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Biotechnology of reproduction in farm and laboratory animals

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“Alles Leben ist lebenswert, das Ziel indes, menschliches Leben und die Tiergesundheit zu verbessern, macht es zuweilen erforderlich, Leben zu opfern, um anderes zu erhalten.”

Albert Schweitzer
In: Teaching of Reverence for Life, Holt, Rinehart, Winston, 1965

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Contents:

1. Research Projects and Objectives:

Methods of assisted reproduction are an essential tool for basic research in life sciences, the propagation of valuable farm animals, and for clinical applications in human medicine. Artificial insemination and embryo transfers are routinely applied for decades and have improved the breeding of cattle, horses and other economically valuable farm animals worldwide. Cryopreserved gametes and embryos serve as genomic resource and backup of laboratory animal models and endangered wildlife species. *In vitro* fertilization and assisted fertilization of oocytes by techniques like partial zona dissection (PZD) and intracytoplasmic sperm injection (ICSI) are established and frequently used treatments to cure fertility problems in humans.

My scientific track in this research field started with a project of assisted reproduction in pigs. Experimental fertilization of *in vivo*- or *in vitro*-matured porcine oocytes with frozen-thawed epididymal or fresh ejaculated sperm cells by ICSI paved the way to a successful production of embryos. In the study fresh and frozen sperm cells were used for *in vitro* fertilization of oocytes with and without previous activation by Ca ionophore (publication 1).

Based on our preparatory results the next step was the practical application of our *in vitro* findings, which resulted in the birth of a live piglet generated by ICSI and consecutive embryo transfer. Although the efficiency was very low, the experiment demonstrated as proof of principle that this specific fertilization technique is applicable in pigs (publication 2).

During in our experiments we observed differences in the properties of the *zonae pellucidae* of *in vitro*- and *in vivo*-generated pig embryos. These qualitative differences and their impact on our experimental results provided the basis for our next investigation . Results revealed that the oviductal environment influences properties of the embryonic *zona pellucida* considerably, resulting in a strong impact on *in vitro* fertilization rates. Interestingly, unknown factors provided *in vivo* by the oviduct could be transmitted via co-culture of dissected oviduct tissue for improving the quality of the *zona pellucida* (publication 3).

After I have moved from the University of Göttingen to the University of Veterinary Medicine in Vienna, my research focus has also changed species and I began to work with laboratory mice. Methods of assisted reproduction play an important role in

laboratory animal sciences on a variety of reasons. In addition to its scientific importance, this field of research contributes significantly to the principle of the 3Rs [1].

Technical, reproductive and animal welfare aspects were investigated in a study to evaluate the feasibility of a repeated embryo transfer in mice. According to the Directive 2010/63EU point (25) “The number of animals used in procedures could be reduced by performing procedures on animals more than once, where this does not detract from the scientific objective or result in poor animal welfare” [2]. Embryo transfer is a routine and frequently applied technique in laboratory animal facilities. In a comprehensive investigation we were able to provide evidence that a second birthing of murine surrogate mothers is possible without technical problems or any increasing burden to the animals. As a result of this study it was shown that a reduction of the number of recipient mice up to 50% may be possible. Furthermore, its application can reduce some of the risk of hygienic contamination in research facilities by minimizing the required import of recipient mice from external sources (publication 4).

Superovulation of mice induced by hormonal treatment is a standard procedure and involved in many methods of assisted reproduction. It has been shown for important inbred strains of mice that superovulation is more efficient in prepubescent mice compared to sexual mature females. Using juvenile females as donors for oocytes and embryos, therefore, can drastically reduce the number of required animals which promotes “Reduction” as one of the 3Rs principle (publication 5, 6). However, it was noted in our observations that for the production of early embryos when superovulation is accompanied by mating to adult males, the procedure could impact the animal’s wellbeing and may have the potential to induce stress to the juvenile females. Therefore, we compared the ovulation and fertilization rate of superovulated prepubescent and adult female mice while simultaneously measuring their behavior and stress hormone levels.

In order to analyze the impact of treatment involved in different methods of assisted reproduction we applied the measurement of non-invasive stress hormones by the analysis of corticosteroid metabolites in collected feces. It is well known that adrenocortical activity depends on the circadian rhythm and can be affected by several internal and external factors. In a long-term observation over 26 months we could demonstrate an age related effect on stress hormone levels in untreated mice, influenced by their strains (genetic background) and the seasons (publication 7). Our results point out that appropriate consideration of these factors can improve the reproducibility of measured stress hormone levels in scientific studies.

2. Project Background and Results:

There is a strong link between research in the field of human reproductive medicine and biotechnology of reproduction in farm and laboratory animal species: failures in human reproduction are often cured by techniques developed in laboratory animal models. Slaughtered farm animals make germ cells easily available for *in vitro* experimentation and methods established in laboratory animals are taken over as routine in farm animals or vice versa. Artificial insemination, *in vitro* fertilization, embryo transfer and cryopreservation of germ cells or early embryos are all well documented examples.

After the first experiments to fertilize an egg by injection of a sperm cell in sea urchins and *Xenopus laevis* the technique was also applied to hamsters as the first mammalian species [3]. The first live offspring was generated by this method in cattle and rabbits, and shortly after the birth of the first human baby was reported [4]. Other mammalian species (mice, horse, sheep) followed soon for the successful application of this technique. However, there was a lack of available information about ICSI in pigs. In 1999 only three studies were published describing attempts to fertilize porcine oocytes by ICSI with no consistent success. Therefore, the aim of our research was to establish a successful ICSI protocol for porcine oocytes (publication 1).

In vitro matured porcine oocytes are easily available from slaughterhouse ovaries and sperm cells can be obtained from stations of artificial insemination. We aimed at creating a ‘porcine’ protocol for ICSI which could be used for training purposes and as a model system for the improvement of human ICSI. In our initial studies we compared the feasibility of *in vivo* and *in vitro* matured porcine oocytes fertilized by frozen or fresh sperm cells for ICSI. To further investigate the success of ICSI we also examined the effects of additional oocyte activation by Ca ionophore. The highest cleavage rate (14%) was achieved with fresh in vitro-capacitated spermatozoa injected into in vivo-matured oocytes. No favorable effect of Ca ionophore treatment was observed.

The most promising protocol was chosen for practical application, resulting in the generation of a live born piglet (publication 2). This was one of the first published reports about the generation of a live porcine offspring via ICSI. The most challenging problem however was the generation of an appropriate number of viable embryos, given the complications imposed by the high rate of polyspermy and parthenogenesis. Pigs require at least five live fetuses to retain pregnancy [5]. To overcome the effect of low implantation numbers we administered oestradiol benzoate from day 10 to day 16 following embryo

transfer to the recipient sow. This precaution prevented the lysis of the *corpora lutea* and perpetuated the pregnancy even with a small number of implantations, which resulted in the birth of a live piglet.

After this success we continued with further *in vitro* studies with porcine oocytes and embryos in order to narrow the factors which influence properties of the *zona pellucida* and its impact on the success of fertilization and embryo development (publication 3). The cross linking of the three *zona pellucida* glycoproteins ZP1, ZP2 and ZP3 is important to prevent polyspermic fertilization and protects the developing embryo until hatching and implantation. The *zona pellucida* of *in vitro* derived and fertilized oocytes seems to be prone to proteolytic digestion. We could show that the quality of the porcine *zona pellucida* significantly improved by a simple co-culture of oocytes/embryos with excised tissue from the oviduct of sows.

After changing my research focus to the field of laboratory animal science, an investigation of the feasibility of repeated use of surrogate mothers in mice was conducted. Embryo transfer in mice is a crucial technique, used for many purposes such as the generation of new genetically modified lines, revitalization of archived lines and rederivation of hygienically contaminated lines. Embryo transfer is also the method of choice for fast expansion of a valuable strain by *in vitro* fertilization and to overcome the reproductive failure of affected mutants. With regard to the 3Rs (here: reduction) repeated use of successfully birthing surrogate mothers would be desirable. The repeated use of surrogate mothers would not only reduce the number of mice in a procedure but save space in the animal facility and reduce the hygienic risk of pathogen introduction via live animal imports. In contrast to virgin females, for the second embryo transfer recipients can be selected according to their reproductive success of the first litter.

The 2010 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 that the animals recover completely from the first procedure and first and second procedures are of only mild or moderate severity [2]. The degree of severity for surgical embryo transfers in mice is classified as moderate. At the time there was no available evidence for a complete recovery of surrogate mothers which would satisfy the second precondition for repeated use of females for this purpose, according to the current national and international laws [6]. We addressed in our study the reproductive outcome of transfers and post-surgical behavior of surrogates after embryo transfer. Additionally, post-operative pain was measured by analysis of stress hormone metabolites in feces. The results

supported the assumption that the repeated use of mice as recipients for embryo transfer is technically possible, achieves comparable pregnancy rates and litter sizes. Recovery time after surgery was normal and measurement of stress hormones provided no indication of enhanced pain or suffering after the second round of transfers (publication 4).

The maximization of oocytes or embryos per donor is mandatory in order to reduce the number of animals required in experimental procedures like transgenesis, *in vitro* fertilization or archiving of mouse models. For this purpose hormonal stimulation is required (superovulation) and it is commonly applied for laboratory mice. Unfortunately, females of the predominantly used mouse strain C57BL/6 don't react well when superovulated as sexually mature adults. Therefore, prepubescent animals of about four weeks of age are routinely used in hormonal stimulation and subsequently mated to adult males. The question arises whether this procedure may induce pain and suffering in the juvenile female mice. For a harm-benefit analysis of experimental procedures the assessment of a possible harm level and its severity classification is essential. Our results revealed that superovulation and mating of mice before puberty has no harmful impact on the animal's wellbeing (publication 5). However, the yield of produced oocytes was significantly increased in comparison to adult C57BL/6 females, resulting in a reduction of animal numbers required in an experiment.

These results could be confirmed and defined in a retrospective study under routine conditions. In that study we also found that the yield of oocytes and produced embryos after *in vitro* fertilization is higher for prepuberal donors compared to adult mice (publication 6).

For an assessment of the degree of pain and suffering we frequently utilize the level of stress hormones measured as corticosteroid metabolites in feces. Due to the experimental use of mice with different age the question arose if the level of stress hormones is subject to age-dependent changes. To improve the reproducibility of measured data of this parameter we collected feces and analyzed the glucocorticoid metabolite secretion over a period covering nearly the complete life time of a mouse without applying any experimental treatment. The results demonstrated an age related impact on glucocorticoid metabolite secretion which was further influenced by the investigated strain type and by external seasonal effects (publication 7).

3. Overview of Publications:

Subject categories, ranking and impact factors according to Journal Citation Reports® Science Edition 2015

1) Intracytoplasmic injection (ICSI) of in vivo or in vitro matured oocytes with fresh ejaculated or frozen-thawed epididymal spermatozoa and additional calcium-ionophore activation in the pig.
Kolbe, T. and Holtz, W.
Theriogenology (1999) 52 (4): 671-682.
Subject category: Veterinary Sciences Ranking: 18/138
Impact factor: 1,838

2) Birth of a piglet derived from an oocyte fertilized by intracytoplasmic sperm injection (ICSI).
Kolbe, T. and Holtz, W.
Animal Reproduction Science (2000) 64, 97-101.
Subject category: Agriculture, Diary & Animal Science Ranking: 15/58
Impact factor: 1,377

3) Differences in proteinase digestibility of the zona pellucida of in vivo and in vitro derived porcine oocytes and embryos.
Kolbe, T. und Holtz, W.
Theriogenology (2005) 63:1695-1705.
Subject category: Veterinary Sciences Ranking: 18/138
Impact factor: 1,838

4) Repeated use of surrogate mothers for embryo transfer in the mouse.
Kolbe, T., Palme, R., Touma, C., Rülicke, T.
Biology of Reproduction (2012) 86(1):1-6.
Subject category: Reproductive Biology Ranking: 5/29
Impact factor: 3,471

5) Impact of superovulation and mating on the wellbeing of juvenile and adult C57BL/6N mice.
Kolbe, T., Sheety, S., Walter, I., Palme, R., Rülicke, T.
Reproduction, Fertility and Development (2014) Epub ahead of print.
(2016) 28: 969–973.
Subject category: Zoology Ranking: 22/161
Impact factor: 2,135

6) Productivity of superovulated C57BL/6J oocyte donors at different ages.
Kolbe, T., Landsberger, A., Manz, S., Na, E., Urban, I., Michel, G.
Lab Animal (NY) (2015) 44(9):346-349.
Subject category: Veterinary Sciences Ranking: 88/138
Impact factor: 0,681

7) Lifetime dependent variation of stress hormone metabolites in feces of two laboratory mouse strains.
Kolbe, T., Palme, R., Tichy, A., Rülicke, T.
PLoS One (2015) 10(8):e0136112.
Subject category: Multidisciplinary Sciences Ranking: 11/63
Impact factor: 3,057

4. Publications:

1) Intracytoplasmic injection (ICSI) of in vivo or in vitro matured oocytes with fresh ejaculated or frozen-thawed epididymal spermatozoa and additional calcium-ionophore activation in the pig.
Kolbe, T. and Holtz, W.
Theriogenology (1999) 52 (4): 671-682.
Subject category: Veterinary Sciences Ranking: 18/138
Impact factor: 1,838

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INTRACYTOPLASMIC INJECTION (ICSI) OF IN VIVO OR IN VITRO MATURED OOCYTES WITH FRESH EJACULATED OR FROZEN-THAWED EPIDIDYMAL SPERMATOZOA AND ADDITIONAL CALCIUM-IONOPHORE ACTIVATION IN THE PIG

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ABSTRACT

In Experiment 1, we performed intracytoplasmic sperm injection (ICSI) of frozen-thawed epididymal and fresh ejaculated in vitro-capacitated spermatozoa into in vivo and in vitro-matured porcine oocytes. Within each group, oocytes were sperm-injected, sham-injected or served as handling controls. After subsequent in vitro-culture for 48 h the number of unchanged, fragmented und cleaved oocytes was recorded. The best result (14% cleaved after ICSI vs 2 and 0% with the sham injection and handling controls; $P < 0.01$) was achieved with fresh in vitro-capacitated spermatozoa injected into in vivo-matured oocytes. In vitro-matured oocytes displayed high fragmentation rates. In Experiment 2, in vitro matured oocytes were injected with freshly ejaculated in vitro-capacitated spermatozoa, followed by a 5 min-exposure to 0 (control), 50 or 100 μM calcium-ionophore. Comparable groups were sham injected or served as handling controls. It became apparent that Ca-ionophore treatment after injection of spermatozoa was ineffective at 100 μM, where at 50 μM a significant reduction in cleavage rate was observed (6 vs 26% with untreated controls, $P < 0.01$). Fluorescence staining with Hoechst 33342 revealed that in most cases of sperm-injected oocytes that remained unchanged after 48 h of in vitro-culture, sperm heads had not decondensed. Only few oocytes had continued to the pronucleus stage. In this context no favorable effect of Ca-ionophore was to be observed.

Key words: intracytoplasmic sperm injection, ICSI, pig, Ca-ionophore, activation

INTRODUCTION

The purpose of this investigation was to accomplish the fertilization of porcine oocytes by way of intracytoplasmic sperm injection (ICSI) and to produce embryonic stages suitable for embryo transfer. In 1962, Hiramoto (23) was the first to record embryological development

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following injection of sperm cells into the eggs of sea urchins. In 1974, the technique was applied to amphibians (6). The first ICSI into mammalian eggs was conducted by Uehara and Yanagimachi in 1976 (47). Since the birth of the first human babies derived from ICSI (38, 39, 49, 50) a range of investigations and clinical applications has been conducted both in humans and animals. In farm and laboratory animals, there are reports on the birth of young in rabbits (25), mice (2, 29, 30), cattle (21), horses (36) and sheep (8). However, there is a paucity of information about ICSI in pigs. Catt and Rhodes (7) were the first to report attempts to inject porcine sperm cells into oocytes, staining them 12 h after injection to verify the presence of pronuclei and sperm tails in the vitellus. In 2 recent investigations (28, 31), round spermatids or round spermatids and fresh ejaculated spermatozoa were injected into in vitro-matured porcine oocytes. In the latter work, cleavage occured 48 h after treatment in 73% of the sperm-injected and in 67% of the sham-injected oocytes. Subsequent in vitro-culture for 7 d resulted in 45% blastocysts derived from sperm injected oocytes and 12% blastocysts derived from sham injected oocytes.

