1 **1. Title page.**

2 **OPTIMIZATION OF THE BALANCE BETWEEN EFFORT AND YIELD IN UNILATERAL**
3 **SURGICAL TRANSFER OF MOUSE EMBRYOS**

4 **KEYWORDS:** Surgical transfer, mouse embryos, reduction, optimization, effort/yield

5 Pablo González-Jara, Tomás Fontela, Esther López-Mimbela, Marta Cereceda, Daniel Del
6 Olmo and Manuel Moreno

7 Animal Facility, Centro de Investigaciones Biológicas, Consejo Superior de Investigaciones
8 Científicas (CIB-CSIC), Madrid, Spain

9

10 Correspondence should be addressed to Pablo González-Jara

11 Postal address: Centro de Investigaciones Biológicas (CIB-CSIC)

12 Ramiro de Maeztu 9, 28040-Madrid

13 Spain

14 Email: pablogjara@cib.csic.es

15

16

17

18 **Short Title: Optimizing surgical transfer of mouse embryos**

2. Abstract

Surgical transfer of embryos is carried out daily in animal facilities worldwide for the rederivation of mouse strains/lines, among other purposes. Current protocols described in laboratory manuals recommend to perform mouse embryo transfer using a high number of embryos, typically in the range of 15 up to 25. To optimize the use of resources it is necessary to estimate and relate the effort required and the yield obtained. Here, we analyse the balance between the number of embryos transferred (effort) and the yield, as the number of born pups obtained from surgical embryo transfer. To do this, we have analyzed data obtained during rederivation of near a hundred lines of mice to a new animal facility. Our results confirm that the use of increasing numbers of embryos per transfer increases the yields of born pups, as was described previously in the literature, but they also highlight the disproportionated effort required, i.e., in the number of embryos that needed to be transferred. Estimate of the mean expected yields of surgical transfers and their comparison with the actual observed yields indicated that the balance between effort and yield is optimized when using lower numbers of embryos than in currently used protocols, in the range of 8-12. Given the heterogeneous nature of the data presented and analyzed here, which come from a population of mice that may be considered as representative of any animal facility, our optimization approach could help save resources in similar facilities and improve the yields of embryo transfer procedures.

3. Introduction

Surgical transfer of mouse embryos to the oviduct or uterine horn of a pseudo-pregnant mouse is a commonly used technique that has proven to be useful in a number of applications in animal facilities worldwide, such as the production of transgenic mice and of specific pathogen free (SPF) animals by rederivation.¹⁻³ Typically, with this technique a variable number of embryos from a donor strain/line are transferred to a pseudo-pregnant recipient female housed under SPF conditions. As a result, the number of embryos produced and handled for embryo transfer in a conventional facility may be very large. Apart from the number of animals sacrificed as embryo donors, the technique requires a considerable investment in time and economic resources, and diverse technical skills. Good examples of these are superovulation through injection of gonadotrophins in donor females to improve embryo production, the maintenance of the stock of vasectomized males that will be used to induce pseudo-pregnancy of foster mothers, or the need to ensure proper synchronization between the oestrous cycles of the recipient females and the donor counterparts.⁴⁻⁶ Tuning of all these processes is crucial to optimize resource investment in day-to-day embryo transfer procedures.

One of the factors that is pertinent to the efficient production of live offspring in an animal facility through routine embryo transfer is the number of embryos that need to be transferred in these procedures. It was shown previously that the yield of a transfer, i.e. the number of pups in the offspring, increases proportionally with the number of fertilized eggs⁷ or embryos transferred.⁸ With the intention of reaching the maximal uterine capacity of the foster mother it is thus usual to use large numbers of embryos in a transfer, typically in the range of 15 up to 25.^{5,8-12} However, the use of such a high number of embryos may increase the effort, considered here as the number of embryos invested per transfer to obtain a single pup. In this work we have revisited this issue with the aim of fine tuning and improving the number of embryos that need to

be transferred by conventional unilateral surgery, using the experience gathered in our facility. With this goal we have analyzed data accumulated during the rederivation of 93 lines of mice to a new facility during a period of ten months, in the years 2013-2014. Analysis of data from transfers that used between 5 and 25 embryos per transfer focused on finding the optimal balance between the effort provided and the yield obtained. Our analyses show that this balance was optimal using lower numbers of embryos than those that are currently used. Data presented and analyzed here come from the heterogeneous population of mice housed in our facility, which may be considered as representative of any other animal facility. Therefore, we think our approach and our findings can help to improve the effectiveness of surgical embryo transfer procedures in similar facilities.

