemented by s.c. injection of meloxicam (0.05 mg/100 g body wt; Metacam; Boehringer Ingelheim), was not applied in the present study, because it might change the postsurgical burden and mask the measurement of distressing effects. All embryo transfers were conducted by the same experienced person (T.K.). To avoid further stress for the animals, we generally do not remove the Michel clamp during pregnancy and lactation. Remaining clamps in reused females were removed during anesthesia of the second surgery. Embryos were transferred unilaterally into the right oviduct, which is routine in our lab. Reproductive performance of the treatment groups was recorded for pregnancy rate and litter size at birth.

Pseudopregnancy

To assess the impact of the first surgery and of the advanced age of females to be used as surrogate mothers, we also investigated the efficacy of inducing pseudopregnancy. However, to be independent from the preparation of embryo donors, we used another set of animals without embryo transfers. To mirror experimental groups 1A and 1B, younger virgin ICR females at 8–16 wk of age (group 3) and surrogate mothers after weaning of their first litter at 16–21 wk (group 4), respectively, were permanently mated to vasectomized males and checked daily for a vaginal plug (Table 1). Before mating, animals of this part of the present study were maintained only in pairs to avoid the Lee-Boot effect possibly affecting females in large groups [17]. The number of days necessary until positive plug check was recorded for both treatment groups.

Stress Evaluation by Noninvasive Corticosterone Measurement

Corticosterone metabolite concentrations were measured in the feces of the animals as a noninvasive approach to assess the levels of stress that might result from repetition of the surgery necessary for embryo transfer [18]. In all experimental females, voided feces were collected individually three times daily (0900, 1700, and 2100 h). To get baseline values, we started with the collection 1 day before mating to the vasectomized male. Embryo transfer was conducted in the morning, and feces were collected starting 1700 h of that day and continued for the next 2 days (see Fig. 2). During this period, the mice were housed on cotton sheets instead of wood bedding as described previously [19] with minor modifications. Samples were frozen (−20°C) and analyzed using a 5α-pregnane-3β,11β,21-triol-20-one enzyme immunoassay as described in detail by Touma et al. [20, 21].

Behavioral Screening

To assess any distress or discomfort due to the embryo transfer, females were also monitored for changes in their behavior. Embryo transfers took place in the morning at approximately 0900 h. Behavior was observed hourly starting after the embryo transfer at 1000 until 1500 h and on the following day at 0900 and 1100 h. Several parameters were evaluated postoperation for the mice using score sheets: grooming, body posture when awake and during sleep, locomotion, activity, food intake (food was offered in a Petri dish on the floor to facilitate intake), and nest building. Behavior was assessed in comparison to the behavior of untreated mice of the ICR breeding colony (referred to as normal) of corresponding age and sex by experienced animal technicians who did not know the treatment group of the mice. Normal behavior and appearance were noted with zero points, and obvious changes in behavior and appearance were noted with one point for each of the listed parameters. Points were summarized and compared between experimental groups.

Histology

From each transfer group, six surrogate mothers were killed after weaning of their litter. The Michel clamp was removed, and a 1-cm^2 piece of skin containing the wound from the surgery was dissected. A corresponding piece of peritoneum was isolated from the site of surgery. Additionally, the ipsilateral and contralateral ovary and oviduct were collected. All tissue samples were fixed in 4% formaldehyde solution for 48 h and then embedded in paraffin (Histocomp; Vogel) using automated embedding equipment (Shandon Excelsior; Thermo Scientific). Paraffin sections (thickness, 3 μm) were cut crosswise to the direction of the incisions and stained with hematoxylin and eosin for routine morphological examination. Descriptive and comparative analysis of the sections was performed under light microscopy, taking into consideration inflammatory responses, integrity of treated tissues and organs, and scarring.

Statistics

Pregnancy rate and plugging rate were compared by chi-square test. Litter size and scoring points were compared by ANOVA. Concentrations of corticosterone metabolites were compared in a linear model by ANOVA between the same time points among the groups.