In the first part of the present investigation, frozen-thawed epididymal or fresh ejaculated in vitro-capacitated sperm cells were injected into in vivo- and in vitro-matured porcine oocytes, followed by 48 h of in vitro culture. In a second series of experiments, based on the observation of Catt et al. (9) that treatment of sperm injected porcine oocytes with calcium-ionophore may enhance cleavage, ICSI was combined with Ca-ionophore treatment at 2 different concentrations, to enhance the cleavage rate.

MATERIALS AND METHODS

In Experiment 1, in vitro-matured oocytes were obtained by slicing 2- to 8 mm-follicles from ovaries collected from prepuberal gilts within 2 h after slaughter. Cumulus-oocyte-complexes (COC) with more than 3 layers of cumulus cells and uniformly granulated cytoplasm were matured for 46 to 48 h in TCM 199 (# M-5017; Sigma, St. Louis, MO USA) containing 26.2 mM $NaHCO_3$ and 10% porcine follicular fluid (10, 18) modified by adding 20 IU PMSG and 20 IU hCG (Intervet, Tönisvorst, Germany), 1 mM sodium pyruvate, 0.5 mM cysteamine (22), 10µg/mL insulin, 10µg/mL transferrin, 10 ng/mL sodium-selenite (ITS-solution, # I-1884, Sigma; 3), and 70 mg/L kanamycin (Sigma-Aldrich, Irvine, UK). Maturation occurred in a water-jacketed incubator (Biosafe eco, Integra Biosciences, Fernwald, Germany) at 39 °C under an atmosphere of 5% CO_2 in air and 100% humidity. After 46 to 48 h of in vitro-maturation COC from all ovaries were pooled in fertilization medium (TALP-PVA; 4, 22, 44) modified by increasing the $CaCl_2$ concentration to 4.7 mM and adding 70 mg/L kanamycin, 4 mM caffeine and 20 mM HEPES. Cumulus cells were removed by transferring COC to a 20-µL hyaluronidase solution (1 mg/mL hyaluronidase from bovine testis, # H-3506 [Sigma] and 0.01 mg/mL soy bean trypsin inhibitor # T-9003 [Sigma] in TALP-PVA) in 500-µL Eppendorf-tubes. After vortexing for 20 sec, the denuded oocytes were washed and kept in fertilization medium until sperm injection.

In vivo-matured oocytes were obtained from slaughtered prepuberal gilts that had been treated with 1500 IU PMSG followed, 72 h later, with 500 IU hCG. They were killed 38 h after the hCG injection. Ovaries were removed and COC were collected by puncturing

preovulatory follicles 8 to 14 mm diameter, with a 23-g hypodermic needle mounted on a 5-mL syringe. The COC were placed in fertilization medium and were denuded as described for in vitro-matured oocytes.

Epididymal spermatozoa were obtained by castration of 3 boars of proven fertility. The spermatozoa were removed from the distal segment of the caudae epididymides by retrograde flushing from the ductus deferens, using lactose-egg yolk-extender as flushing medium. Semen was pellet-frozen (100-µL pellets containing 13 to 16 × 10^6 sperm cells) on dry ice in the lactose-egg yolk-extender supplemented with 6% glycerol (52). For thawing, 1 pellet of each of the 3 boars was dropped into 3 mL of an OLEP-thawing extender (31) at a temperature of 45 °C. The semen was maintained at 37 °C in a water bath until use. Capacitation of frozen-thawed semen was omitted because it has been shown to be a dispensable procedure in the context of IVF (1, 44, 52).

Ejaculated spermatozoa were obtained from 3 fertility-proven boars and were collected by the gloved-hand method. The collected semen was diluted with BTS-extender (MiniTueb, Tiefenbach, Germany) and pooled. A volume of 15 mL was centrifuged at 500 g for 10 min. The pellet was resuspended in 6 mL TCM 199 with 26.2 mM $NaHCO_3$, 1 mM sodium pyruvate, 2.9 mM potassium lactate, 3.05 mM glucose, 0.4% BSA (# A-9647, Sigma, Deisenhofen, Germany), 1% porcine follicular fluid and 70 mg/L kanamycin. The pH of the medium was adjusted to 7.8. After two-fold centrifugation and resuspension, the spermatozoa were allowed to capacitate for 90 min at a concentration of 0.2 × 10^8 cells/mL in an incubator at 39 °C under an atmosphere of 5% CO_2 in air and 100% humidity (17, 46, 54).

Intracytoplasmic sperm injection was conducted with the aid of a pair of micromanipulators (Leitz, Wetzlar, Germany) under an inverted microscope (IM 35, Zeiss, Göttingen, Germany). To accomodate a porcine spermatozoon, the inner diameter of the injection pipettes (CustomTips, Eppendorf, Hamburg, Germany) had to be 6 to 7 µm, which is larger than is commonly used for ICSI in humanes (5 µm). A 20-µL drop of PVP solution (10%, Gück, Hamburg, Germany) was placed in the center of a Petri dish (50 mm diameter, Falcon, Becton Dickinson, UK) and was surrounded by 3 groups of 10-µL drops of fertilization medium. One group of drops was for sperm injection, another group for sham injection and the third group for the handling controls. All drops were covered with mineral oil (# M-8410, Sigma-Aldrich, Steinheim, Germany). Every drop of fertilization medium was occupied by a single oocyte. Then 4 µL of sperm suspension were transferred to the PVP-drop. For sperm injection, a motile spermatozoon was located in the PVP-drop, and the midpiece was crushed with the injection pipette to immobilize it. The spermatozoon was then aspirated into the injection pipette tail-first. After moving to a drop of fertilization medium containing an oocyte, the oocyte was fixed with the holding pipette so that the polar body was in a 12 or 6 o'clock position, to avoid damage to the metaphase chromosomes during the process of injection. Zona pellucida and oolemma were punctured with the injection pipette containing the immobilized sperm cell, and a small amount of cytoplasm was aspirated to verify penetration of the oolemma. The spermatozoon was then injected into the center of the oocyte (8, 12, 47). For sham injection, the oocyte was penetrated with an

injection pipette containing no sperm cells, and the cytoplasm was aspirated and returned as if a sperm cell had been injected. The oocytes of the handling group were transferred to the Petri dish just like the others, but were returned for further in vitro-culture without treatment.

In Experiment 2, in vitro-matured oocytes that were injected with ejaculated spermatozoa, sham-injected or left untreated were incubated for 5 min after injection in fertilization medium containing no Ca-ionophore (control), 50 μM Ca-ionphore or 100 μM Ca-ionophore. Ca-ionophore (A23187; Sigma) was dissolved in DMSO as 2 mM stock solution and was diluted to the desired concentration with fertilization medium prior to use.

After their respective treatments, the oocytes were washed in NCSU 23-medium (40) modified by addition of 10μg/mL insulin, 10μg/mL transferrin, 10 ng/mL sodium-selenite (ITS-solution, # I-1884, Sigma, St. Louis, USA; 3) and 70 mg/L kanamycin and then placed into 400 μL of that medium in a 4-well-dish (Nunclon Delta, Intermed, Denmark). After 48 h of culture at 39°C under an atmosphere of 5% CO_2 in air and 100% humidity, numbers of unchanged, fragmented and symmetrically cleaved oocytes were recorded. They were stained with Hoechst 33342 fluorescence stain (5) and examined under a fluorescence microscope at X 400 (Axioskop, Zeiss, Goettingen, Germany). The fluorescence stain permitted differentiation between symmetrically cleaved oocytes with nuclei in each blastomere and those with some blastomeres devoid of a nucleus. The latter were considered fragmented rather than normally cleaved. Uncleaved oocytes were stained with Hoechst 33342 to investigate the fate of the spermatozoon.

The different treatments of the first experiment were conducted simultaneously, each set of treatments being replicated 6 or 7 times to reach a group size of 100 for each of the 6 in vitro-matured oocyte groups and 50 for each of the 6 in vivo-matured oocyte groups. In Experiment 2, the same pattern was followed, injecting sperm (ICSI), sham injecting or just handling oocytes, followed by exposure to 0, 50 or 100 μM Ca-ionophore directly afterwards. Treatments were replicated 5 times until a group size of 50 oocytes was arrived at.

For statistical analysis the data of the replications were pooled. Differences in percentage of oocytes cleaving, fragmenting or unchanged were assessed by Chi-square test.

RESULTS

As shown in Figure 1 A of the in vitro-matured oocytes (IVM-oocytes) injected with frozen-thawed epididymal spermatozoa in Experiment 1, 16% showed genuine cleavage, characterized by nucleated blastomeres. By comparison, cleavage had occured in 14% of the ooctes in the sham injection group and in 4% of oocytes in the handling group. A high proportion of oocytes was fragmented (34, 36 and 42%, respectively). The proportion of unchanged oocytes was the same for each of the three groups (50, 50 and 54%, respectively).

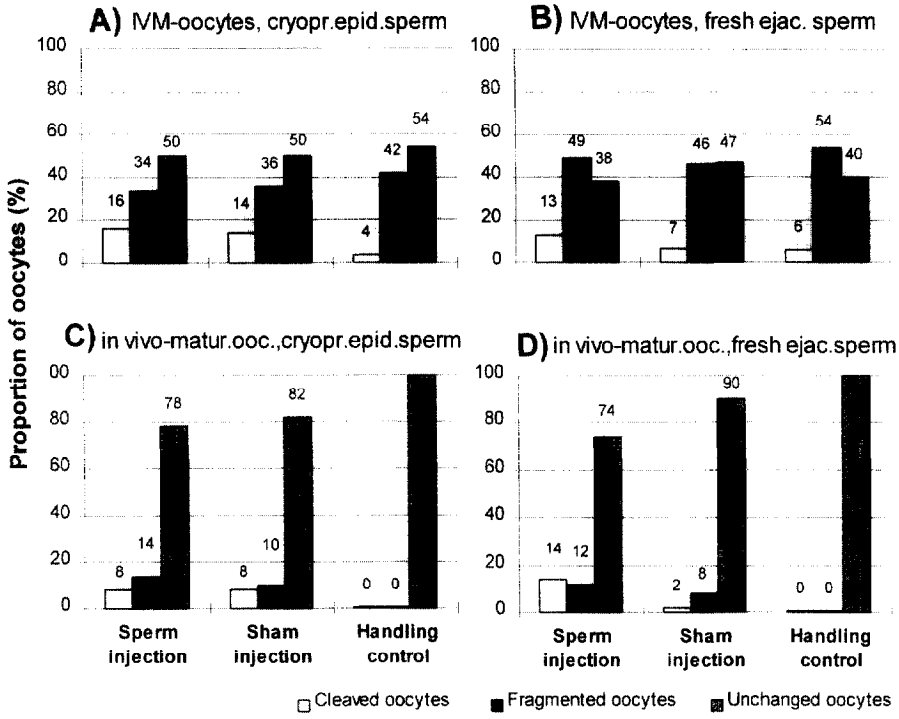

Figure 1. Appearance (cleaved, fragmented, unchanged) of oocytes after intracytoplasmic sperm injection using in vitro-matured (A and B; n = 100) or in vivo-matured (C and D; n = 50) oocytes and frozen-thawed epididymal spermatozoa (A and C) or fresh ejaculated spermatozoa (B and D).

When injecting unfrozen ejaculated spermatozoa into IVM-oocytes (Figure 1 B), the results were almost identical: 13, 7 and 6% cleaved, 49, 46 and 54% fragmented, and 38, 47 and 40% unchanged, respectively.

With in vivo-matured oocytes a different pattern was observed. Again, cleavage rates were low; 8, 8 and 0% for frozen-thawed epididymal (Figure 1 C) and 14, 2 and 0% for fresh ejaculated spermatozoa (Figure 1 D), respectively. None-the-less, the cleavage rate after injection of freshly ejaculated spermatozoa was significantly higher than after sham injection or just handling of the cells (14 vs 2 and 0%; $P < 0.05$). The proportion of fragmented oocytes occurring upon injection of spermatozoa into in vivo-matured oocytes (14, 10 and 0% for frozen-thawed epididymal and 12, 8 and 0% for fresh ejaculated spermatozoa, respectively) was considerably lower than with in vitro-matured oocytes ($P < 0.01$). As a

consequence, in in vivo-matured oocytes the proportion of unchanged oocytes was significantly higher (78, 82 and 100% for frozen-thawed epididymal and 74, 90 and 100% for fresh ejaculated spermatozoa, respectively) than in IVM-oocytes ($P < 0.05$).

The ratio of cleaved oocytes after sperm injection vs cleaved oocytes after sham injection and handling controls reflects the effectiveness of ICSI. Using frozen-thawed epididymal spermatozoa there was no difference in cleavage rate between sperm injected and sham injected oocytes, but a substantially lower cleavage rate in the handling controls (Figure 1 A: 16, 14 and 4% and Figure 1 C: 8, 8 and 0%; $P < 0.05$). In that respect it was irrelevant whether the oocytes were in vitro-matured or in vivo-matured. A considerable difference was observed between sperm injection and sham injection with frozen-thawed epididymal (Figure 1 A and C) and freshly collected in vitro-capacitated spermatozoa (Figure 1 B and D); A vs B: 16/14 vs 13/7 and C vs D: 8/8 vs 14/2. After injection of the latter into in vivo-matured oocytes, significantly more oocytes cleaved than in the respective controls (sham injection and handling controls; $P < 0.01$). This indicates that ICSI with freshly collected spermatozoa was distinctly more effective than with frozen-thawed epididymal spermatozoa. The most encouraging result was obtained when injecting fresh ejaculated spermatozoa into in vivo-matured oocytes; D: 14% cleavage vs only 2% cleavage with sham injection.

Figure 2 summarizes the results of the experiment involving Ca-ionophore treatment. In oocytes not exposed to Ca-ionophore the cleavage rate was significantly higher after sperm injection than after sham injection or in the handling control groups (26 vs 8 and 2%, respectively; $P < 0.01$).

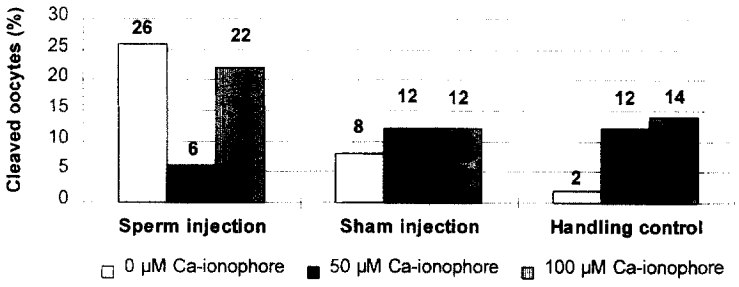

Figure 2. Cleaved oocytes (%) after intracytoplasmic sperm injection of in vitro-matured oocytes with fresh ejaculated spermatozoa and subsequent activation by Ca-ionophore (50 oocytes in each of the 9 groups).

In Experiment 2, the results of an additional activation of IVM-oocytes by exposure to Ca-ionophore following the injection of a spermatozoon were ambiguous: cleavage rate was substantially decreased at a Ca-ionophore concentration of 50 µM (6 vs 26%; $P < 0.01$) but

only marginally at 100 μM (22 vs 26%; P > 0.05). After sham injection the effect of Ca-ionophore treatment on cleavage rate was insignificant (12 and 12% for oocytes treated with 50 and 100 μM, respectively, vs 8% without). In the handling controls, the cleavage rates after Ca-ionophore treatment closely resembled those observed after sham injection (12 and 14%, respectively).