4. Materials and methods

Animals

The CIB animal facility is a state-of-the-art installation (<http://www.cib.csic.es/facilities/scientific-facilities/animalarium>). Human care and use of mice in the rederivation programme in this facility were approved by the relevant local ethics committees for animal experimentation, and procedures were carried out in accordance with European Union guidelines and Spanish legislation. The set of donor lines to be rederived consisted of a diversity of genetically modified mice from various sources, generated on a C57BL/6 genetic background. Lines are owned by the different CIB scientists that use the facility. All lines have in common their C57BL/6 genetic background and they differ only in the transgenic construction they carry. SPF CrI:CD-1 (ICR) mice first purchased from Charles River, France, and then generated in-house were used as either recipient females or vasectomized males. The microbiological status was evaluated for the list of agents recommended by the Federation of European Laboratory Animal Science

Associations¹³ and included in the mouse FELASA complete PRIA panel in CRL Diagnostic Services (Wilmington, MA, USA) on a sample from the purchased males and females. Afterwards a standard program based on sentinel screening, 2 mice housed per cage, one cage per single rack, were performed quarterly (FELASA basic PRIA panel) and yearly (FELASA complete PRIA panel), respectively. From the beginning to the end of the rederivation program all sampled animals, whether purchased or sentinels tested negative. A diurnal rhythm was maintained with a 12/12 h light-dark cycle and with artificial light from 07:00 h. Animals were housed at 21-22°C and relative humidity at 50±10%. Mice were fed with a standard autoclavable mouse diet supplied *ad libitum* (D40, SAFE, Augy, France) and with sterilized tap water. Vasectomized and breeding males were individually caged. Females were housed in type 3 cages (Tecniplast, Italy) in groups of 8-10 animals from weaning until they were coupled with males. Cages were provided with sterilized Lignocell Select Fine (Rettenmaier Iberica, Barcelona, Spain) bedding material and environmental enrichment, according to standard welfare recommendations.

Embryo transfer

Collection of embryos and their surgical transfer were performed according to standard procedures.¹⁰ Superovulation was induced in 4-5 week-old donor females by i.p. injection of 5 IU PMSG (Sigma-Aldrich, San Luis, MO, USA), followed 47 hours later by 5 IU hCG (Sigma-Aldrich, San Luis, MO, USA). Female mice were immediately single mated with the breeding males. The presence of vaginal plug was determined the next morning prior to 10:00 h. Females with a vaginal plug were sacrificed 1.5 days post-coitum, oviducts were isolated and embryos were obtained by flushing the oviducts with 100 µl of PBS + 0.5% BSA buffer kept at 37°C. The morphological qualities of the two-cell stage embryos to be transferred was routinely

assessed by visual inspection under binocular lens (Leica M80, Wetzlar, Germany) with a magnification of 60x based on the experience of qualified technical staff of the animal facility. After 10 washes, embryos were surgically transferred to pseudo-pregnant females. For that purpose, 6-8 week-old nulliparous CD-1 recipients mated the previous night with vasectomized males were used. Transfers were planned according to the availability of embryos. Embryos (from a minimum number of 5 and a maximum of 25 per transfer) were transferred unilaterally to the left oviduct through the infundibulum. Surgery procedures were performed specifically by two highly skilled technicians of the animal facility team. Both of them are well-trained and get comparable results performing embryo transfer. Surgery was performed after induction of anaesthesia with 5% isoflurane (Abbvie Farmacética, SL., Madrid, Spain) in O₂ and 2% for maintenance. Mice eyes were protected with an ophthalmic lubricating ointment (Lipolac, Angelini Pharmaceutical, Barcelona, Spain) to prevent corneal drying and damage during surgery. Each surgery lasted typically 10-15 minutes. Before recovery from anaesthesia animals were treated with 100 µl buprenorphine (0.1mg/kg) (Buprex, Pharmaceuticals Limited, Berkshire, UK) by i.p. injection and transferred to a peaceful environment for 30 minutes. Afterwards housing in standard conditions was maintained until offspring birth.

