Protocol for Providing Additional Pseudo-Pregnant Recipient Mice for Embryo Transfer and Intra-Uterine Insemination by Plugging in the Middle of the Day

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Summary
The fact that 10% of female mice enter oestrus and allow mating in the middle of the day is an old observation that has been more or less forgotten. We here show that this old knowledge can be used to improve the efficacy of both embryo transfer and insemination protocols. The present technical paper shows that rapid re-arrangements of mating cages, to achieve pseudo-pregnant recipients in the middle of the day, can be of great advantage in emergency situations. Such emergency situations occur repeatedly, i.e. when a scientist has forgotten to re-arrange her/his mating cages, and the last important male suddenly has become ill and may die within a few hours. A rapid technique for uterine artificial insemination in mice in such situations is extremely valuable. An artificial intra-uterine insemination requires only a minimum of planning, a minimum of instrumentation and a minimum of surgical training. The artificial insemination must be performed shortly after mating due to rapid constriction of the utero-tubual junction (UTJ). This means that the timing of the insemination is very important. We here show that the success rate for embryo transfers, when using recipients plugged in the middle of the day, was the same as for ordinary overnight mating protocols. In addition, it should be noted that the success rate (frequency of pregnancies) for uterine inseminations was 55% if using F1 recipients of C57BL/6J (considerable lower if using recipients of inbred C57BL/6J), which is amazingly high, since inseminations in mice is known to be tricky to perform in a reproducible manner.

Introduction
The laboratory mouse is the dominant model animal in biomedical research, and the continuously increasing number of transgenic strains that are distributed between laboratories all around the world has forced animal houses to use safe procedures for the import of new mouse strains. One of the safest import procedures is via embryo transfer, which means that most animal houses with high requirements concerning health status have established this procedure. In turn, this means that a number of single-caged vasectomised males are available at many animal houses all around the world, as well as a continuously ongoing breeding of "clean" recipient female mice of suitable strains. A common complication when working with embryo transfers is that occasionally insufficient numbers of pseudo-pregnant mice are obtained. We have noted that such situations often can be rescued by allowing the vasectomised males to mate with a new set of females at the middle of the day (the same day as the plugged mice should be used). The goal of the present study is to show that such "emergency-mated" mice are good recipients both for embryo transfers and intra-uterine inseminations.

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Materials and Methods

Mice

Two different strains/crossings were chosen for this investigation, inbred C57BL/6J mice and a F1-crossing between NFR/N and C57BL/6 mice (NFR/NxC57BL/6J). The C57BL/6J mice were originally bought from BOM Mice, Ry, Denmark and the NFR/N mice (an inbred strain of NMRI) were originally obtained from NIH, Bethesda, Maryland, USA. Mice used for insemination experiments were bred and kept in the barrier animal facility at BMC, Lund University, Sweden. Mice used in embryo transfer experiments were bred and kept at the animal house of the Department of Pathology at Lund University, Sweden. The animals were fed with irradiated standard breeding rodent diet (Lab For R 36, Lactamin AB, Sweden) and sterile water *ad libitum* in a photoperiod of 12 h light:12 h dark. The mice used in this study were 4-6 months of age and a health monitoring program was done according to European Laboratory Animals Sciences Association recommendations.

Ethical Permission

Vasectomies, embryo transfers and inseminations were performed according to the rules of the Swedish Animal Welfare Agency (approved permission M-12504 to R.Mattsson for embryo transfer and vasectomy in mice, and approved permission M-20404 to R.Mattsson for insemination in mice).

Vasectomy

Vasectomies were performed on 20 males. The surgical operations were in principle performed according to the standard method for scrotal vasectomy (*Nagy et al, 2003*), as follows. Isoflurane (3%) was used for anaesthesia and buprenorphine (3mg/ml) 0.4ml s.c. as preoperative analgesia. The mice were put on a warm blanket during the whole procedure. An aseptic preparation preceded the vasectomy, which was done by one scrotal incision. After blunt dissection and opening at the midline of the scrotal sac, the vas deferens on each side was found. Two ligatures with Vicryl® (6-0) were placed 0.5cm apart on each vas deferens and the tissue between the two ligatures was removed. The testicular tunics were closed with one (Vicryl® 6-0) suture on each side. A continuous suture (Vicryl® 6-0) was placed in the skin. After 2 weeks of recovery the males were placed in separate cages and allowed access to females for 4 weeks to ensure successful vasectomy (no pregnancy).

Preparation of Fertilized 1- and 2-Cell Embryos

One-cell embryos were prepared according to the standard method (*Nagy et al, 2003*). Results presented in the present paper refer to transfers of embryos obtained after super-ovulation of young females of C57BL/6J background following mating with males of the same strain (data obtained from Lund Transgenic Core Facility). In most cases the 1-cell embryos were allowed to divide in vitro (cultivation overnight in medium M16 at 37 C and 5% CO2) to the 2-cell stage prior to transfer.

Embryo Transfer via Oviduct

Embryo transfers via oviduct were performed according to the standard procedure (*Nagy et al, 2003*). Normally, 2-cell embryos were transferred into the right oviduct of a recently plugged pseudo-pregnant recipient mouse (day 0.5 of pseudo-pregnancy, or plugged in the middle of the day, i.e. within 1-2 hours prior to transfer).