Oocytes showing no cleavage response after sperm injection (n = 12, 18 and 24 in the 0, 50 and 100 μM Ca-ionophore treatment groups, respectively) were subjected to Hoechst fluorescence staining. As shown in Table 1, without Ca-ionophore treatment 44% of the oocytes contained metaphase II chromosomes and 11% a female pronucleus. In the remaining oocytes, no female chromosomal material could be found. Where sperm heads were discernable (77%), most of them had not undergone decondensation. Only 11% of the inspected oocytes displayed partially decondensed sperm heads. None of them was fully decondensed. In most of the Ca-ionophore treated oocytes (70 and 94% in the 50 and 100 μM Ca-ionophore groups, respectively) no metaphase chromosomes or female pronuclei were observed and in none of the visible sperm heads decondensation of the chromosomal material had taken place.

Table 1. Chromosomal status of the oocyte and appearance of the sperm head in uncleaved oocytes after sperm injection, 5 minutes of exposure to Ca-ionophore at concentrations of 0, 50 or 100 μM and 48 hours of in vitro-culture (percentage within treatment group)

Nuclear state of gametes		Ca-ionophore (μM)		
Female	Male	0	50	100
Metaphase II	Not visible	11	10	0
Metaphase II	Condensed	33	10	6
Pronucleus	Condensed	11	10	0
Not visible	Condensed	22	50	59
Not visible	Partially decondensed	11	0	0
Not visible	Not visible	11	20	35

DISCUSSION

Intracytoplasmic injection of 2 types of spermatozoa (frozen-thawed epididymal and fresh ejaculated) into two types of oocytes (in vitro-matured and in vivo-matured) was performed.

The frequent occurrence of fragmentation of in vitro-matured oocytes is an indication of their instability. This interpretation is supported by the frequent occurrence of parthenogenic cleavage in the handling controls. This was not observed when working with in vivo-matured oocytes. In vitro-matured and fertilized (IVMF) porcine oocytes are known to possess a high parthenogenic potential (4, 37, 54) and a tendency for polyspermy (15, 16,

55). With ICSI the problem of polyspermy is avoided. We suspect that the artificial environment provided during the in vitro-maturation period of the oocytes was inadequate. Staining with lacmoid after in vitro-maturation revealed that more than 80% of the oocytes had proceeded to the metaphase II-stage. Nuclear maturation thus had taken place. Presumably the imperfect cytoplasmic maturation was responsible for the high rate of parthenogenesis and fragmentation. For whatever reason, in this investigation better results were achieved with in vivo-matured oocytes. Similar observations were reported in the context of in vitro-fertilization of porcine oocytes (43).

In the present investigation frozen-thawed epididymal spermatozoa were used for intracytoplasmic sperm injection because they had been shown to be best suited for in vitro-fertilization in pigs without the necessity of in vitro-capacitation (37, 44). Epididymal spermatozoa are said to lack the decapacitating factors that need to be removed during capacitation (26, 27), which may or may not be meaningful in the context of ICSI. By way of comparison, ICSI was conducted with spermatozoa taken from freshly collected ejaculates and capacitated in vitro. The superiority in fertilization potential of fresh ejaculated in vitro-capacitated spermatozoa indicates that the type of sperm cell has an impact on the success rate with ICSI. This was not necessarily to be expected, because the sperm head is injected into the cytoplasm of the oocyte without having to penetrate barriers such as cumulus oophorus, zona pellucida or vitelline membrane.

The fact that the cleavage rate after injection of frozen-thawed spermatozoa into either in vitro- or in vivo-matured oocytes was no higher than after sham injection, where the handling controls showed essentially no cleavage activity, suggests that it is the injection process that provides the cleavage-inducing impuls rather than the deposition of the sperm cell in the cytoplasm. The unusually high cleavage rate encountered with sham injected oocytes of the frozen-thawed epididymal semen-group may possibly be explained by the fact that sham injection was always conducted after injection of the sperm cell without changing pipettes. Because sham injection involved aspiration of ooplasm, tiny amounts of extender remaining in the pipette might have entered the cell. Substances such as glycerol and egg yolk may thus have been introduced, inducing parthenogenic cleavage of sham-injected oocytes. This hypothesis needs verification.

In the group injected with spermatozoa derived from freshly ejaculated in vitro-capacitated semen the cleavage rate of the sham injected oocytes was as low as that of the handling controls (Figure 1 B and D). Kim et al. (28), injecting fresh ejaculated spermatozoa into in vitro-matured porcine oocytes, observed a cleavage rate of 73% after sperm injection and 67% after sham injection. The difference between parthenogenically cleaving and genuinely fertilized oocytes became more evident after subsequent in vitro-culture. After 7 to 9 d of culture following sham injection, 12% of the oocytes developed to the blastocyst stage, as compared with 45% after actual sperm injection. The cleavage rate of 13% after ICSI observed in our own investigation was lower than that reported by Kim et al. (28). The same, however, applied to sham injection where, in our study, the cleavage rate amounted to 7%. The difference between our own findings and those reported by Kim et al. (28) may be due to differences in source of oocytes, in vitro maturation system or other factors.

Staining of sperm-injected oocytes that had remained uncleaved after 48 h of culture with Hoechst 33342 revealed more than 30% sperm heads that had not decondensed. Since the flagellum of the injected spermatozoon had been crushed prior to sperm-injection to immobilize it, the sperm decondensing factor (male pronucleus growth factor, MPGF) present in the oocyte's cytoplasm should have reached the male nucleus via the damaged cell membrane (14), thus facilitating decondensation. With human ICSI, the proportion of spermatozoa that do not decondense is comparable to that in our study (13). The authors hold incomplete cytoplasmic maturation of the oocytes and technical imperfections responsible.

Catt et al. (9) report that of sperm-injected porcine oocytes not subjected to Ca-ionophore, 55% were activated, where Ca-ionophore treatment resulted in 67% activation. The corresponding fertilization rates were 31 and 63%, respectively. These findings are based on a small set of data and the published abstract does not supply procedural detail. Ca-ionophore increases influx of extracellular calcium into the ooplasm (24, 46) leading to exocytosis of cortical granules (11). In contrast to Catt et al. (9), we observed no improvement in cleavage rate when exposing sperm-injected oocytes to 100 μM Ca-ionophore (26 vs 22% in the controls). The fact that exposure to 50 μM Ca-ionophore even reduced cleavage rate to a mere 6% might be explained by incomplete exocytosis of cortical granules as suggested by Wang et al. (52). In the bovine Ca-ionophore A23187 treatment has been shown to be indispensable for successful ICSI (20). In sheep, the mechanical stimulus of injection is sufficient to activate oocytes (19). The pig seems to be somewhere in-between: although the mechanical stimulus of a sham injection leads to a low percentage of cleavage, actual injection of a spermatozoon is more effective. The following-up of sperm injection with Ca-ionophore treatment did not enhance the cleavage rate. In the controls, Ca-ionophore treatment without sperm injection seemed to induce parthenogenesis. It has been described in the literature that in the pig both Ca-ionophore treatment (34, 41) and electrical stimulation of oocytes (42) may lead to parthenogenic activity. Our observation, indicating that sperm injection, sham injection and handling control groups displayed cleavage with either 50 or 100 μM Ca-ionophore, confirms this. The activation affects primarily the female chromosomes, less so the injected spermatozoa (Table 1).

With little information on ICSI in the pig available (7, 9, 28), the present investigation was of exploratory nature and may serve as a basis for future studies. The improvement in cleavage rate observed during the second experiment may have to do with a gain in technical experience acquired with time. Additional activation by Ca-ionophore affected the chromosomes of the oocyte but did not enhance decondensation of the injected sperm head. Cleavage rate was not enhanced by Ca-ionophore treatment.

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Birth of a piglet derived from an oocyte fertilized by intracytoplasmic sperm injection (ICSI)

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Abstract

This report is of a live piglet derived from an embryo produced by intracytoplasmic sperm injection and carried to term. In vivo-matured oocytes were injected with in vitro-capacitated spermatozoa and after 40 h of culture, 2 to 4-cell-stage embryos were transferred to the oviducts of oestrus induced gilts 24 h after the expected ovulation time. Pregnancy of the surrogate mothers was maintained by estrogen treatment on days 10 to 16 after transfer. The single piglet that was born to a mother that had received 32 embryos was underweight, though healthy and lively.

Keywords: Reproductive technology; Pig; Intracytoplasmic sperm injection; ICSI; Oocytes; Embryo

1. Introduction

Since the birth of the first human babies derived from intracytoplasmic sperm injection (ICSI) (Palermo et al., 1992, 1993;Van Steirteghem et al., 1993a,b) ICSI-offspring has been produced in mice (Ahmadi et al., 1995; Kimura and Yanagimachi, 1995; Lacham-Kaplan and Trounson, 1995), rabbits (Hosoi et al., 1988), cats (Pope et al., 1997), cattle (Goto et al., 1991) and sheep (Catt et al., 1996). There is a paucity of information with regard to ICSI in pigs (Iritani et al., 1991; Catt and Rhodes, 1995; Catt et al., 1997; Lee et al., 1998; Kolbe and Holtz, 1999). The best result achieved in our own group amounted to 26% cleavage rate (Kolbe and Holtz, 1999). All attempts to transfer cleaved embryos failed to deliver pregnancies. This short communication reports on our recent success in producing

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a single live piglet upon transfer of sperm-injected oocytes. This, to our knowledge, is the first reported offspring of its kind.

2. Materials and methods

The ICSI technique applied in this investigation is described in detail in Kolbe and Holtz (1999). Briefly, oocytes aspirated from follicles of 8 to 14 mm diameter of oestrus-induced prepuberal crossbred (German Landrace, Yorkshire, Hampshire, Pietrain) gilts 38 h after an ovulation-inducing hCG-injection, were subjected to intracytoplasmic injection of spermatozoa taken from freshly collected ejaculates of fertility-proven boars and capacitated for 90 min in TCM 199 with 26.2 mM $NaHCO_3$, 1 mM sodium pyruvate, 2.9 mM potassium lactate, 3.05 mM glucose, 0.4% BSA (# A-9647, Sigma, Deisenhofen, Germany), 1% porcine follicular fluid and 70 mg/l Kanamycin (# K-0129, Sigma-Aldrich, Irvine, UK) at a pH of 7.8. In four separate sessions, 83, 71, 73 and 95 oocytes, respectively, obtained from between 5 and 10 donors at a time, were sperm-injected.

The sperm-injected oocytes were cultured for 40 h in 400 μl of North Carolina State University (NCSU) 23 medium modified by the addition of 10 μg/ml of transferrin, 10 ng/ml of sodium selenite (ITS-solution, # I-1884, Sigma, St. Louis, USA) (Barnes and Sato, 1980) and 70 mg/l of Kanamycin at 39°C under a moisture-saturated atmosphere of 5% CO_2 in air. Morphologically intact 2 to 4-cell-stage embryos were selected and transferred to oestrus-induced prepuberal gilts of similar breed and age 24 h after assumed ovulation. Oestrus induction was accomplished by i.m. injection of 1000 IU of equine chorionic gonadotropin (eCG; Intergonan, Intervet, Boxmeer, Netherlands), followed, 72 h later, by 500 IU of human chorionic gonadotropin (hCG; Ovogest, Intervet). The transfers were conducted 66 h after the hCG-injection as described by Wallenhorst and Holtz (1999), sperm-injected oocytes being equally distributed to both oviducts. Three recipients received 13, 16 and 23 embryos in the 2 to 4-cell-stage, respectively. The fourth recipient received 32 embryos that had been exposed to TALP-PVA medium (with HEPES) containing 50 μm Ca-ionophore (Sigma, Deisenhofen, Germany) for 5 min following sperm injection. All recipients received a daily i.m. injection of 5 mg oestradiol benzoate (1 ml Menformon, Intervet, Boxmeer, Netherlands) from day 10 to 16 after transfer to induce maintenance of corpora lutea regardless of the establishment of pregnancy (Pope et al., 1986, 1990).

3. Results

Of the total of 322 in vivo-matured oocytes injected with ejaculated, in vitro-capacitated spermatozoa, between 16 and 34% (mean 26%) proceeded to 2-cell-stage embryos with morphologically normal appearence within 40 h of in vitro culture. Between 6 and 10% were fragmented, the remaining oocytes remained uncleaved. Of the four recipients, three did not remain pregnant. Only the recipient that had received 32 Ca-ionophore-treated embryos remained pregnant and farrowed 114 days later. It produced a single male piglet of body weight 770 g and fetal membranes weighing 150 g. In spite of a body weight of about half what would have been expected, the newborn appeared lively, noisily moving about

in search of the udder and suckling at regular intervals. On the second day after parturition the piglet was crushed and bit by the sow, suffering a massive haematoma and a deep neck wound. The wound was sutured and the piglet was separated from its mother and bottle-fed. Despite these efforts it succumbed to its injuries 2 days later. An autopsy indicated that all organs were normal and apparently functional.

4. Discussion

The recipient giving birth to a single piglet happened to be the one that had received embryos exposed to Ca-ionophore after ICSI. Ca-ionophore has been shown to enhance influx of extracellular Ca-ions into the ooplasm (Hoshi et al., 1992; Suzuki et al., 1994) leading to exocytosis of cortical granules (Cran and Cheng, 1986). Findings reported elsewhere (Kolbe and Holtz, 1999) suggest that in all probability, this concurrence is coincidental because in that investigation Ca-ionophore did not affect the fertilization rate of sperm-injected oocytes.

In earlier, non-published attempts with a similar approach, between 48 and 60 morphologically normal sperm-injected oocytes were transferred to six recipients. The transfers were carried out within 5 h after ISCI, i.e. without allowing for observation of in vitro development. None of the six recipients remained pregnant. When considering that the average cleavage rate under comparable conditions was 16% (Kolbe and Holtz, 1999) only 8 to 10 viable embryos were to be expected. With an embryonic mortality of 60% experienced when transferring porcine embryos (Polge, 1982; Pope et al., 1986; Schlieper and Holtz, 1986, 1992; Stein-Stefani and Holtz, 1987; Brüssow, 1990; Blum-Reckow and Holtz, 1991) this being even higher when transferring IVF-embryos (9% survival to the blastocyst stage reported by Yoshida et al., 1990), chances that more than 5 embryos, the critical number required to establish pregnancy in pigs (Polge et al., 1966), would have survived are slim. With this in mind, in the present experiment the sperm-injected oocytes were cultured for 40 h to enable us to conduct a preselection. Only 2 to 4-cell-stage embryos with a morphologically sound appearence were transferred. To make sure pregnancy is sustained regardless of the number of embryos surviving, oestradiol benzoate was injected from day 10 to 16 after transfer. The effectiveness of this treatment in maintaining luteal function in non-pregnant gilts had been confirmed under the prevailing conditions (Holtz and Kolbe, unpublished).

With only one transferred embryo surviving through parturition it would have been expected that the piglet would exhibit an above-average birth weight. To the contrary, the newborn was underweight. The reason for this is open to conjecture. This accomplishment may serve as encouragement for further studies.