RESULTS

Parameters of Reproduction

In all embryo transfers, pseudopregnancy was confirmed by observation of a swollen ampulla. The second embryo transfer in reused recipients was not any more difficult compared to the procedure in virgin females. No adhesions or scars of the ovarian fat pad, the ovarian bursa, or other tissues were found, and the ovarian infundibulum was easily accessible for the second transfer. The embryo transfers with young or old virgin females (groups 1A and 2) resulted in pregnancy rates of 95.7% and 95.5%, respectively. After the second embryo transfer (group 1B), 87.5% of surrogate mothers became pregnant. The difference of approximately 8% was not significant (chi-square test). Moreover, we observed no

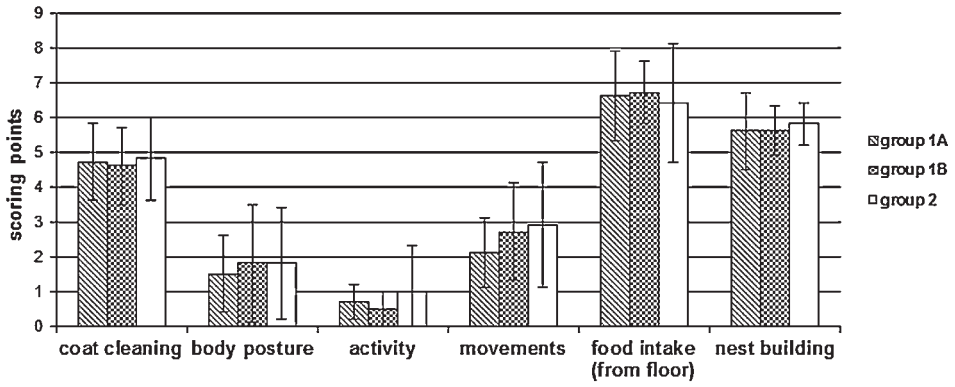

FIG. 1. Behavior scoring after embryo transfer. Values are presented as the mean ± SEM. Group 1A, virgin young recipients; group 1B, reused old recipients; group 2, virgin old recipients.

significant differences regarding litter size (mean ± SEM) among all groups. However, the time to become pseudopregnant differed significantly between young virgin and aged, reused females, and the latter had a highly variable rate of plugging. Results of all treatment groups, including days to induce pseudopregnancy, are summarized in Table 1.

Behavior

Recovery in terms of behavior of the treated females was completed in all three groups within 3 h (Fig. 1). Coat was clean and dry, and body posture and movements were regular. Mice showed normal activity, and food consumption was observed from the cage lid and from the floor. On the following morning, all females had built a nest from the offered nesting material. Scoring points for welfare assessment were compared by ANOVA and did not reveal significant differences among the groups (Fig. 1).

Stress Hormone Metabolites

The monitoring of corticosterone metabolites in the feces allows noninvasive assessment of postexperimental pain and suffering. The mice generally exhibited a strong individual variation of the measured values. In the interval before mating and embryo transfer, older mice (groups 1B and 2) showed a more pronounced diurnal fluctuation compared to the young recipients (Fig. 2). These diurnal fluctuations were less distinct after embryo transfer for the group of young recipients (group 1A) and for the same mice after the second embryo transfer (group 1B). Only the old virgin recipients (group 2) showed an enhanced corticosterone metabolite excretion pattern the day after embryo transfer. However, the measurements generally provided no indication of strong and sustained pain or suffering after the first or the second embryo transfer (ANOVA) (Fig. 2).

Histology

Crosscutting and staining of the peritoneum did not reveal any obvious scar tissue (Fig. 3). The skin was sometimes deformed by the Michel clamp, but all tissue layers were reconstructed in all investigated probes (Fig. 4). Histological examination of ovaries and oviducts revealed that the bursa of the treated ovary had not reclosed after the embryo transfer(s). However, no adhesions that might impede a second embryo transfer were visible on treated organs (Fig. 5).