Preparation of Spermatozoa for Intra-Uterine Insemination

A simple and practical protocol was used for the preparations of spermatozoa. The male mice were sacrificed by exposure to CO_2 and the testes removed. The cauda epididymidis and vas deferens was dissected free of fat and other tissues at 37°C. Six cuts were made in the vas deferens with a sharp pair of scissors and three cuts were done in cauda epididymidis. The vas deferens and cauda epididymidis were placed immediately in 100 μL Whittingham's solution (*Nagy et al, 2003*) supplemented with 30mg/ml bovine serum albumin (BSA) at 35°C. To minimize temperature fluctuations the

spermatozoa preparation was kept at room temperature for at least 10 minutes to allow spermatozoa to swim out into the medium (*Sato et al, 2001*). The sperm concentration was checked by counting the sperm in a Bürker-chamber. The total number spermatozoa/insemination was 12.5-25x10^3 per injection with the concentration of 2.5-5x10^6 sperm/ml. The motility was estimated to > 50%.

Intrauterine Insemination Performed in the Middle of the Day

Intra-uterine insemination was performed on adult, hormonally untreated, females plugged by vasectomised males in the middle of the day. The mice were put together at 12:00 h and checked one hour later. The plugged females were immediately taken to surgery for insemination. Isoflurane (2%) was used for anaesthesia and buprenorphine (3mg/ml) 0.4 ml s.c. as preoperative analgesia. The mice were put on a warm blanket during the whole procedure. The hair was shaved and the skin was swabbed with ethanol 70%. After drying, plastic drapers (Obsite^R) were placed on the skin and a dorsal incision was made just caudally to the diaphragm. After blunt dissection an incision through the peritoneum was made on each side. Gently the fat around the ovaries was picked up and one ovary at a time gently pulled out of the wound. The uterus was inspected and in most cases filled with fluid. An injection needle (0.7mm) was used to penetrate the uterine wall so that the needle of a Hamilton syringe could be passed into the lumen of uterus. Aliquots (0.5 μL) of the sperm solution were injected in each horn of the uterus. The ovaries were put back in place and one suture (Vicryl® 6-0) was used to close the peritoneum. A continuous suture (Vicryl® 6-0) was made in the skin before the females were wrapped in prewarmed paper and left to recover consciousness.

Results

1. Frequency of vaginal plugs and egg yield in the middle of the day

In our animal house a fairly high frequency of ordinary adult females will allow copulation at the middle of the day (10-15%). This means that if 40 females are put with 20 vasectomised mice a normal yield would be 4-6 plugged females, which is sufficient for a successful embryo transfer or insemination. As shown in Table 1, the ovulation frequency and yield of viable eggs is very low in the C57BL/6J strain, while a fairly good ovulation frequency and yield of eggs are obtained in the F1 females. This means that a F1 hybrid between C57BL/6J and another strain (in this case NFR/N) is the best choice for intra-uterine inseminations.

Table 1. Ovulation Frequency and Egg Yield in Untreated Adult Mice Plugged with Vasectomised Males in the Middle of the Day

Pseudo-pregnant recipient strain	Mating period	N	Successful ovulations in plugged mice (%)	Mean egg yield in ovulating mice ± S.E
C57BL/6J	Day time	12	50	1.8 ± 0.8
NFR/N × C57BL/6J	Day time	12	75	5.2 ± 1.8 P=0.026*

*P-value calculated with Mann-Whitney U test. Significant difference in egg yield (4 hours after copulation) between C57BL/6J and NFR/N x C57BL/6J mice plugged at day time.

2. Frequency of successful pregnancies in pseudo-pregnant recipient mice subjected to embryo transfer of 2-cell embryos (C57BL/6J) via oviduct

The data presented in Table 2 below refers to data obtained at Lund Transgenic Core Facility during a period of 18 months (2006-2007). Around 70% of all embryo transfers (embryos obtained from various transgenic mice on C57BL/6J background) done during this period resulted in a successful pregnancy, and the average litter size was around five. Each recipient mouse received 15 two-cell embryos in one oviduct. The data demonstrate that adult F1 females that are plugged by vasectomised males in the middle of the day are equally good recipients for embryo transfers as those used for embryo transfer after ordinary overnight matings.

3. Frequency of successful pregnancies in pseudo-pregnant recipients subjected to intra-uterine insemination shortly after copulation at the middle of the day

The data presented in Table 3 shows that our "emergency protocol" for intra-uterine inseminations actually is very efficient if using F1 hybrids between C57BL/6J and NFR/N, while it is less useful for inbred C57BL/6J mice, which is consistent with the data showing low numbers of ovulated eggs in C57 BL/6J mice plugged in the middle of the day (see Table 1).

Discussion

Most animal houses with high health status requirements have an established procedure for intake of mice via embryo transfer, which means that a stock of single-caged vasectomised mice is available. These males are used for production of pseudo-pregnant recipient mice, which normally are obtained by allowing the males to go with adult females overnight, and the females are checked for vaginal plugs the following morning. Such pseudo-pregnant females are suitable recipients for one- or two-cell embryos on the day for detection of the plug. Emergency situations (low number of "overnight plugs") do occasionally occur.