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Differences in proteinase digestibility of the zona pellucida of in vivo and in vitro derived porcine oocytes and embryos

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Abstract

Embryo transfer practicioners know very well that, in a variety of species, there are differences between in vitro- and in vivo-derived embryos. It is assumed that these differences are results of suboptimal in vitro conditions leading to cytoplasmic and nuclear imperfections that will result in decreased embryo viability. In the present investigation the resistance of the zona pellucida of in vivo- and ex vivo-derived porcine embryos to a proteolytic enzyme is addressed. Ovulated but unfertilized oocytes, in vitro and in vivo-derived embryos of various developmental stages were exposed to a 0.5% pronase solution. The zonae of ovulated oocytes and in vivo-derived embryos at various stages of development took much longer to be digested than zonae of comparable in vitro-stages. Residence of in vitro derived embryos in an oviduct, no matter whether excised or in situ, significantly increased the zona resistance to pronase digestion. Embryonal stages normally residing in the uterus exhibited a distinctly decreased zona resistance to pronase. The culture of IVF embryos in an excised oviduct brought about a 6- to 14-fold increase in time required for zona digestion. A 24 h residence of IVF-derived embryos in the oviduct of a live recipient resulted in a digestion time for the zona of, on average, 48 h versus 1.4 h in the controls. Individual differences were substantial. The observations suggest that there must be structural changes in the zona pellucida or some sort of protective coating

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deposited while ova or embryos reside in the oviduct supplying protection against the action of proteolytic enzymes.

Keywords: Zona pellucida; Porcine; Embryo transfer; IVF; Digestion

1. Introduction

The zona pellucida acts as a protective coat enveloping oocyte and embryo until zona hatching prior to implantation. Some of the known purposes of the zona pellucida are the control of osmotic pressure [1], selective binding of spermatozoa to its surface, manifestation of the zona reaction and provision of an impermeable shell preventing polyspermia once the cortical granules have released their enzymes [2]. It effectuates the individuality of the embryo by preventing aggregation of individual embryos [3] and facilitates survival in the oviduct [4,5]. In the course of events the quality of the zona is modified and, amongst other things, it hardens by changing the structure of the three zona proteins [2]. In the mouse, the composition of the glycoproteins constituting the zona pellucida was found to be dissimilar for oocytes and embryos [6].

This investigation addresses differences in response of the zona pellucida to exposure to the enzyme pronase in oocytes matured and fertilized either in vivo or in vitro. Pronase has been shown to be more effective than trypsin in digesting the zona [7]. Its effect involves hydrolysis of the zona proteins ZP1 and ZP2 [8]. The effect of exposure of in vitro-produced embryos to an oviduct environment either in vivo or in vitro was also tested for zona digestibility.

2. Materials and methods

2.1. General procedures

Oocytes and embryos used in this investigation were obtained from prepuberal crossbred gilts (Large White × Landrace crossed with Pietrain or Hamphire). To obtain germinal vesicle (GV) stage oocytes, they were slaughtered at a body weight of 85–105 kg. Immediately after electric stunning and exsanguination the reproductive tracts of the gilts were removed [9] and kept on a warming plate at 39 °C. Oocytes were aspirated within 30 min after slaughter by puncture of 2–8 mm follicles. To obtain more advanced stages (metaphase II (MII) oocytes and embryos of up to the morula stage), gilts of 80–95 kg were estrus induced by i.m. injection of 1500 IU eCG (Intergonan, Intervet, Boxmeer, Netherlands) followed, 72 h later, by an ovulation-inducing injection of 500 IU hCG (Ovogest, Intervet). The gilts were slaughtered at varying intervals after the hCG treatment and the reproductive tracts were collected and handled as described above. In vivo matured MII oocytes were aspirated from 8 to 14 mm follicles of estrus induced non-inseminated gilts slaughtered 38 h after the hCG treatment. Tubal oocytes

and zygotes were recovered from estrus induced non-inseminated or inseminated gilts, respectively, after flushing of their oviducts 64 h after hCG treatment with 10 ml of phosphated buffered saline (PBS) containing 2% fetal calf serum (FCS, Gibco, Life Technologies, Eggenstein, Germany). Oocytes and embryos residing in the uterus were obtained either 88 h (2–8 cell embryos) or 136 h (morulae) after hCG treatment by filling each uterine horn with 40 ml of flushing medium and massaging the contents out twice. Flushings were screened under a stereo microscope equipped with a warming stage set to 39 °C.

For in vitro maturation of GV oocytes, cumulus-oocyte-complexes (COC) with more than three layers of cumulus cells and uniformly granulated cytoplasm were matured for 44–46 h in tissue culture medium 199 (TCM, # M-5017, Sigma, St. Louis, USA) containing 26.2 mM $NaHCO_3$ and 10% porcine follicular fluid [10,11], to which 20 IU/ml eCG and 20 IU/ml hCG (Intergonan and Ovogest, respectively), 1 mM sodium pyruvate, 0.5 mM cysteamine [12], 10 μg/ml insulin, 10 μg/ml transferrin, 10 ng/ml sodium-selenite (ITS-solution, # I-1884, Sigma, St. Louis, USA) [13] and 70 mg/l Kanamycin (Sigma-Aldrich, Irvine, UK) were added. Maturation took place in a water-jacketed incubator (Biosafe eco, Integra Biosciences, Fernwald, Germany) at 39 °C under a moisture-saturated atmosphere of 5% CO_2 in air. To produce fertilized oocytes, in vitro matured oocytes were incubated with frozen-thawed epididymal sperm [14] at a concentration of 10^6 cells/ml in 100 μl drops of Tyrode's medium with albumin, lactate and pyruvate supplemented with polyvinylalcohol (TALP-PVA). The $CaCl_2$ concentration had been increased to 4.7 mM and 70 mg/l Kanamycin and 4 mM caffeine were added [12,14,15]. After 6 h of incubation at 39 °C, oocytes were washed in NCSU 23-medium [16] modified by the addition of 10 μg/ml of insulin, 10 μg/ml of transferrin, 10 ng/ml of sodium-selenite [13] and 70 mg/l of Kanamycin (mNCSU 23). They were then placed into 400 μl of that medium in a 4-well dish (Nunclon Delta, Intermed, Denmark) to be cultured at 39 °C under a moisture-saturated atmosphere of 5% CO_2 in air for 48 h.

The end-point of each of the three experiments henceforth described consisted of the exposure of the respective oocytes and embryos to pronase as follows. They were incubated individually in 30 μl-drops of PBS devoid of potassium and magnesium containing 0.5% pronase E (# P-8811, Sigma, Deisenhofen, Germany) kept in a Petri dish (Greiner, 35 mm × 10 mm, Solingen, Germany) under mineral oil (# M-8410, Sigma, Deisenhofen, Germany). They were inspected visually under an inverted microscope at 100× at 1 min intervals until 10 min and at 10 min intervals thereafter, until the zona pellucida had completely disappeared. The time required for the zona to disappear was recorded.

2.2. Experiment 1: the effect of pronase on the zona pellucida of in vivo and in vitro derived oocytes and embryos

GV stage oocytes, ovulated oocytes, in vivo-matured follicular oocytes, in vitro-matured oocytes, zygotes (displaying an extruded second polar body) and 2–8 cell embryos as well as in vivo derived morulae were exposed to 0.5% pronase, and the time required for complete dissolution of the zona pellucida was recorded.

2.3. Experiment 2: the effect of pronase on the zona pellucida of in vitro-derived 2–8 cell embryos cultured in excised oviducts

Oviducts were removed from prepuberal gilts that had been estrus-induced by injection of 1000 IU eCG followed, 72 h later, by 500 IU hCG and slaughtered 88 h after the hCG-treatment. The reproductive tracts were removed from the gilts immediately after stunning and exsanguination. After ligation close to the utero-tubal junction and at the fimbrial end, oviducts were excised, thoroughly washed in Dulbecco's PBS and submerged in 10 ml of tissue culture medium 199 supplemented with 70 mg/l of Kanamycin in a covered 10 cm diameter culture dish. After punturing the wall with a hypodermic needle, 7–9 IVF-derived 2–8 cell embryos were deposited in the lumen with the aid of a fine-bore glass pipette about 3 cm away from the puncture hole. After 24 h of in vitro culture at 39 °C under a moisture-saturated atmosphere of 5% CO_2 in air, the ligatures were cut and the embryos were flushed out with 5 ml of PBS supplemented with 2% FCS. By way of comparison, an identical number of the IVF-derived 2–8 cell embryos were cultured in 4-well dishes (Nunclon Delta, Intermed, Denmark) in 400 μl of mNCSU 23-medium at 39 °C under a moisture-saturated atmosphere of 5% CO_2 in air.

2.4. Experiment 3: the effect of pronase on the zona pellucida of in vitro-derived 2–8 cell embryos accomodated in the oviduct of a temporary recipient for 24 h

Prepuberal gilts were estrus induced as in Experiment 2. The animals were anesthetized with azaperone and metomidate [17] 88 h after the hCG injection. Through a midline incision ovaries were inspected and, if ovulation had occured, in vitro-produced 2–8 cell embryos were transferred to both oviducts [18]. The reproductive tract was repositioned, the incision was closed in two layers and an antibiotic was administered (240 mg trimethoprim, 600 mg sulfadimidin and 600 mg sulfathiazol; 6 ml Vetoprim 24%, Mallinckrodt Veterinary, Burgwedel, Germany). After 24 h, the animals were re-opened under anesthesia and the transferred embryos were flushed from the oviducts with 10 ml PBS. Simultaneously, a similar number of IVF-derived 2–8 cell embryos were cultured in vitro for 24 h in 400 μl of mNCSU 23-medium in a 4-well dish (Nunclon Delta, Intermed, Denmark) kept in an incubator at 39 °C under a moisture-saturated atmosphere of 5% CO_2 in air.

2.5. Experimental design and statistical analyses

In vivo- and in vitro-derived oocytes and embryos of different developmental stages were subjected to the zona digestion assay. The material resulted from 4–8 experimental days until enough specimen of each stage of development had been collected (28–82 per group).

Experiment 2 was replicated four times to obtain a total number of 46 specimens (8–18 per oviduct). Experiment 3 was replicated four times with one recipient gilt at a time.

In every instance the time required for complete enzymatic removal of the zona pellucida was visually recorded. Data from different replications were pooled and the differences in time until complete zona dissolution were tested for significance by the Mann–Whitney *U* test (SSPS for Windows 6.1, 1994, SSPS Inc.).

3. Results

3.1. Experiment 1

The results of the comparison of in vivo- and in vitro-derived oocytes and embryos are summarized in Table 1. The time required to digest the zona pellucida of GV-oocytes was 1.4 ± 0.0 (S.E.M.) min. Metaphase II-oocytes showed the same low resistance to pronase digestion regardless of the mode of maturation; in vivo-matured 1.4 ± 0.1 (S.E.M.) min, in vitro-matured 1.8 ± 0.0 (S.E.M.) min ($P > 0.05$).

Table 1
Time (min) required for complete digestion of the zona pellucida of in vivo-derived (top) and in vitro-derived (bottom) oocytes and embryos (means and S.E.M.)

Stage of development	In vivo-derived			In vitro-derived		
	n	Mean	S.E.M.	*n*	Mean	S.E.M.
GV-oocyte	60	1.4	0.0	–	–	–
M II-oocyte	28	1.4[a]	0.1	60	1.8	0.0
Ovulated unfertilized oocyte	35	47.1[b]	7.3	–	–	–
Fertilized oocyte (zygote)	35	177.4[c]	10.8	61	1.5[d]	0.1
2–8 Cell embryo	82	111.9[e]	2.7	60	1.8[f]	0.8
Morula	28	4.0	0.8	ND	ND	ND

ND: not determined; a:b, c:d, e:f: $P < 0.01$, Mann–Whitney *U* test.

The zonae of ovulated oocytes collected from the oviduct 64 h after hCG treatment took significantly ($P < 0.01$) longer to be digested (47.1 ± 7.3 (S.E.M.) min) than zonae of M II-oocytes (1.4 ± 0.1 (S.E.M.) min). In fertilized oocytes (zygotes) collected from the oviducts of inseminated gilts 64 h after hCG treatment, the resistance of the zona to the lytic effect of pronase had increased dramatically: 177.4 ± 10.8 (S.E.M.) min digestion time. By contrast in in vitro-fertilized oocytes, the zona remained highly susceptible to pronase: 1.5 ± 0.1 (S.E.M.) min ($P < 0.01$). With the commencement of embryonic development, resistance of the zona against the digesting enzyme subsided: 111.9 ± 2.7 (S.E.M.) min for 2–8 cell stages, although it was still significantly higher than in the comparable in vitro stage (1.8 ± 0.1 (S.E.M.), $P < 0.01$). Zonae of embryos collected from the uterus in the morula stage were almost as susceptible to pronase digestion as they were prior to ovulation: 4.0 ± 0.8 (S.E.M.) min. Pronase susceptability of the zonae of 2–8 cell embryos derived from in vitro-fertilization, was not increased over that of M II or in vitro fertilized oocytes: 1.8 ± 0.1 (S.E.M.) min ($P > 0.05$).

3.2. Experiment 2

The zonae of in vitro-derived 2–8 cell embryos that had undergone culture in an excised oviduct took, on average, 13.1 ± 0.9 (S.E.M.) min to be completely digested (Table 2). By comparison, the zonae of control embryos cultured in mNCSU 23-medium for a similar period of time, disappeared within 1.3 ± 0 (S.E.M.) min ($P < 0.01$).

Table 2
Time (min) required for complete digestion of the zonae pellucidae of 2–8 cell IVF-derived embryos cultured for 24 h either in an excised oviduct or in modified NCSU 23-medium

Replicate	Excised oviduct			Culture medium		
	Number of embryos	Mean	S.E.M.	Number of embryos	Mean	S.E.M.
1	8	10.0	0.6	8	1.6	0.1
2	10	8.8	0.5	9	1.3	0.1
3	18	12.4	0.7	27	1.2	0.0
4	10	21.0	1.7	12	1.5	0.0
Mean		13.1[a]	0.9		1.3[b]	0.0

a:b: $P < 0.01$, Mann–Whitney U test.

Table 3
Time (min) required for complete digestion of the zonae pellucidae of 2–8 cell IVF-derived embryos cultured 24 h in a recipient's oviduct or in modified NCSU 23-medium

Replicate	Recipient oviduct			Culture medium		
	Number of embryos	Mean	S.E.M.	Number of embryos	Mean	S.E.M.
1	15	128.3	15.1	11	1.9	0.0
2	12	4.6	0.2	8	1.4	0.2
3	7	2.0	0.3	9	1.2	0.1
4	9	7.6	0.8	8	1.0	0.0
Mean		48.0[a]	13.0		1.4[b]	0.1

a:b: $P < 0.01$, Mann–Whitney U test.

3.3. Experiment 3

Transfer of 2–8 cell IVF-derived embryos to oviducts of live recipients where they remained for 24 h, resulted an increase in pronase digestability of the zona that was, however, highly variable (Table 3). In the oviducts of one recipient the zonae took 128.3 ± 15.1 (S.E.M.) min to be completely digested, compared to 1.9 ± 0 (S.E.M.) min in the corresponding in vitro controls (68-fold increase). In three other recipients, digestion time was not increased quite as dramatically, yet it was elevated 1.7–7.6 times over controls. In every case, differences between in vivo and in vitro culture were significant ($P < 0.01$), even after excluding the recipent with the extraordinary increase.

4. Discussion

The exposure of in vivo- and ex vivo-derived porcine oocytes and embryos to a 0.5% pronase E solution showed that the zonae of ovulated oocytes and in vivo-derived embryos at various stages of development took much longer to be digested than zonae of comparable in vitro-stages. Residence of in vitro derived embryos in an oviduct, both in vitro and in situ, significantly increased the zona resistance to pronase-digestion.