DISCUSSION

Embryo transfer in mice is one of the most important methods of assisted reproduction and is used for several applications in biomedical research. The procedure prescribes

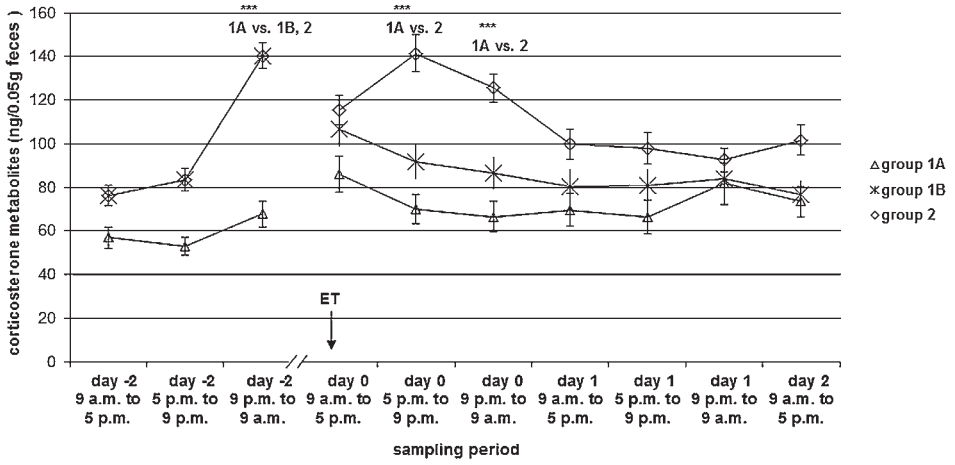

FIG. 2. Noninvasive stress monitoring via corticosterone metabolite concentrations. Embryo transfer was conducted at 0900 h on Day 0. Values are presented as the mean ± SEM. Group 1A, virgin young recipients; group 1B, reused old recipients; group 2, virgin old recipients. ***$P < 0.001$, ANOVA.

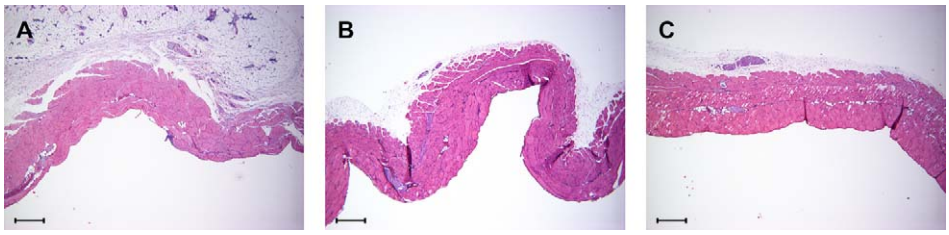

FIG. 3. Hematoxylin and eosin-stained peritoneum crosscut to the surgical cut. **A**) Group 1A, virgin young recipients. **B**) Group 1B, reused old recipients. **C**) Group 2, virgin old recipients. Bar = 500 μm.

that surrogate mothers are only used once for that purpose. To investigate the feasibility of a second embryo transfer, we reused recipients immediately after weaning of their first litter. In doing so, we not only assessed the results of the embryo transfers with regard to the pregnancy rate and the litter size but also the degree of possible impairment (histology) and symptoms of suffering (behavior and stress hormone metabolites) after the first and second surgeries.