Statistical analyses

All the analyses were performed by grouping the data into five categories, according to the number of embryos employed for each single embryo transfer (1st: 5-8 embryos; 2nd: 9-12; 3rd: 13-16; 4th: 17-20; 5th: 20-25). Grouping the data increased sample size within categories and corrected for potential bias due to heterogeneity of data. Pregnancy rates among categories were compared by the Chi-Square test of association. Subsequent analyses of birth rates, and effort-yield comparisons were carried out after removing the incidence of cannibalism and births

by caesarean section from the pool of data. Comparison of yield (number of pups born per each single transfer) and effort (number of embryos invested per transfer to obtain each single pup) among categories was performed using both ANOVA and the non-parametric Kruskal-Wallis tests. Although raw data within two out of the five categories did not meet the criteria of normality and homoscedasticity, similar results were essentially obtained, independently of the test used. *Post-hoc* pairwise by the Least Significant Difference (LSD) method was used to compare yield and effort from each category with the remaining categories. In addition, to measure the linear dependence between effort and yield the Pearson correlation coefficient was used. To estimate expected yields the ratio between the mean value of embryos used for a transfer in each category and that of the first category was first obtained. Then, the mean expected yield was estimated as the product of multiplying this ratio by the mean observed yield in the 1st category. Comparison of the observed yield values with the estimated expected value for each category was performed using the Student *t*-test against the null hypothesis of equal means. Statistical analyses were performed using STATA v14.0 (StataCorp, TX, USA).

5. Results

The optimal number of mouse embryos to be used in day-to-day unilateral surgical transfers was inferred from the analysis of the set of 391 transfers performed in our facility during rederivation of 93 transgenic lines. On average 4.21 ± 0.25 (mean $\pm$ SEM) transfers per line were carried out. The set of transfers rendered on average 85.2% gestations (**Table 1**). Pregnancy rates were similar among the five categories of transfers ($\chi^2_{(4)} = 0.52$; $P = 0.97$). These rates were comparable to those reported by other authors for unilaterally ovariectomized females in similar conditions⁷⁻⁸ and showed, as previously described, no changes in efficiency

associated with increasing the number of embryos used per transfer. In our surgical conditions no implantation-related fatalities, i.e., foster mothers dying after transfer, were found.

Births assisted by caesarean section affected 1.2% of gestations, and incidence of cannibalism postpartum affected 3.9% of the litters. Absolute numbers of embryos transferred, live pups born and their associated success rates according to five categories are shown in **Table 2**, after removing births by caesarean section and cannibalism-related incidences from the data set of pregnancies. It is worth noting the significant decline of success rates as the number of embryos transferred increase, from 1st to the 5th category, as determined by the Spearman rank order correlation coefficient ($r_s = -1$, $P < 0.05$). Mean values of effort, and mean observed and expected yields are summarized in **Table 3**. Mean observed yields significantly increased as the number of embryos transferred increased (3.57 ± 0.40 in category 1st to 7.43 ± 0.61 in category 5th) (mean $\pm$ SEM). Similarly, mean effort values increased from the 1st category onwards (2.32 ± 0.29 to 3.77 ± 0.39) (mean $\pm$ SEM), the correlation between yield and effort being strongly positive ($R^2 = 0.91$; $P = 0.011$). Comparisons performed using a Kruskal-Wallis test showed significant differences in yield among categories ($\chi^2_{(4)} = 16.20$; $P = 0.003$). A similar result was obtained when effort was analyzed ($\chi^2_{(4)} = 24.50$; $P < 10^{-3}$). *Post-hoc* pairwise LSD comparisons for each of the two variables between the values of each category and the remaining categories indicated significant differences, with the exception of those comparisons that were performed between contiguous categories (data not shown). When observed yield values were compared with its estimated mean expected yield within each category under the null hypothesis of equal means, significant decreases ($t = -2.13$; $P \leq 0.035$) were found from the 2nd category onwards.