In vitro-derived embryos are, as a rule, less viable than embryos flushed from the reproductive tract of a donor animal. Apart from cytoplasmic or nuclear deficiencies, alterations of the zona pellucida might play a role in this context. In this investigation a significant difference in the resistance of the zona pellucida to protease digestion of in vivo and in vitro derived oocytes and embryos was observed. The increased resistance taking effect after in vivo fertilization might be associated with exocytosis of cortical granules effectuated by sperm penetration [19–22]. On the other hand, a similar, though less pronounced effect was observed after oocytes had resided in the oviduct. After in vitro fertilization no comparable zona-hardening was observed. Factors known to induce zona hardening other than sperm penetration are age [23–25] and the presence of specific glycoproteins in the oviduct [26]. In the present context, age of the oocyte did not seem to play a role as the in vitro matured oocytes of comparable age did not show the zona resistance displayed by IVM oocytes. Secretion of oviduct-specific proteins has been observed in mouse [27,28], hamster [29], rabbit [30,31], sheep [32,33], cow [34] and sow [26]. Brown and Cheng [26] report an interaction of two estrus-related glycoproteins with the zona pellucida in the porcine oviduct, which is in agreement with observations in mice by Kapur and Johnson [27]. Hedrick et al. [35] describe three macromolecules, other than the zona proteins ZP1, ZP2 and ZP3, located on the surface of ovulated porcine oocytes. The tubal environment has been shown in a number of species to exert an effect on the surface of the zona pellucida when coculturing embryos with oviduct cells [36–40] or oviduct tissue [41,42] or maintaining them in an organ culture system [43–45]. Zona hardening has been observed in bovine IVF embryos transferred to oviducts [46,47] much in the way observed for the pig in the present study.

Kouba et al. [48] demonstrated that oviduct proteins avert binding of spermatozoa to the zona and decrease polyspermia, a frequently occurring phenomenon in porcine IVF systems [49,50], but they did not observe a reduced zona solubility. This finding is assertive with our observation of increased zona resistance acquired in the oviduct. Maybe there are different proteins responsible for reduction of polyspermia and zona hardening. This argument emphasizes the need for further biochemical analyses of the oviduct fluid. The lack of these proteins in in vitro systems seems to be responsible for the susceptibility of the zonae to pronase [51]. Reduced sperm binding and penetration was already demonstrated after incubation of sperm with oviduct fluid [52] or coculture with oviduct cells [53]. In the in vivo situation, sperm penetration triggers exocytosis of a proteinase from the cortical granules to the perivitelline space, leading to changes in zona structure [6,54] bringing about the effect of zona hardening [55]. Conceivably, in the in vitro situation exocytosis is slow and incomplete, leading to a high incidence of polyspermia [56,57]. The problem of polyspermia in the porcine IVF system seems not only to be due to insufficient in vitro maturation but also to proteins lacking in the environment. Recently, oviduct-specific proteins were isolated and added to the media for in vitro-maturation and -fertilization [58]. Reduced polyspermia and fewer sperm binding to the oocytes were observed. The importance of zona properties for successful fertilization is also mentioned in the context of IVF operations in bovines although in these polyspermia is not considered a problem [59,60]. Apparently, after fertilization resistance against proteinases is not required any longer and fades away gradually, as observed in the in vivo-collected 8-cell and morula

stages. This contradicts the existence of a lytic effect in the uterus [61] corroborating findings of Menino and Wright [62] or could be explained as a dilution of proteins no longer secreted by the uterine environment.

We attempted to simulate the natural situation by either incubating IVM/IVF embryos in excised ligated oviducts cultured in vitro (Experiment 2) or transferring them to the oviduct of donors gilts (Experiment 3). In both situations zona resistance was prolonged compared with in vitro controls. The pronounced individual variability in pronase resistance displayed when transferring embryos to recipient oviducts is difficult to explain. Maybe there was not enough contact between embryos and oviduct proteins following the manipulation of the environment. Diffences between in vitro organ culture and in vivo transfer resulting in different oviduct fluid composition might explain the difference in the results of Experiments 2 and 3. Nonetheless, in all cases the zona gained in resistance to pronase digestion. We assume that proteins present in the oviduct are involved in the hardening of the zona. Other factors, such as ageing or sperm penetration, are acting in a comparable way on embryos of both experimental and control groups. The enzyme resistance of the zona observed in Experiment 3 when transferring embryos to recipient oviducts showed extreme individual variability which might be due to individual reactions of recipients or to interference with the tubal environment during the procedure of surgery and transfer.

In follow-up experiments it should be attempted to identify and isolate the oviduct proteins responsible for the zona to acquire resistance to protease. This might be helpful in obtaining better control of polyspermia in porcine in vitro systems.

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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

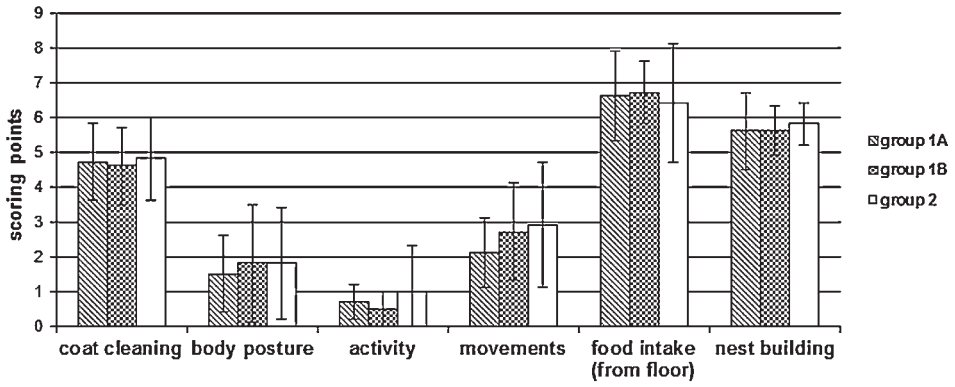

FIG. 1. Behavior scoring after embryo transfer. Values are presented as the mean ± SEM. Group 1A, virgin young recipients; group 1B, reused old recipients; group 2, virgin old recipients.

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

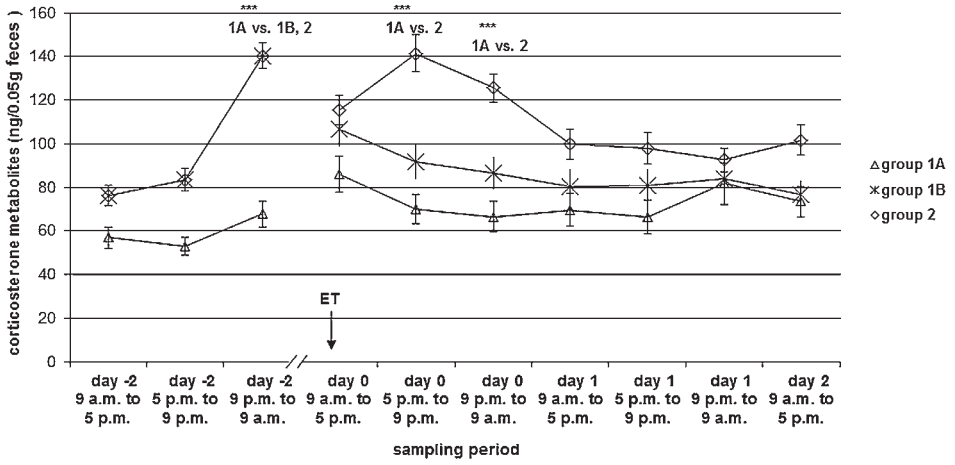

FIG. 2. Noninvasive stress monitoring via corticosterone metabolite concentrations. Embryo transfer was conducted at 0900 h on Day 0. Values are presented as the mean ± SEM. Group 1A, virgin young recipients; group 1B, reused old recipients; group 2, virgin old recipients. ***$P < 0.001$, ANOVA.

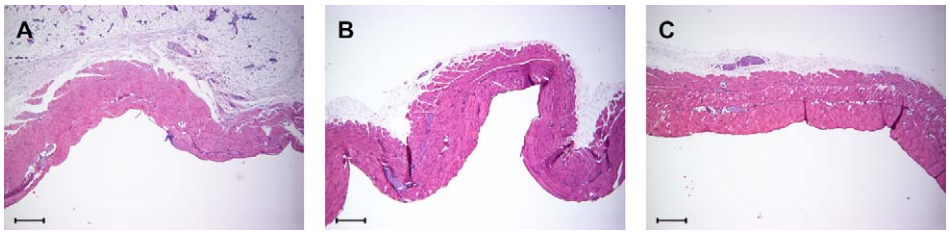

FIG. 3. Hematoxylin and eosin-stained peritoneum crosscut to the surgical cut. **A**) Group 1A, virgin young recipients. **B**) Group 1B, reused old recipients. **C**) Group 2, virgin old recipients. Bar = 500 μm.

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.

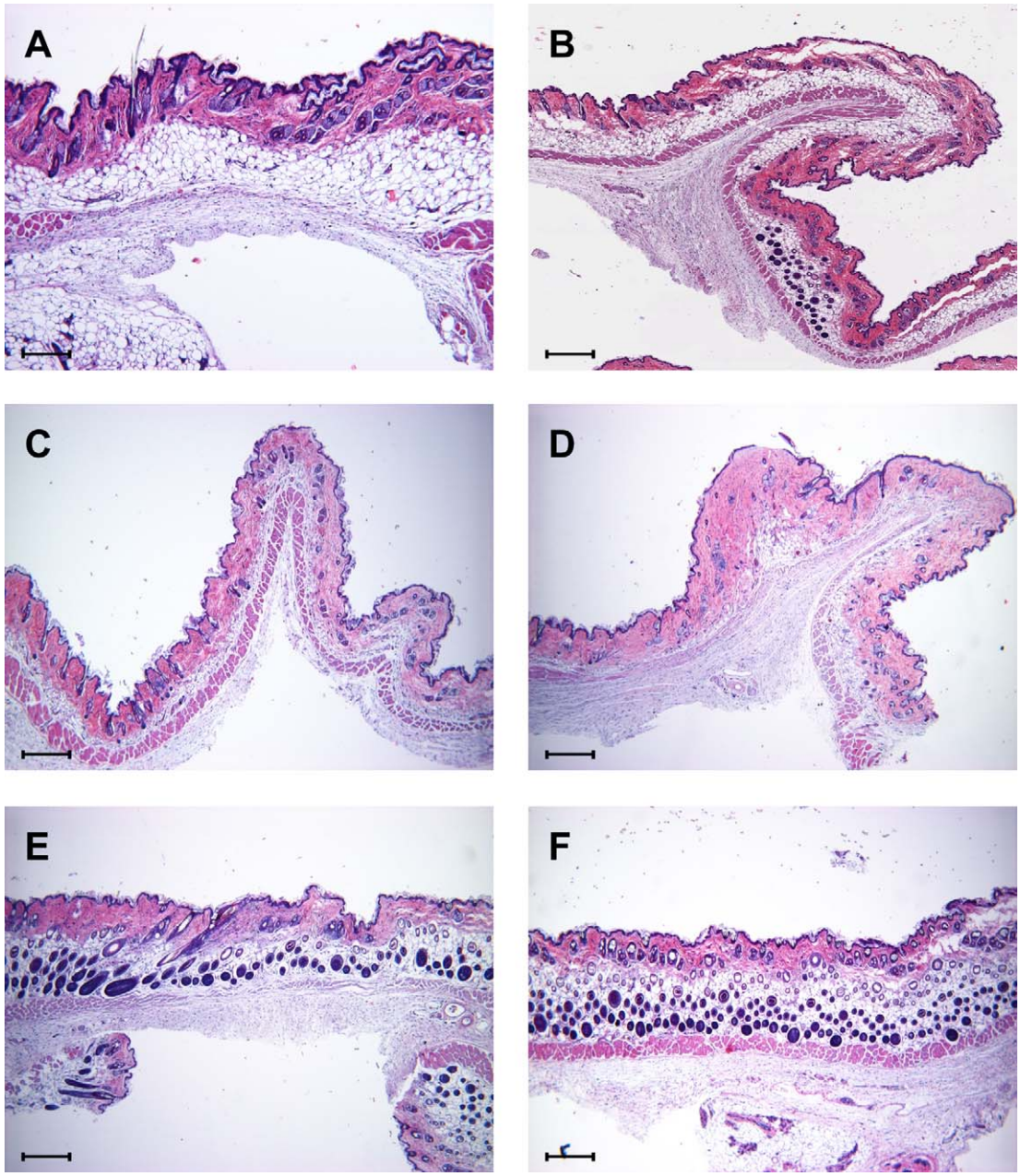

FIG. 4. Hematoxylin and eosin-stained skin crosscut to the surgical cut. **A** and **B**) Group 1A, virgin young recipients. **C** and **D**) Group 1B, reused old recipients. **E** and **F**) Group 2, virgin old recipients. Bar = 500 μm.

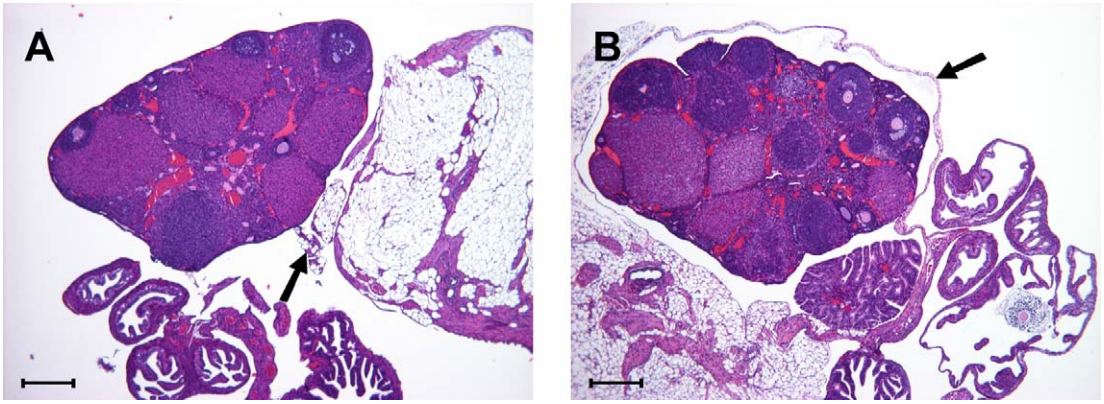

FIG. 5. Hematoxylin and eosin-stained ovary and oviduct from group 1B (reused old recipients). **A**) Treated ovary and oviduct. Arrow indicates remnants of bursa. **B**) Untreated ovary and oviduct. Arrow indicates intact bursa. Bar = 500 μm.

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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5) Impact of superovulation and mating on the wellbeing of juvenile and adult C57BL/6N mice.

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Impact of superovulation and mating on the wellbeing of juvenile and adult C57BL/6N mice

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Abstract. Superovulation of mice is routinely used to increase the number of obtainable ova per female. Because of the better outcome, prepubescent females are preferentially used. Here, we provide results of the impact of superovulation and mating on the wellbeing of juvenile compared with adult C57BL/6N mice. Two groups of mice (3–4 weeks vs 7–8 weeks old) were superovulated and mated. Observation of mating behaviour showed that reluctant adult females tended to fight the male's approach, whereas juveniles preferred to take flight. Faeces were collected daily for the analysis of stress hormones. There was no difference in the levels of glucocorticoid metabolites either between age groups or between treated animals and their controls. Histology after mating revealed intact vaginal mucosa without any detectable lesions in all animals regardless of age. In contrast to adults, almost all juveniles were synchronised in oestrus and produced significantly more ova. Taken together, our results reveal no increased welfare problem from using juvenile mice for superovulation and mating. Considering the higher yield of fertilisable oocytes and zygotes, it is advisable to use C57BL/6N prepubescent mice in order to reduce the number of donor females required.

Additional keywords: distress, mating behaviour, oestrous cycle, oestrus synchronisation, sexual maturation, vaginal histology.