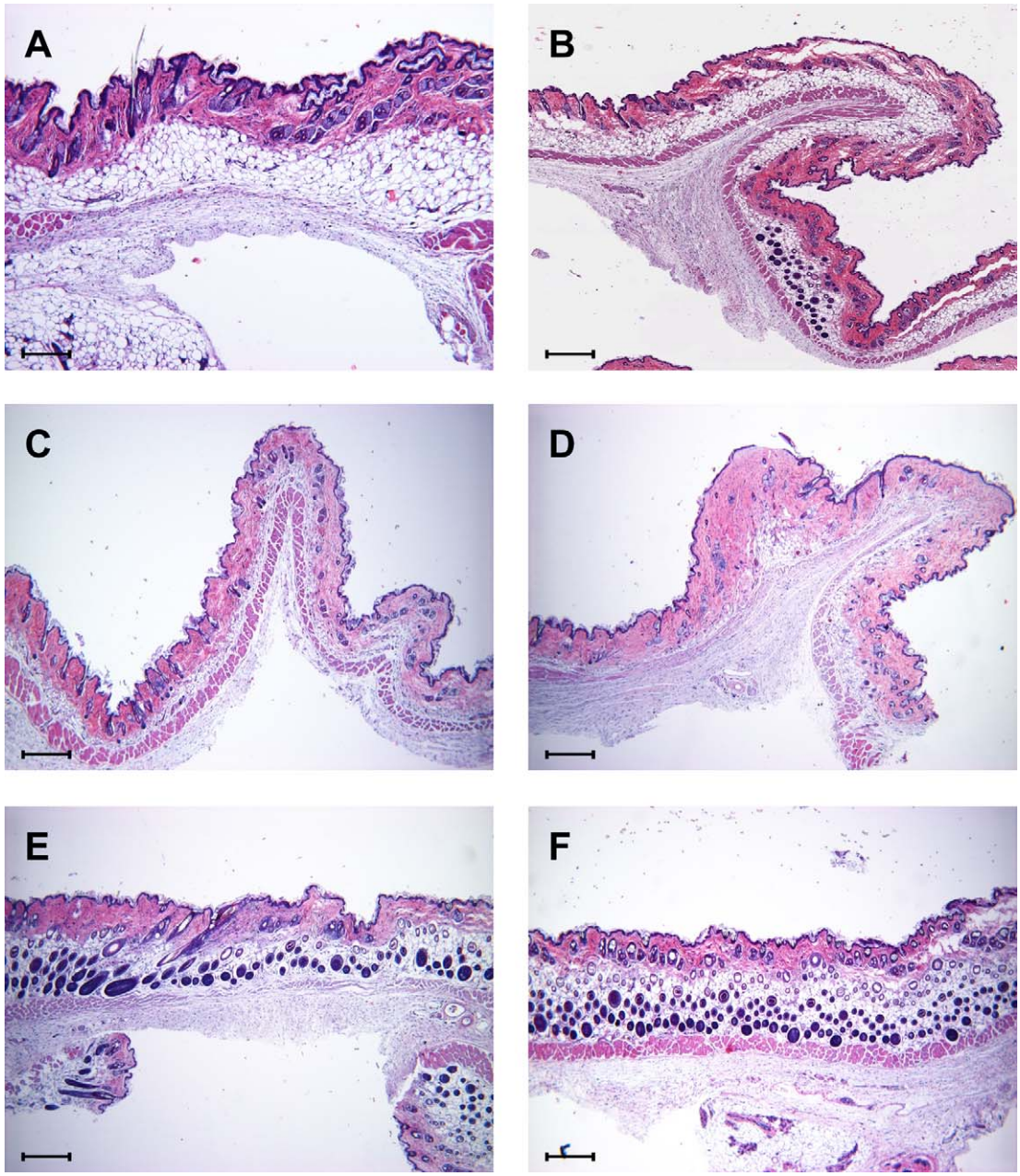

FIG. 4. Hematoxylin and eosin-stained skin crosscut to the surgical cut. **A** and **B**) Group 1A, virgin young recipients. **C** and **D**) Group 1B, reused old recipients. **E** and **F**) Group 2, virgin old recipients. Bar = 500 μm.

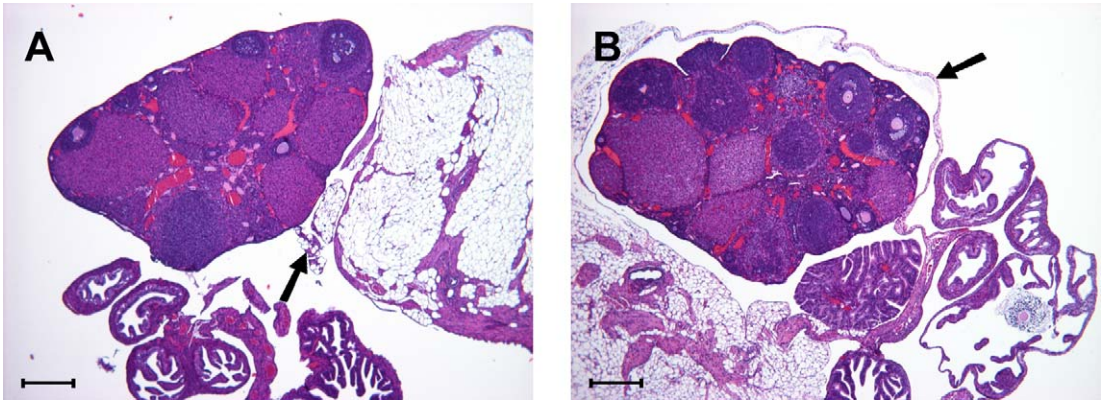

FIG. 5. Hematoxylin and eosin-stained ovary and oviduct from group 1B (reused old recipients). **A**) Treated ovary and oviduct. Arrow indicates remnants of bursa. **B**) Untreated ovary and oviduct. Arrow indicates intact bursa. Bar = 500 μm.