6. Discussion

Our analyses clearly show that increasing the number of embryos used for a transfer entailed not just an increase in the yield of live born pups, as already shown,⁸ but also a disproportionate larger increase in the effort made, represented by the number of embryos invested to obtain a single pup (see the percentage of observed *versus* expected yield reduction in last row of Table 3). It is interesting to note that a similar result to this one shown by us was observed for unilateral transfers performed with high numbers of embryos (15 and 25) when mean yield data in table 2 from the paper by Johnson *et al.*⁸ were used to estimate expected yields. Since the experiments were performed using the more productive strain ICR both as a donor and receptor of embryos it seems a general conclusion that for resource optimization the use of a lower numbers of embryos than those commonly used is recommended in unilateral surgical transfer. The idea that surgical transfer should be performed using a high number of embryos comes from works published a couple of decades ago when the technique was routinely performed with up to 25 in the case of unmanipulated embryos.⁹ Even though more recent protocols advise to use smaller numbers of embryos per transfer, ≈ 15 ,¹⁰ the idea of using high embryo numbers is widespread⁵. The results presented here demonstrate that the reduction of the number of embryos used in unilateral embryo transfers improve resource investment.

Current protocols claim that more than 50-75% of unmanipulated embryos should develop to normal fetuses when transferred into the oviducts,⁹⁻¹⁰ although this claim seems to apply only to transfers performed with small numbers of fertilized eggs/embryos (see⁷⁻⁸) and unilaterally. Our success rates were lower than the described range limits. Differences in success rates may be related, among other factors, to the genetic background of the strains employed as donor and receptor and its compatibility interactions. In our case the donor lines were a heterogeneous set of transgenic lines engineered in a C57BL/6 genetic background while the recipient females were CD-1. To rule out that the births rates of some transgenic lines when they are rederived

could be lower than that in natural births, we compared litter size of natural births with those obtained after rederivation in a subset of 24 of the transgenic lines used in this study, and no correlation was found (data not shown). In our facility, natural births from wild type C57BL/6J give on average litters of 7.16 ± 1.28 (mean $\pm$ SD) pups against 13.16 ± 2.13 (mean $\pm$ SD) in the case of CD-1. Taken into account a 75% success rate, a transfer of 15 embryos would render a number of pups still far from the uterine capacity of foster CD-1 females. Even more, transferring 25 embryos, the maximal number transferred in this study, should at theoretical 75% render a number of pups close to the maximal uterine capacity of a naturally pregnant CD-1 female seen in our colony, 18-20 pups per single litter. However, in spite of some births of up to 12 pups for the 4th and 5th categories of transferred embryos, average yield of transfers were far below the theoretical expected values. Taken into account that no transuterine migration of embryos has been demonstrated for unilateral transfer in mice,¹² it is likely that by performing the surgical transfer bilaterally we may get yields closer to the maximum uterine capacity of fosters CD-1.

Although a more economical and non invasive method, nonsurgical embryo transfer (NSET) in mouse, has been described,¹⁴⁻¹⁵ conventional surgery remains already the option of choice to perform embryo transfer. The apparent low success rate of NSET as compared to conventional surgery may dissuade researchers to use this method¹⁶ (but see also¹⁷). In addition, in spite of the usefulness of NSET for the transcervical transfer of blastocysts, embryos at earlier stages of development may be only transferred to oviducts by conventional surgery. Development of pre-implanted embryos from the two-cell stage to the blastocyst stage entails a marked reduction in the number of embryos obtained from the donor female. Therefore, even at equal efficiency, NSET involve the use of a higher initial number of embryo donors than in surgical transfer.

In conclusion, our results show that the pregnancy rate is independent of the number of embryos transferred while success rate is, pointing out that the yield maximizes when using low number of embryos per single unilateral transfer, the ideal number of embryos to transfer being somewhere between the first (5-8) and the second (9-12) category. Reducing the number of embryos used per transfer will reduce the number of females used as embryo donors. By performing the transfer with ten embryos we estimated the reduction of the number of donors in 1.5-2.5 times. Saving on embryos to transfer may also be useful when strains undergoing rederivation produce low numbers of embryos or in cases where the available number of donors is scarce. The 1.5-2.5 reduction of the number of donors together with the repeated use of the surrogate mothers as described by Kolbe et al.¹⁸ will reduce the number of animals needed for surgical transfer procedures fulfilling the principle of the 3Rs. As the set of transgenic lines presented here may be considered as representatives of the mice population kept in a standard animal facility dedicated to biomedical research, our approach may be extensive to similar facilities.