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Introduction

Preimplantation mouse embryos are often required for biomedical research. All transgenic and assisted reproductive technologies applied in mice depend on oocytes and early embryos. For experimental and ethical reasons, it is desirable to harvest as many oocytes or embryos per donor as possible and consequently females are routinely superovulated by administration of exogenous hormones. Protocols for superovulation differ slightly between institutions with regard to hormone doses, the origin of the hormones and the exact time of injection during the daylight phase in the facility (Edgar *et al.* 1987; Legge and Sellens 1994; Johnson *et al.* 1996; Luo *et al.* 2011). The interval of 46–48 h between the pregnant mare serum gonadotrophin (PMSG) and human chorionic gonadotrophin (hCG) injection is undisputed. However, several publications reported that there are strong strain-dependent differences regarding the optimal age of the females for superovulation (Gates and Bozarth 1978; Hoogenkamp and Lewing 1982; Sugiyama *et al.* 1992; Ozgunen *et al.* 2001; Luo *et al.* 2011). For the widely used inbred strains C57BL/6J and C57BL/6N, the best results in terms of the number of harvested ova and embryos are achieved with juvenile females superovulated immediately after weaning and before puberty (Sugiyama *et al.* 1992; Byers *et al.* 2006; Luo *et al.* 2011). Furthermore, the quality of superovulated oocytes was not affected by the donor age, demonstrated by a similar percentage of fertilised oocytes (Luo

et al. 2011). Thus, the use of premature B6 females has become the standard procedure. However, reproduction before sexual maturity is unnatural and therefore the question arises if superovulation and mating of juvenile mice may induce pain and suffering in the animals. The recent discovery of a juvenile pheromone that exerts a powerful inhibitory effect on male mating behaviour suggests an inherent protective action of sexually immature mice (Ferrero *et al.* 2013). Therefore, the age-specific burden of the procedure should be considered for juveniles. In the present study we investigated and compared the possible distress induced by superovulation and subsequent mating to juvenile and adult females by observation of their mating behaviour, measurement of stress hormones and histology of the vaginal area.

Materials and methods

Animals (specific pathogen free) were housed in Makrolon cages (Tecniplast, Buguggiate, Italy) under standard laboratory conditions (room temperature 21 ± 1°C; relative humidity 40–55%; photoperiod 12 : 12 h, light cycle from 0600 to 1800 hours) and supplied with a standard breeding diet (V1126; Ssniff Spezialdiäten GmbH, Soest, Germany) and tap water *ad libitum*. Mice were treated according to the institutional accredited guidelines. Experimental procedures were discussed and approved by the ethics committee of the University of Veterinary Medicine Vienna and granted by the national authority according to §26 of Law for Animal Experiments under licence number BMWF-68.205/0258-II/3b/2011.

Twenty-two juvenile (3–4 weeks, approx. 10 g) and 22 adult (7–8 weeks, approx. 16 g) C57BL/6N (Charles River Laboratories, Sulzfeld, Germany) females were separated at delivery into individual cages. For mating, 12 experienced adult males of the same strain were used.

Observation of mating behaviour and measurement of stress hormones

Mice of both age groups were delivered separately for three experimental rounds. The first two rounds consisted of 16 mice each (eight juveniles and eight adults) and were used for the assessment of the experimental burden by hormone levels and mating behaviour. The eight mice from each age group were randomly divided into experimental ($n = 5$) and control ($n = 3$) mice. Controls were not treated or mated and only used to evaluate baseline stress hormone levels. On Day 6 after arrival at 0900 hours, the experimental mice of both the young and the adult groups were treated for superovulation by an intraperitoneal (i.p.) injection of 5 IU PMSG (Folligon; Intervet, Vienna, Austria) and 48 h later 5 IU hCG (Chorulon; Intervet). Immediately after the second hormone application on Day 8, the females were mated with adult, experienced B6N males for 2 h in the male's cage. During mating, the mating behaviour of females and males was monitored. The following parameters were evaluated: male mounts, unsuccessful mating attempts, female flight and female repulsion. The last two parameters were defined by the female's (inferred) intent: 'female flight' was when the females ran laps along the cage wall while maintaining a few centimetres between the males and themselves while 'female repulsion' consisted of the females rearing on their back paws, raising their front paws in defiance and baring their teeth. However, if the male continued to advance, in all observed cases the female reverted to flight rather than actually fighting the male.

Concentrations of corticosterone metabolites were measured in the faeces of the animals in a non-invasive approach to assess the levels of stress that might result from superovulation and mating (Touma *et al.* 2003, 2004). As the peak of the excretion of corticosterone metabolites in faeces of mice is delayed by 8 to 10 h from the initial stress impulse (hormone application and mating; Touma *et al.* 2004), voided faeces were collected daily (1800–2100 hours) starting at the day of arrival (Day 1) to the day of mating (Day 8) and stored frozen (−20°C) until analysis. To avoid influences on the animal's circadian rhythm, a red light was used as the light source during collection. There were no care activities during the 8-day period of faeces collection to exclude confounding factors. For faeces collection, the mice were temporarily housed individually on paper towels without wood bedding. Samples were extracted with methanol and analysed using a 5α-pregnane-3β,11β,21-triol-20-one enzyme immunoassay as described in detail by Touma *et al.* (2003, 2004).

Superovulation, mating and vaginal histology

For the third round of experiments, 12 mice (six juveniles and six adults) were used to investigate both results of superovulation and vaginal histology. All mice from each age group were superovulated and mated overnight. The next morning, the females were killed by cervical dislocation and checked for a vaginal plug and swollen ampullae as an indication of successful mating and ovulation. After flushing of the dissected oviducts, fertilised, unfertilised and degenerated oocytes were counted for each donor.

The vagina and cervix of six juveniles and six adults were dissected immediately after isolation of the oviducts (24 h after hCG injection) and fixed *in situ* in buffered 4% formaldehyde solution for 48 h, then embedded in paraffin (Histocomp; Vogel, Giessen, Germany) using automated embedding equipment (Shandon Excelsior; Thermo Scientific, Waltham, MA, USA). Serial paraffin sections (thickness 3 μm) of vagina–cervix were cut longitudinally and stained with haematoxylin and eosin for routine morphological examination. Descriptive and comparative analysis of the sections was performed under light microscopy by a blinded investigator, taking into consideration inflammatory responses, integrity of treated tissues and organs and scarring.

Statistical analysis

Differences in mating behaviour between age groups and the number of fertilised, unfertilised and degenerated ova produced by juvenile and adult females after superovulation and mating were compared by *t*-test. Daily stress hormone levels of juvenile and adult mice measured non-invasively in the faeces were analysed by ANOVA with repeated-measurements followed by Tukey's honest significant difference (HSD) post hoc tests. Values were considered significantly different if $P \leq 0.05$.

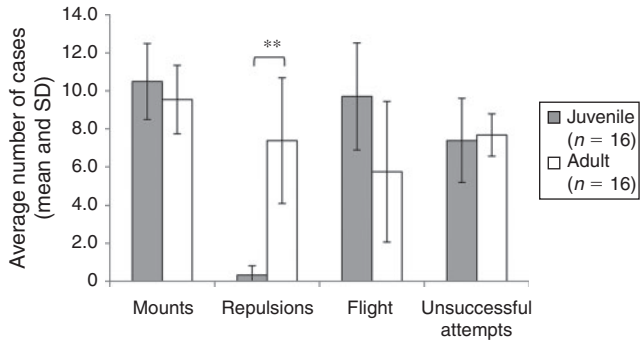

Fig. 1. Difference in mating behaviour between juvenile and adult C57BL/6N mice (**$P \leq 0.01$).

Results

Mating behaviour and levels of corticosterone metabolites

The number of attempted mounts by the males was almost equal in both groups. The reaction of females, however, revealed differences, mostly evident in the repulsive behaviour in adults ($P \leq 0.05$; Fig. 1) and in attempts to escape in juveniles.

No significant differences were observed in the levels of stress hormone metabolites between experimental groups and their controls or between juvenile and adult animals (Fig. 2). All measured animals experienced equal levels of distress, independent from hormone application or mating. Note that hormone treatment and mating were in the morning on Days 6 and 8, respectively, ~9 h before faeces collection in the evening.

Results of superovulation and histological analysis

Overall, significantly more ova were obtained from juvenile females compared with the adult group ($P \leq 0.05$; see Table S1, available as Supplementary Material to this paper), regardless of fertilisation. Although all animals were hormonally treated for superovulation, vaginal epithelial morphology was different between the two age groups: in all five successfully superovulated juveniles, a fully cornified stratified squamous epithelium was found, indicating that they were in oestrus after superovulation (Fig. 3). The sixth juvenile (JV3), from which no ova were obtained, was determined to be in proestrus. Even though all adults ovulated (suggesting that they were in oestrus), only three of the six had a vaginal histology that also corresponded to that cycle stage. Moreover, fertilised oocytes were only isolated from females AV4 and AV5, even though both were histologically not in oestrus (Fig. 3 and Table S1) and only female AV5 was plugged. In contrast to the adults, the results of superovulation of juveniles corresponded to their vaginal histology. Three of six treated juveniles were plugged (JV1, JV4, JV6) and all but one (JV3) had swollen ampullae and a high number of flushed ova. In four of the five successfully superovulated juvenile donors, fertilised ova were isolated (Table S1).

The vaginal histology revealed no indications of lesions or other injuries to the vaginal mucosa in any of the 12 examined mice.

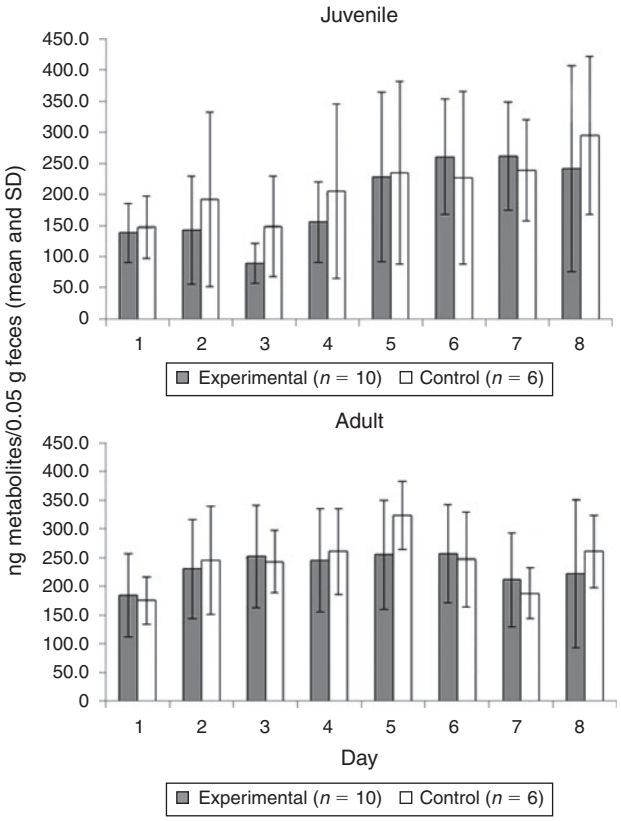

Fig. 2. Daily stress hormone levels of juvenile and adult C57BL/6N mice measured non-invasively in the faeces. Note the time points of possible stress impulses on Day 6 (PMSG injection) and Day 8 (hCG injection and mating).

Discussion

In this study, the effect of donor age in C57BL/6N mice was evaluated with regard to the distress of the females induced by the hormonal treatment and mating and the response to superovulation. We assessed the females' mating behaviour, stress hormone metabolites, genital lesions and vaginal epithelial morphology to determine the individual ovarian cycle. The number of unfertilised and fertilised oocytes per treated donor was counted and the results confirmed the general assumption of better results after superovulation of juvenile B6N mice (Table S1).

Many factors affect the outcome of superovulation treatment in mice, ranging from environment (Miyoshi *et al.* 1993), time schedule for injections and oocyte collection (Vergara *et al.* 1997), hormone doses (Edgar *et al.* 1987) and the age of donors related to the bodyweight (Gates and Bozarth 1978; Sugiyama *et al.* 1992; Ozgunen *et al.* 2001; Luo *et al.* 2011). Furthermore, the receptivity of the ovaries for exogenous hormones is genetically controlled (Spearow 1988; Spearow and Barkley 1999) and it was already shown more than 50 years ago that the outcome of superovulation is clearly age dependent (Zarrow and Wilson 1961). These observations led to the conclusion that every mouse strain has an optimal age for superovulation which is usually either 'premature'–'prepubertal' or 'mature'–'postpubertal'. For the widely used inbred strain C57BL/6J, the age at

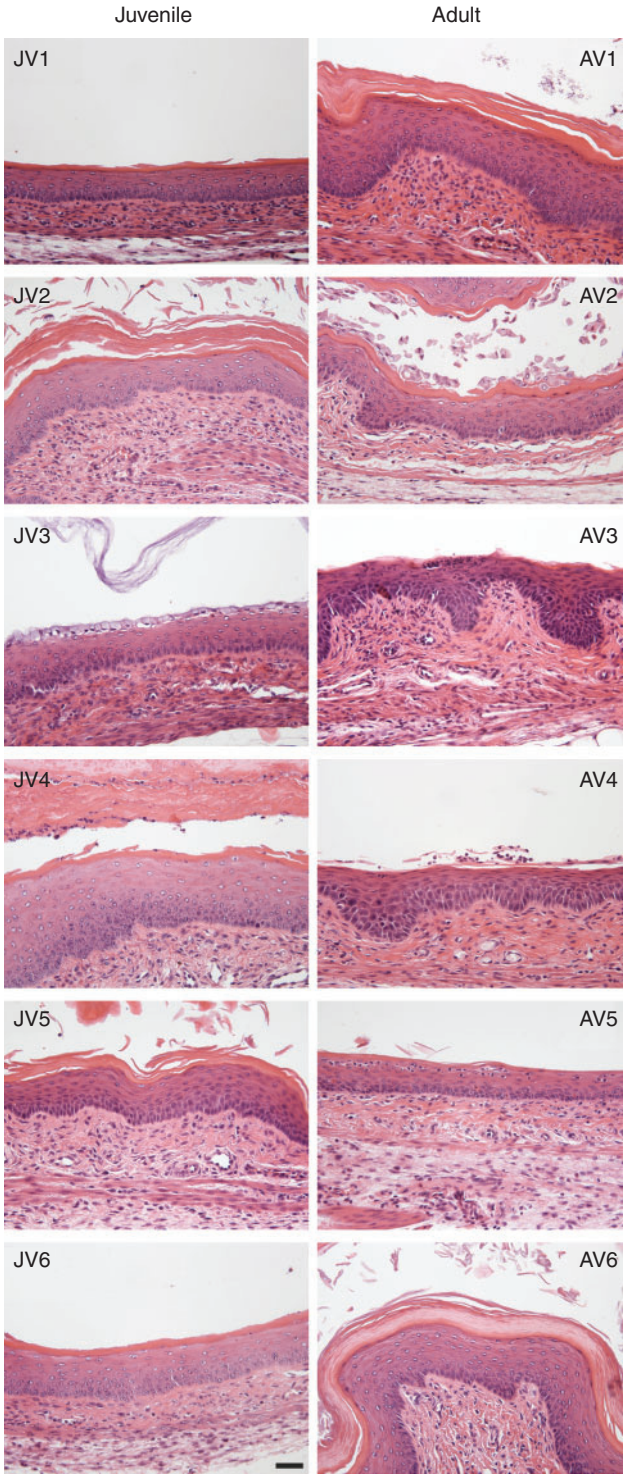

Fig. 3. Vaginal epithelium of juvenile (JV) and adult (AV) C57BL/6N mice 24 h after superovulation and mating. JV1, JV2, JV4, JV5 and JV6 are in oestrus, JV3 in proestrus. AV1, AV2 and AV6 are in oestrus, AV5 in metestrus, AV3 and AV4 in diestrus. Staining with haematoxylin & eosin (H&E). Scale bar = 50 µm.

vaginal opening indicating sexual maturation is ~34 days (Yuan *et al.* 2012) and the optimal donor age is suggested to be around 21 days (Sugiyama *et al.* 1992; Byers *et al.* 2006; Luo *et al.* 2011). Our results confirm these references insofar as premature superovulated donors produced significantly more oocytes (Table S1). However, is it justifiable in terms of the animal welfare to treat juvenile mice with hormones and mate them with a distinctly older and heavier male? To our knowledge, there are no data available about the impact of this approach on the wellbeing of the treated animals.