In the present paper we show that pseudo-pregnant mice plugged in the middle of the day are equally suitable for embryo transfers as are ordinary "overnight plugged" recipients. This means that a low yield of pseudo-pregnant recipient mice after overnight plugging, which often will lead to the

Table 2. Pregnancy Frequency and Litter Size in Embryo-Transferred Pseudo-pregnant Recipient Mice Plugged either at Night or in the Middle of the Day

Pseudo-Pregnant strain	Mating period	N	Mean pregnancy frequency (%) ± S.E	Mean litter size ± S.E**
NFR/N × C57BL/6J	Overnight	243	74.3 ± 3.0	5.4 ± 0.34
NFR/N × C57BL/6J	Day time	21	68.9 ± 7.3 P=0.444*	4.7 ± 0.94 P=0.313*

*P-values calculated with Mann-Whitney U test. No significant differences in pregnancy frequency or litter size between embryo-transferred recipient mice plugged at night or during the day. Each recipient mouse was transferred with 15 two-cell embryos in one oviduct/recipient on the day for detection of the vaginal plug. Embryo transfer data from Lund Transgenic Core Facility (transfers of various transgenic mice on C57BL/6J background) over a 24 months period (2005-6).
**Litter size of pregnant mice only.

Table 3. Pregnancy Frequency and Litter Size in Inseminated Recipient Mice Plugged with Vasectomised Males in the Middle of the Day

Sperm donor and recipient strains	Mating period (recipients)	n	Mean pregnancy frequency (%)	Mean litter size ± S.E**
C57BL	Day time	10	10	1.0
NFR/N × C57BL	Day time	18	55	5.0 ± 0.8 P=0.027*

*P-value calculated with Mann-Whitney U test. Significant difference between C57BL and the NFR/N x C57BL mice subjected to intra-uterine insemination.

**Litter size of pregnant mice only.

cancelling of a planned embryo transfer, actually can be rescued by putting new females to vasectomised males during the day. In fact, it is often easy to obtain pseudo-pregnant females plugged during daytime since around 10% of the females normally will be plugged after spending just 1-2 hours together with the vasectomised males at noon. When the recipients are used for conventional embryo transfers, the amount of ovulated eggs is of no importance, and any strain that easily get plugged in the middle of the day should be possible to use.

Our results also demonstrate that females plugged in the middle of the day are not only suitable as recipients for conventional embryo transfers, but they can also immediately be used for insemination via uterus. So far, it has been quite tricky for ordinary laboratories to perform reliable inseminations in mice, although protocols for intra-vaginal, intra-uterine, intra-oviductal and intra-bursa inseminations have been published (*Leckie et al, 1973, Sato et al, 2002*). The advantage with the present protocol is that a very quick insemination can be performed (within an hour) without the aid of a microscope, providing that vasectomised mice and recipient females are available. A one-hour time interval was selected because previous observations had shown that constriction of the UTJ by muscular contraction and elongation of microvilli occurred after this time interval (*Suarez ,1987*).

In fact, emergency situations do repeatedly occur in animal houses, when an important mouse strain cannot be maintained for various reasons. Commonly, this is because the female has become too old to get pregnant and the last male has an age-related illness and must be sacrificed. Still, it may be possible to save the strain if sperms are dissected out from the cauda epididymidis and vas deferens of the sacrificed male. We believe that the method described here can be a useful tool for rescuing such strains.

A drawback with the use of older recipient mice plugged in the middle of the day is that they tend to ovulate a fairly low number of eggs (especially C57BL/6J mice) and that only 50-70 % of the mice show a proper ovulation. As previously mentioned, this does not matter in the case of ordinary embryo transfers, but will lead to low litter sizes in the case of inseminations. It is also obvious that one should assume with a success frequency that is around 40-50% for inseminations (if using hybrid recipients), but this should still be enough for saving important mouse strains. It should be noted that fairly old recipient mice have been used in the insemination studies presented in this paper (4-6 months of age). The problem with a low ovulation frequency can easily be overcome by selecting the youngest mice for "middle of the day plugging" in those cases when an "emergency insemination" should be performed.

In the long term perspective we hope that the simple insemination protocol we describe here for intra-uterine inseminations should also be feasible for frozen spermatozoa. This probably means that we need to optimize a number of parameters, such as using age matched recipients of suitable strains (strains with optimal ovulation frequency in the day time), such as certain strains of blind albino mice, which are not affected by the light cycle in the animal house.

Insemination may not be the first choice of method for saving a mouse strain if there is no extreme emergency situation, since good in vitro fertilization protocols do exist, and there might be time to contact, and get help from, experts in this field (*EMMA, 2008*). If that time is not available, we suggest that an insemination protocol should be tested. As previously mentioned, only a limited number of insemination protocols have been developed for rats and mice (*De Repentigny, 1996; Sato et al, 2002; Nakatsukasa E, 2001*) and we believe that the protocol presented here would be one of the easiest to follow.

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