Most importantly, the procedure of a second oviduct transfer is possible without any technical problems as a result of the first surgery. The histology of the ovary and oviducts only showed remnants of the opened ovarian bursa, and the fimbriated end of the intact uterine tube was always easily accessible. Pregnancy rates and litter size after embryo transfer in reused surrogate mothers was comparable to both young and age-matched virgin females.

Histological examination 6 wk after the first surgery did not reveal the site of the first incision of the peritoneum (Fig. 3). The skin was unavoidably deformed around the wound by the Michel clamp, but wound healing was completed without obvious scar formation (Fig. 4).

Because the second embryo transfer was also performed into the right oviduct, the same tissue areas were affected by the surgery. To measure the unbiased impact of the second procedure on the well-being of the animals, no analgesia was used in all treatments. The behavioral scoring after embryo transfer did not reveal indications for pain and distress in any treatment group. These results are supported by the noninvasive measurement of fecal corticosterone metabolites.

The method of noninvasive stress measurement has been successfully validated and applied several times in mice [18–22]. In accordance with the results of Touma et al. [23], a clear diurnal rhythm of corticosterone metabolite levels was observed, but in the present study, a strong variation of individual measurements was also observed in all groups. Consequently, the mean values calculated within each group and per measuring point showed a relatively large SEM (Fig. 2). To assess the impact of the embryo transfer, pre- and posttreatment values of corticosterone metabolite levels were compared among the groups. No postsurgery increase in corticosterone metabolites was observed in either the first or the second embryo transfer, suggesting no strong or sustained burden for the animals. Comparison of corticosterone metabolite levels between the first and the second embryo transfer (groups 1A and 1B) showed no differences. A significant increase of stress hormone concentrations after surgery was seen exclusively in group 2 (i.e., aged surrogate mothers used for the first time) (Fig. 2). The reason for this change of corticosterone metabolite levels is unknown but is beyond the scope of the present study. The important finding here is that a second embryo transfer did not result in increased concentrations of corticosterone metabolites as a indication for elevated distress.

To summarize, our results demonstrate the feasibility of reusing surrogate mothers for a second embryo transfer. Neither the practical procedure nor the outcome is significantly different from that in virgin females. Moreover, no obvious indications of welfare problems due to hyperalgesia during the second embryo transfer were observed. Although we have not examined the feasibility of performing a third embryo transfer, our data suggest that a second reuse of surrogate mothers is a technical possibility. However, due to the advanced age of the animals after weaning of the second litter and the possibility of pronounced difficulties with inducing pseudopregnancy in multiply reused females, we do not suggest the use of females for a third embryo transfer.

By reusing surrogate mothers, it is possible to avoid importing additional females into the animal facility and, therefore, to save the time and space for their appropriate adaptation to the new environment. A potential disadvantage to reusing surrogate mothers is the unpredictable rate of plugging. This could complicate experimental planning, and some of the projected savings may be offset by having to mate more females to obtain the required number of pseudopregnant females. Further research is needed to find out if this problem can be solved by a short recovery phase after weaning of the first litter, by selection for mice in proestrus by vaginal smear or the appearance of the vagina [24], and by use of the Whitten effect [25] to stimulate follicle growth.

The use of surrogate mothers for diagnostic testing is not appropriate in open-cage animal husbandry but is a common procedure in facilities equipped with individual ventilated cages. If these animals can be reused as embryo recipients, another approach to investigate the hygienic quality of the offspring is desirable. Eliminated littermates with inappropriate genotype can be used instead of the surrogate mother for diagnostic purposes. However, researchers should be aware that exposure of newborn mice could induce neonatal tolerance to some viruses and may cause false-negative results [26].

Embryo transfer is an important method for several applications in transgenesis and assisted reproduction. Therefore, reusing surrogate mothers for a second transfer will reduce substantially the number of animals used in these research areas.

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