Especially for nulliparous female mice, the interaction with the male conspecific for mating is a completely new experience and C57BL/6N mice are described to have 'high' and 'goal-focussed' copulative behaviour (McGill 1962; Carola *et al.* 2008). The recently discovered secretion of a juvenile pheromone from the lacrimal gland that is released into tears of sexually immature mice (Ferrero *et al.* 2013) could potentially interfere with the mating of freshly weaned females by affecting male mating behaviour. The pheromone, termed exocrine-gland secreting peptide 22 (ESP22), inhibits sexual behaviour of adults towards young. Its expression is strongly increased in juveniles of both sexes and decreases promptly near puberty (Ferrero *et al.* 2013). However, it is not known if the secretion of ESP22 is possibly changed or suppressed by the artificial sexual maturation in superovulated juvenile females, which would circumvent this protection.

Our study revealed that juveniles and adults differ in their mating behaviour. Juvenile and adult females had different strategies to counteract the approach of the males before they were willing to mate (Fig. 1). The adult group seemed to balance their counterattacks with flight reflexes and choose between fight and flight equally, whereas the juvenile group had to resort to escaping almost every time the male approached uninvited. After willingness for mating was established, the number of successful mounts and unsuccessful mating attempts was comparable between both age groups, suggesting that the male's mating behaviour was not inhibited or changed in the juvenile female group.

For the assessment of distress induced by hormone treatment and mating we compared levels of faecal corticosterone metabolites between treated and untreated juvenile and adult females (Fig. 2). The observation of the frequently used self-rewarding behaviours like nest building or burrowing as indication for pain and suffering are not very applicable for this approach because the animals will usually be killed soon after hormone treatment and mating. Despite the observed differences in mating behaviour between the adult and juvenile B6N females, there was surprisingly no increase in the levels of stress hormone metabolites in any treatment group at any time point. The non-invasive measurement of corticosterone metabolites in the faeces has been proven to be a good indicator of distress in laboratory mice (Touma *et al.* 2003; Gurfein *et al.* 2012). However, compared with previous data, the level of corticosterone metabolites was generally increased in all mice of this study including controls, possibly due to the impact of not allowing the mice time to adapt to the new environmental and experimental conditions after delivery. Starting the experiment immediately after arriving was necessary to ensure that juvenile

females were still prepubescent at the time point of superovulation. In addition, the strong diurnal changes of corticosterone concentrations reach the maximal values in the first third of the dark phase, i.e. the period used for faeces collection of this study (Touma *et al.* 2004). We can also not exclude a strain-specific impact on the results of corticosterone metabolites, although C57BL/6J mice are known for a lower sensitivity to subchronic mild stress and for a lower level of anxiety-like behaviour than other inbred strains (Ducottet and Belzung 2005; An *et al.* 2011).

The histology of reproductive organs showed no lesions on the vaginal mucosa of either juvenile or adult females. However, the expected synchronised induction of oestrus after hormonal treatment was only seen in the juvenile group. Although a low level of ovulation was induced in all six treated adults, females of this group varied in the cycle phases demonstrated by the individual characteristics of the vaginal epithelium. Since cytological changes of the uterine mucosa reflect the underlying endocrine events of the respective cycle phase, the observed discrepancy between adult females after superovulation indicate an interfering impact of the endogenous ovarian cycle on the hormonal treatment (Fig. 3).

In summary, our results reveal a difference in the mating behaviour but no evidence for increased distress or injuries when using juvenile C57BL/6N mice for superovulation and mating. In contrast to juveniles, the vaginal histology indicated an incomplete synchronisation and oestrus induction in hormone-treated adult mice. Considering the higher yield of oocytes and zygotes, it is advisable to use juvenile mice of this strain in order to reduce the number of required donor females.

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6) Productivity of superovulated C57BL/6J oocyte donors at different ages.
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Productivity of superovulated C57BL/6J oocyte donors at different ages

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Superovulation is often used to increase the number of oocytes that can be collected from donor females for *in vitro* fertilization. Donor age can affect the quantity and quality of oocytes produced during superovulation, and in some strains of mice juvenile females are optimal donors. The authors reviewed donor and oocyte records from a breeding program to evaluate how donor age affects the number and fertilization efficiency of oocytes collected from C57BL/6J mice. Generally fewer oocytes per donor were collected from females aged >32 d than from females aged 21–32 d. Fertilization efficiency of oocytes generally declined with donor age when oocytes were fertilized with fresh or with stored sperm. These findings suggest that the use of younger C57BL/6J donors, instead of older donors, can reduce the number of donors needed for IVF procedures.

Transgenic and assisted reproductive technologies often use experimental mouse models in techniques that involve *in vitro* fertilization (IVF). Such technologies require a large number of high-quality oocytes and early embryos, which are obtained from female donors through non-survival surgeries. There is an ethical imperative to reduce, when possible, the number of animals used in research[1], and this can be accomplished in IVF by maximizing the number of high-quality oocytes that can be collected from a donor in order to minimize the number of donors used. Researchers often accomplish this by inducing superovulation, or the production of multiple oocytes, through administration of gonadotropin hormones.

Superovulation protocols of different facilities vary slightly with respect to their hormone doses, the origin of applied hormones and the time of injection relative to the facility's light cycle[2–5]. Most protocols are consistent, however, in the timing of administration of human chorionic gonadotropin 46–48 h after administration of pregnant mare serum gonadotropin[6]. Many factors influence the efficacy of superovulation in mice, including temperature and humidity[7], the schedule of hormone injections and oocyte collection[8], the doses of hormones[2] and the age[9] and body weight of female donors[4,10–12]. Genetic factors also influence how ovaries respond to exogenous hormones[13,14].

The effect of the age of a donor on the efficacy of superovulation varies by strain[5,10–12,15]. The widely-used inbred strain C57BL/6 produces its highest output of embryos per donor when juvenile females are superovulated soon after weaning, near 20 d or 21 d of age, and before puberty, near 48 d of age[5,11,16]. For this reason, premature C57BL/6 females are often used as oocyte and embryo donors, but little research shows how donor age affects the quality of oocytes used for *in vitro* fertilization (IVF).

Sperm quality can also affect the success of IVF. Recent developments and improvements in cryogenic preservation have enabled breeding programs to freeze and store sperm for use in later procedures, such as embryo rederivation, rapid colony expansion and revitalization of mouse strains[17–21]. In light of these developments, however, little research has explored how donor age affects the fertilization efficiency of oocytes when fertilized by fresh sperm or by stored sperm that has been frozen and thawed. To this end, we reviewed records of donors and oocytes used for IVF at a transgenic breeding and rederivation program

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to evaluate how a donor's age affects the efficacy of superovulation and the fertilization efficiency of oocytes when fertilized by fresh sperm or stored sperm.

METHODS

Husbandry and records

All oocytes and sperm in this analysis were collected from mice in at the animal facility of the Charité Universitätsmedizin (Berlin, Germany) for rederivation protocols that often use use IVF and embryo transfer techniques to breed mice of specific strains that are specific-pathogen-free. All mice were housed in same-sex groups of 3–8 mice, in type III Makrolon cages with access to standard breeding diet (V1126, Ssniff Spezialdiäten GmbH, Germany) and tap water *ad libitum*. Cages were kept at a room temperature of 21 ± 1 °C and at 40–55% relative humidity, under a14-h:10-h light: dark cycle (with the lights coming on at 6:00 a.m.).

Our database included records from 268 different dates, during which we collected a total of 194,107 oocytes from 7,567 female mice and we collected sperm from a total of 1,770 male mice. During these dates we carried out 169 IVF procedures using fresh sperm from 331 male mice and 43,675 oocytes from 1,858 female mice; 104 IVF procedures using stored sperm from 283 male mice and 26,905 oocytes from 923 female mice; and 487 IVF procedures using both fresh and stored sperm from 1,156 male mice and 123,527 oocytes from 4,787 female mice. All mice were bred from stocks within the animal facility of the Charité Universitätsmedizin (Berlin, Germany), which are renewed every 8–10 generations.

All breeding and procedures were carried out in strict accordance with National and European guidelines for animal experiments, with approval by the ethics commission of the regulatory authorities of the city of Berlin, Germany, the Landesamt für Gesundheit und Soziales (registration number H0190/02).

Our retrospective analysis used records from only female C57BL/6J mice aged 21–60 d. We analyzed a subset of records from 4,268 female C57BL/6J mice to assess how donor age affects the efficacy of superovulation. We also analyzed a subset of records from 4,381 female C57BL/6J mice whose oocytes were fertilized *in vitro* to assess how donor age affects fertilization efficiency with either fresh sperm (n = 2,021) or stored sperm (n = 2,360).

In vitro fertilization

To collect sperm, we euthanized male mice by cervical dislocation and surgically removed the caudae epididymis. We extracted sperm cells by puncturing the caudae 1–2 times with a 26-gauge needle (B. Braun, Melsungen, Germany) and soaking them in a cell culture dish with a diameter of 35-mm (Greiner Bio-One, Frickenhausen, Germany) that had been prepared with methyl-beta-cyclodextrin (MBCD) preincubation medium and mineral oil (Sigma-M8410, Sigma-Aldrich, Munich, Germany) according to previously described methods[20]. Sperm was drawn with the needle from the caudae into the MBCD and then was incubated for 45–60 min at 37 °C in 5% carbon dioxide in an incubator (CB53, Binder, Tuttlingen, Germany). We then discarded the caudae and collected 3 µl of motile sperm from the periphery of the MBCD drop. We either used the fresh sperm immediately or stored the sperm at −196 °C for 1–60 months. Stored sperm were cryopreserved in accordance with previously described methods[17]. Fresh sperm were capacitated in the incubator for 75 min and stored sperm were thawed for 2 min and capacitated for 50–70 min in the incubator.

To collect oocytes, we superovulated female mice by administering an intraperitoneal injection of 5 IU pregnant mare serum gonadotropin (Intergonan, Intervet, Neufahrn, Germany) at 8:00 p.m. and then administering an intraperitoneal injection of 5 IU human chorionic gonadotropin (Ovogest, Intervet, Neufahrn, Germany) 46 h later. We euthanized females 14 h later by cervical dislocation. We surgically removed the oviducts from each female and collected oocyte cumulus complexes from the ampullae into culture dishes 35 mm in diameter (Falcon, VWR International, Dresden, Germany) containing IVF Medium (K-RVFE-50, Cook Medical Europe, Baesweiler, Germany) along with oocytes from 0–5 other donors of the same age and strain, while we counted the number of oocytes collected from each female. In this analysis, 87% of dishes contained oocytes from only 1 or 2 donors.

We carried out IVF with oocyte cumulus complexes using 3 µl of either capacitated fresh sperm (about 3.7×10^6 motile cells) or stored sperm (about 2.0×10^6 motile cells) in cell culture dishes, using previously described methods[20]. We incubated the fertilized oocyte cumulus complexes for 4–5 h at 37 °C in 5% carbon dioxide in the incubator, then discarded degenerated oocytes and washed the remaining oocytes in IVF medium (K-RVFE, Cook Medical Europe, Baesweiler, Germany). We then counted the remaining cells and incubated them for 24 h at 37 °C in 5% carbon dioxide in the incubator. The next day we recorded the number of embryos that had developed to the two-cell stage and either froze them or transferred them into pseudopregnant foster mothers.

Data and statistical analyses

We divided donor records into five groups based on the age of donors at the time of oocyte collection: 21–24 d, 25–28 d, 29–32 d, 33–36 d and >36 d. We calculated the fertilization efficiency of each dish as the percent of oocytes that successfully developed to the two-cell embryo stage within that dish. We then assigned

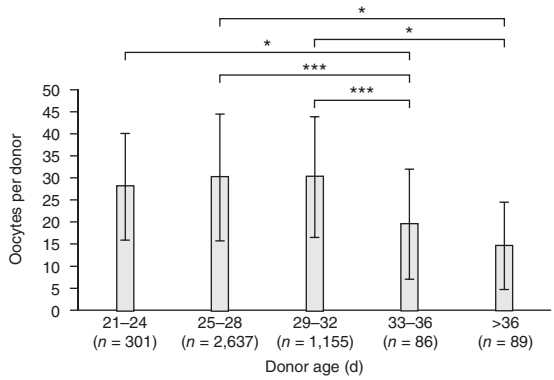

FIGURE 1 | We collected significantly fewer oocytes from donors aged 33–36 d than from donors aged 21–24 d, 25–28 d or 29–32 d. We also collected significantly fewer oocytes from donors aged >36 d than from donors aged 25–28 d or 29–32 d. *P < 0.05; ***P < 0.001.

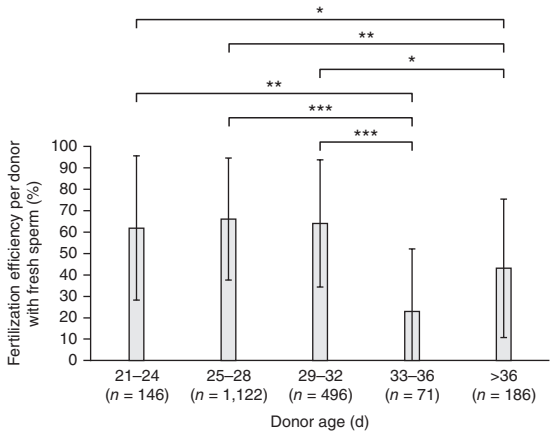

FIGURE 2 | Fertilization efficiency was significantly lower in donors aged 33–36 d and >36 d than in donors aged 21–24 d, 25–28 d or 29–32 d when oocytes were fertilized with fresh sperm. *P < 0.05; **P < 0.01; ***P < 0.001.

that value of fertilization efficiency to each donor that contributed to the dish, for statistical analysis.

We carried out a one-way analysis of variance test (ANOVA) to test whether the number of oocytes collected after superovulation differed between age groups of C57BL/6J donors. We also carried out one-way ANOVAs on C57BL/6J donors whose oocytes were fertilized with fresh sperm and on C57BL/6J donors whose oocytes were fertilized with stored sperm, to test whether fertilization efficiency differed between donor age groups when oocytes were fertilized with either type of sperm. We made *post hoc* pairwise comparisons between donor age groups using Bonferroni corrections.

RESULTS

Donor age significantly affected the number of oocytes collected after superovulation ($F_{3,1804}$) = 55.16, P < 0.0001; **Fig. 1**). We collected significantly fewer oocytes from donors aged 33–36 d than from donors aged 21–24 d (P < 0.05), 25–28 d (P < 0.001) or 29–32 d (P < 0.001). We also collected significantly fewer oocytes from donors aged >36 d than from donors aged 25–28 d or 29–32 d (P < 0.05 for both comparisons).

Age significantly affected the fertilization efficiency of donors when oocytes were fertilized with fresh sperm ($F_{3,736}$ = 25.23, P < 0.0001; **Fig. 2**). Fertilization efficiency was significantly lower in donors aged 33–36 d than in donors aged 21–24 d (P < 0.01), 25–28 d (P < 0.001) or 29–32 d (P < 0.001) and was significantly lower in donors aged >36 d than in donors aged 21–24 d (P < 0.05), 25–28 d (P < 0.01) or 29–32 d (P < 0.05) when oocytes were fertilized with fresh sperm.

Age significantly affected the fertilization efficiency of donors when oocytes were fertilized with stored sperm ($F_{3,927}$ = 10.26, P < 0.0001; **Fig. 3**). Fertilization efficiency was significantly lower in donors aged 29–32 d than in donors aged 21–24 d (P < 0.01) or 25–28 d (P < 0.05) when oocytes were fertilized with stored sperm. Fertilization efficiency was also generally lower in donors aged 33–36 d and >36 d than in donors aged 21–24 d, 25–28 d or 29–32 d, but the sample sizes of these older groups were too small to compare statistically.