7. Acknowledgements

The authors wish to thank Pablo Díaz, Francisco Casado, Carlos Escobar, Ester Jiménez, Olivia Barcón, María Herrera y Andrés Rodríguez for technical assistance. We would also like to thank Dr Jesús del Mazo for his research support. We thank Dr Tomás Canto for critical reading of the manuscript.

8. Declaration of conflicting interests

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

9. Funding

This research was sponsored with core funding derived from the user fees of the CIB animal facility.

10. References

1. Whittingham DG. In-vitro fertilization, embryo transfer and storage. *Brit Med Bull* 1979; 35: 105-111.

2. Fray MD, Pickard AR, Harrison M and Cheeseman MT. Upgrading mouse health and welfare: Direct benefits of a large-scale rederivation programme. *Lab Anim* 2008; 42: 127-139.

3. Murphy D. Production of transgenic rodents by the microinjection of cloned DNA into fertilized one-celled eggs. *Methods Mol Biol* 2008; 461: 71-109.

4. Auerbach AB, Norinsky R, Ho W, Losos K, Guo Q, Chatterjee S and Joyner AL. Strain-dependent differences in the efficiency of transgenic mouse production. *Transgenic Res* 2003; 12: 59-69.

5 Heykants M and Mahabir E. Estrous cycle staging before mating led to increased efficiency in the production of pseudopregnant recipients without negatively affecting embryo transfer in mice. *Theriogenology* 2016; 85: 813-821.

6. Luo C, Zuñiga J, Edison E, Palla S, Dong W and Parker-Thornburg J. Superovulation Strategies for 6 Commonly Used Mouse Strains. *J Am Assoc Lab Anim Sci* 2011; 50(4): 471–478.

7. McLaren A and Michie D. Studies on the Transfer of Fertilized Mouse Eggs to Uterine Foster-Mothers. *J Exp Biol* 1959; 36: 40-50.

8. Johnson LW, Moffatt RJ, Bartol FF and Pinkert CA. Optimization of embryo transfer protocols for mice. *Theriogenology* 1996; 46(7): 1267-1276.

9. Hogan B, Beddington R, Costantini F and Lacy E. Recovery, culture, and transfer of embryos and germ cells. In *Manipulating the mouse embryo: a laboratory manual*, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1996, pp. 127-188.

10. Behringer R, Gertsenstein M, Vintersten K and Nagy A. Surgical procedures. In:
Manipulating the mouse embryo: a laboratory manual, 4th ed. Cold Spring Harbor Laboratory
Press, Cold Spring Harbor, NY, 2014, pp. 195-234.

11. Kaneda H, Taguma K, Suzuki C, Ozaki A, Nakamura C, Hachisu A, Kobayashi K, Wakana S
and Shiroishi T. An optimal embryo transfer condition for the effective production of DBA/2J
mice. *Exp. Anim.* 2007; 56(5): 385-388.

12. Rülcke T, Haenggli A, Rappold K, Moehrlen U and Stallmach T. No transuterine migration
of fertilised ova after unilateral embryo transfer in mice. *Reprod Fert Develop* 2006; 18(8): 885-
91.

13. Mähler M (Convenor), Berard M, Feinstein R, Gallagher A, Illgen-Wilcke B, Pritchett-Corning
K and Raspa M. FELASA recommendations for the health monitoring of mouse, rat, hamster,
guinea pig and rabbit colonies in breeding and experimental units. *Lab Anim* 2014; 48(3): 178-
192

14. Marsk L and Larsson KS. 1974 A simple method for non-surgical blastocyst transfer in mice.
J Reprod Fertil 1974; 37(2): 393-398.

15. Green M, Bass S and Spear B. A device for the simple and rapid transcervical transfer of
mouse embryos eliminates the need for surgery and potential post-operative complications.
Biotechniques 2009; 47(5): 919-924.

16. Cui L, Zhang Z, Sun F, Duan X, Wang M, Di K and Li X. Transcervical embryo transfer in mice. *J Am Assoc Lab Anim Sci* 2014; 53(3): 228-231.

17. Bin Ali R, van der Ahé F, Braumuller TM, Pritchard C, Krimpenfort P and Huijbers IJ. Improved pregnancy and birth rates with routine application of nonsurgical embryo transfer. *Transgenic Res* 2014; 23(4): 691-695.

18. Kolbe T, Palme R, Touma C and Rülcke T. Repeated use of surrogate mothers for embryo transfer in the mouse. *Biol Reprod* 2012; 86(1): 19, 1-6.

11. Tables

Table 1. Comparison of pregnancy rates in the five categories in which transfers were grouped, according to the number of embryos used.

Table 2. Birth rates in the five categories in which transfers were grouped, according to the number of embryos used.

Table 3. Comparison of observed effort, and observed and expected yield in the five categories in which transfers were grouped, according to the number of embryos used.

Table 1. Comparison of pregnancy rates in the five categories in which transfers were grouped, according to the number of embryos used.

Pregnancy Rate			
Category ¹	<i>n</i>	Pregnant/Used	% Pregnancy
1st (5-8)	17	15/17	(88.2%)
2nd (9-12)	56	49/56	(87.5%)
3rd (13-16)	159	134/159	(84.3%)
4th (17-20)	103	87/103	(84.5%)
5th (21-25)	56	48/56	(85.7%)
Total	391	333/391	(85.2%)

¹Number of embryos transferred per each transfer were grouped into five categories. Among brackets appear the numbers of embryos transferred in each

Table 2. Birth rates in the five categories in which transfers were grouped, according to the number of embryos used.

Birth Rate				
Category	<i>n</i>	No. of transferred embryos	No. of born pups	Success rate (%) ¹
1st (5-8)	14	97	50	51.54
2nd (9-12)	45	507	233	45.95
3rd (13-16)	123	1782	674	37.82
4th (17-20)	85	1570	523	33.31
5th (21-25)	47	1073	344	32.06
Total	314	5029	1824	36.26

¹ Here success rate is expressed as the ratio between the absolute number of born pups and the absolute number of transferred embryos within category

Table 3. Comparison of observed effort, and observed and expected yield in the five categories in which transfers were grouped, according to the number of embryos used.

Effort and yield					
Category	Transferred embryos (mean $\pm$ s.e.m.)	Effort ¹ (mean $\pm$ s.e.m.)	Observed yield ² (mean $\pm$ s.e.m.)	Expected yield ³	Yield reduction ⁴ (%)
1st (5-8)	6.93 $\pm$ 0.30	2.32 $\pm$ 0.29	3.57 $\pm$ 0.40	3.57	-
2nd (9-12)	11.15 $\pm$ 0.17	2.61 $\pm$ 0.22	5.03 $\pm$ 0.34	5.74	12.44*
3rd(13-16)	14.39 $\pm$ 0.15	3.15 $\pm$ 0.28	5.55 $\pm$ 0.27	7.42	25.10**
4th(17-20)	18.44 $\pm$ 0.15	3.68 $\pm$ 0.34	6.36 $\pm$ 0.34	9.50	33.10**
5th(21-25)	22.96 $\pm$ 0.37	3.77 $\pm$ 0.39	7.43 $\pm$ 0.61	11.83	37.10**

¹ Average number of embryos invested per transfer to obtain a single pup (number of embryos transferred/number of pups obtained)

² Average number of pups obtained per transfer

³ An estimate of the average number of pups that would be expected to be obtained for each category taken into account the mean number of transferred embryos and the yield observed in the 1st category: the ratio between the mean number of transferred embryos used in each category and that of the first category was first obtained. Then, this ratio was multiplied by the mean observed yield in the 1st category

⁴ The proportion that the observed yield decreases with regard to the estimated expected yield

* $t = -2.13$; $P \leq 0.035$

** $t \leq -6.46$; $P \leq 10^{-3}$