DISCUSSION

We reviewed records of female C57BL/6J mice and their oocytes used for IVF at a transgenic breeding facility. Donor age significantly affected the efficacy of superovulation and affected the fertilization efficiency of oocytes from these donors when fertilized by fresh or stored sperm. Younger donors aged 21–32 d generally produced more oocytes and produced oocytes with higher fertilization efficiency than donors aged >33 d.

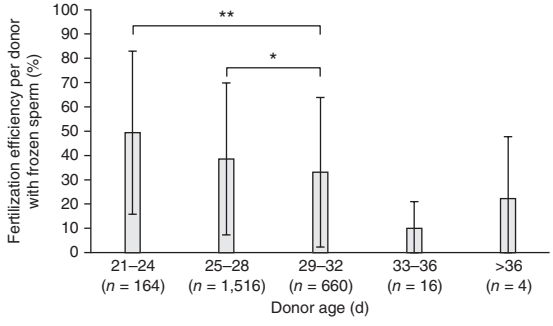

FIGURE 3 | Fertilization efficiency was significantly lower in donors aged 29–32 d than in donors aged 21–24 d or 25–28 d when oocytes were fertilized with stored sperm. Fertilization efficiency was also generally lower in donors aged 33–36 d and >36 d than in donors aged 21–24 d, 25–28 d or 29–32 d, but the sample sizes of these older groups were too small to compare statistically. *P < 0.05; **P < 0.01.

Among younger donors, age did not affect fertilization efficiency when oocytes were fertilized with fresh sperm, but oocytes from donors aged 29–32 d showed significantly lower fertilization efficiency compared with oocytes from donors aged 21–28 d when fertilized with stored sperm.

Previous research suggests that different strains of mice have different optimal ages for superovulation, which might occur before or after puberty[10,12,22], and some studies suggest that 21 d is the optimal age for C57BL/6 mice[5,11,16]. Our findings generally support these previous recommendations and we collected significantly more oocytes from younger donors than from older donors (**Fig. 1**).

The fertilization efficiency of oocytes from immature donors aged 21–24 d was slightly higher than that of oocytes from donors aged 29–32 d only when fertilized with frozen sperm (**Figs. 2** and **3**), although we can offer no clear explanation for this possible trend. Furthermore, the fertilization efficiency of oocytes from donors aged < 36 d was markedly higher than that of oocytes from donors aged 33–36 d, regardless of whether they were fertilized with fresh or stored sperm.

As younger donors produced more oocytes than older donors and as those oocytes showed generally greater fertilization efficiency, the use of young C57BL/6J donors aged 21–32 d instead of older females could reduce the number of oocyte donors needed for protocols that require IVF. Fertilization efficiency with frozen sperm was greatest in oocytes collected from donors aged 21–24 d, so IVF protocols that involve stored sperm might minimize the number of donors needed by using females of only this youngest age range. In these ways, careful age-based selection of donors can reduce the number of animals used in IVF procedures.

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COMPETING FINANCIAL INTERESTS
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7) Lifetime dependent variation of stress hormone metabolites in feces of two laboratory mouse strains.

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RESEARCH ARTICLE

Lifetime Dependent Variation of Stress Hormone Metabolites in Feces of Two Laboratory Mouse Strains

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Abstract

Non-invasive measurement of stress hormone metabolites in feces has become routine practice for the evaluation of distress and pain in animal experiments. Since metabolism and excretion of glucocorticoids may be variable, awareness and adequate consideration of influencing factors are essential for accurate monitoring of adrenocortical activity. Reference values are usually provided by baselines compiled prior to the experiment and by age matched controls. The comparison of stress hormone levels between animals of different ages or between studies looking at hormone levels at the beginning and at the end of a long term study might be biased by age-related effects. In this study we analyzed fecal corticosterone metabolites (FCM) during the lifetime of untreated female mice of the strains C57BL/6NCrl and Crl:CD1. For this purpose feces for each individual mouse were collected every two months over a period of 24 hours, at intervals of four hours, until the age of 26 months. Results of the study revealed that age of the animals had a significant impact on the level and circadian rhythm of stress hormone metabolites. Furthermore, long-term observation of mice revealed a strain specific excretion profile of FCM influenced by strong seasonal variability.

Introduction

Since the non-invasive measurement of hormone metabolites in fecal samples was approved as an alternative for the analysis of plasma glucocorticoid concentrations, it has become a widely accepted technique to diagnose stress response in many animal species (reviewed in [1, 2]). In laboratory rodents for example this approach avoids the stressful blood sampling procedure, which may interfere with an animal's endocrine status. The acute stress response, i.e. the significant increase of glucocorticoid concentration in the blood, often induced by the necessary fixation of an animal and the puncture of a blood vessel, may mask the real physiological condition.

In contrast, the analysis of fecal corticosterone metabolites (FCM) to monitor adrenocortical activity allows for frequent sampling without any disturbance to the animals [3]. However, before such a refined experimental method can be reliably applied, detailed knowledge about possible factors, which may influence glucocorticoid metabolite excretion in feces, is essential.

Corticosterone is the major glucocorticoid in mice and therefore it is widely used as an indicator of pain and distress [4]. Supportive for the monitoring of stress hormones via metabolites is that in mice corticosterone metabolites are predominantly excreted via feces [3]. Collection of feces eliminates any methodological problem related to the strong and immediate endocrine response of mice to blood sampling, which is an important external stressor. However, there are also several other factors, which can potentially act on the corresponding endocrine variables, such as the excretion of corticosterone metabolites under unstressed or stressful conditions.

As common for most hormones the secretion of corticosterone and excretion of its metabolites also follow nycthemeral cycles [3, 5]. Moreover, the excretion pattern of corticosterone metabolites strongly depends on the time of day, resulting in a delay of excretion during the light phase, which is characterized as the time of low activity in nocturnal animals [6]. Therefore, a permanent light-dark rhythm and a specified time for sampling should be selected in order to measure reproducible and comparable experimental data [6, 7].

In addition to circadian rhythmicity differences between female and male mice are also reported to be a concern in the fecal excretion of glucocorticoid metabolites. In contrast to females, male mice excrete proportionally more corticosterone metabolites via their feces, however, overall concentration of fecal corticosterone metabolites is significantly higher in females than in males [3, 6]. Sex differences in stress response, measured as plasma corticosterone levels, are significantly influenced by genetic factors, which result in strain specific differences. These differences are readily seen in standard and recombinant inbred strains [8, 9]. A significant increase in plasma corticosterone concentration was found in unstressed late pregnant mice [10], suggesting an estrogen enhanced adrenocortical sensitivity to ACTH during late pregnancy, similarly to rats where this condition has also been observed [11].

In experiments where food is not standardized or constant, the influence of diet composition and preparation should be considered when corticosterone metabolites are measured as concentration in feces. Feeding of high energy diets will reduce fecal mass excreted by the animals, which may bias readings and result in overestimation of stress hormone secretion [12, 13]. In contrast, permanent food restriction or feeding of a calorie restricted diet may induce physiological stress in rodents, resulting in a daily period of mild hyperadrenocorticism, which can be measured as elevated levels of plasma corticosterone and FCM concentrations [14, 15].

Recent findings about bidirectional communication between the brain and the gut demonstrated accumulating evidence for the significance of postnatal microbial colonization as an environmental determinant for the development of anxiety behavior [16]. Axenic (germfree) mice display lower levels of anxiety and increased motor activity, compared to SPF (specific pathogen free) animals with a commensal microflora. The pattern of both behaviors were 'normalized' by perinatal exposure of germfree newborns to microbiota obtained from SPF mice [17]. Moreover, ingestion of probiotics, such as lactic acid bacteria, appeared to be beneficial to host physiology, which included a reduction of stress-induced corticosterone levels in normally colonized, healthy mice [18]. In contrast, other research has shown that exposure of neonatal rats to Gram-negative bacterial endotoxin results in chronically elevated basal levels, as well as, a higher and prolonged increase of stress induced corticosterone levels in adulthood [19]. These results suggest that exposure to pathogens in early life has a long-term negative impact on neuroendocrine regulation of stress in adult rodents. Such response may be readily seen in rodents born and raised under inadequate hygienic conditions and supports the need for SPF status of laboratory rodents as standard practice to generate reproducible and comparable experimental results.

Changes of basal corticosterone secretion in response to seasonal changes are considered unlikely in laboratory rodents since these animals are under strictly controlled environmental conditions. Furthermore, domestication in a highly standardized vivarium seems to additionally reduce seasonality in laboratory mice compared to wild-caught mice as shown for parameters of reproduction [20]. Indeed, comparable values of basal serum corticosterone were reported in unstressed C57BL/6J mice in spring and autumn [21]. However, the corticosterone secretion of stressed animals differed significantly between the seasons, suggesting a circannual rhythmicity of the adrenal gland reactivity to stressors [21, 22]. Moreover, observed seasonal changes in pain-related behaviors may result from diurnal variations in the activity of nociceptive systems in laboratory mice [20]. Although the underlying chronobiological mechanisms are not yet identified, melatonin (and its ability to entrain neuro-endocrine rhythms) is suggested as a candidate for triggering circannual changes in pain response of laboratory rodents [23, 24].

Compared to circadian variations annual periodicity of corticosterone secretion in laboratory mice is still poorly investigated. Moreover, there is a lack of information on how basal levels of corticosterone or its metabolites in feces of mice fluctuate over their lifetime. Here we present the results of a long term study of two commonly used laboratory mouse strains to determine the concentrations of fecal corticosterone metabolites over their complete life span.

Materials and Methods

Animals

Female mice of the strains C57BL/6NCrl (B6) and Crl:CD1 (born and nurtured in our breeding facility) were housed in Makrolon cages under standard laboratory conditions (room temperature 21 ± 1°C [mean ± SEM]; relative humidity 40–55%; photoperiod 12L:12D), supplied with a standard breeding diet (V1126, Ssniff GmbH, Germany) and tap water *ad libitum*. Cages were equipped with bedding material (Lignocel, J. Rettenmaier & Söhne GmbH, Germany, heat treated) and enriched with cardboard tubes (SDS Deutschland c/o Jung GmbH, Germany) and nesting material (Pur-Zellin; Paul Hartmann AG, Germany). SPF quality of the animals was confirmed by a sentinel program according to FELASA recommendations [25]. The study was discussed and approved by the ethics and animal welfare committee of the University of Veterinary Medicine Vienna in accordance with Good Scientific Practice (GSP) guidelines and national legislation.

Preliminary study

To test the potential effects of experimental housing conditions during fecal sample collection we conducted a preliminary experiment with four eight-week-old B6 and CD1 female mice. Before fecal collection started animals of each strain were grouped together for a one week long adaptation period in our experimental facility. They were then housed individually for the sampling period of seven days. During this time the mice were kept on cotton sheets without wood bedding. In order to identify the most optimal day for unbiased FCM measurement following separation, we collected feces once daily beginning on the next day post separation (9 a.m.) and continued for seven consecutive days, then samples were stored frozen (-20°C) until analysis.

Main study

Two groups of ten B6 and ten CD1 female mice were delivered from our breeding facility to the experimental facility at the age of three weeks. After a one week adaptation period mice were separated for the first time at 11 a.m. into type II cages lined with cotton sheets. Based on

results of our preliminary study (see results of the preliminary study), voided feces collection started on day five after separation of animals, at the following sampling intervals: 3 p.m., 7 p.m., 11 p.m., 3 a.m., 7 a.m. and 11 a.m. (every 4 hours), then stored frozen at -20°C. Afterwards, the mice were returned to their home cages and were housed in groups until the next sampling date. Sampling was performed at the age of 1 month, 2 months and then repeated at every second month until the last of the surviving mice reached the age of 26 months (one CD1 mouse and five B6 mice). This sampling interval yielded a maximum of 84 samples per mouse.

Clean fecal samples of each mouse from each sampling interval were dried, homogenized and 0.05 g of the dry weight (DW) was extracted with 1 ml of 80% methanol. Concentrations of FCM were analyzed by an in-house 5α-pregnane-3β,11β,21-triol-20-one enzyme immunoassay (EIA) that was developed and successfully validated for measuring corticosterone metabolites in mice. For further details of the EIA see [3, 6].

Statistical analysis

For our preliminary study we used ANOVAs with repeated measures, followed by simple linear contrasts with Bonferroni's alpha correction as a post hoc procedure, to estimate the effects of separation on FCM concentrationsover the seven day pre-study period following separation.

For the main study we analyzed the effects of age and seasons on mean FCM concentrations, as well as the coefficient of variation (CV) by using linear mixed effects models for each strain. The daily variation of FCM was estimated by calculating the CV. The CV was determined as ratio of the standard deviation to the mean of measured FCM concentration for each time point. Due to the loss of data points over the 26 months period, we assumed for each model a heterogeneous first order autoregressive (ARH1) covariance matrix as error structure, where the individuals (id) were included as a random factor. In addition, a linear regression analysis was performed for age in order to describe the development of FCM concentration over the life-time of mice. Multiple comparisons were only conducted between seasons (sampling points) using Bonferroni's alpha correction procedure. Shapiro-Wilk-test was performed to test the assumption of normal distribution of mean FCM and CV.

All statistical analyses were performed using IBM SPSS v19. Tests are two-sided and a p-value of $\leq$ 5% (*, $p \leq 0.05$) was considered significant.

Results

Preliminary study

Measured concentration of FCM decreased from day 1 post separation to day 3, suggesting an adaptation to the individual housing conditions. From day 4–7 these measurements leveled off at lower concentrations in both strains (Fig 1). No significant differences were measured on a daily basis between samples of CD1 mice obtained during the seven-day test period. In contrast, B6 mice showed a significant decrease of FCM concentration between day 1 after separation compared to days 4, 5 and 6, however, no significant differences were found between the FCM concentrations of day 3 and the following days. We arbitrarily selected day 5 after separation as most appropriate for the commencement of our 24h-sampling period to obtain FCM concentrations for the main study.

Main study

To address any possible impact of genetic differences we analyzed two commonly used mouse strains. Sampling of feces was performed based on the described experimental protocol until the maximum age of 26 months. Several mice from each group have died spontaneously in the

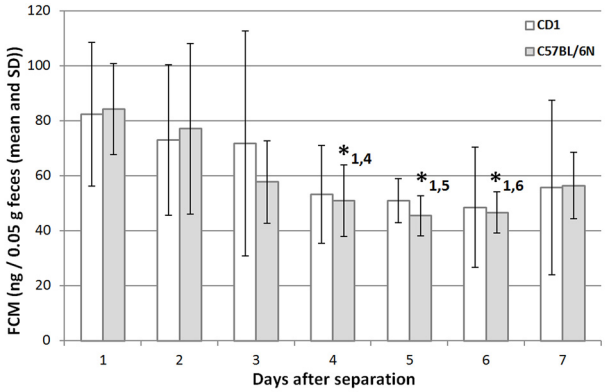

Fig 1. Concentrations of fecal corticosterone metabolites (FCM; mean ± SD) of female C57BL/6N and Crl:CD1 mice over a seven day period after separation. Preliminary study (n = 4; each strain). *, $p \leq 0.05$ for paired samples t-tests between day 1 and days 4, 5 and 6.

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course of our study. Surprisingly, the survival rate of animals of the B6 inbred strain was generally higher compared to the CD1 outbred mice (Fig 2). All but one B6 mice survived until the 10th collection time point, therefore, most of the test animals of this strain reached the minimum of 18 months in age. In contrast, only six of 10 CD1 outbred mice reached the age of 12 months, only three survived to the age of 18 months and only one animal lived to the maximum of 26 months. The short lifetime of CD1 mice resulted in a substantial reduction of sample size for the CD1 group that limited the statistical outcome for the period after 12 months of age.

Figs 3 and 4 provide an overview of the data measured over all time points presenting mean FCM concentrations and CV. Daily mean values of individual FCM concentrations of CD1 mice increased over the test period from 80–200 to 130–500 ng/0.05 g feces (Fig 3). In contrast, the mean values in B6 mice stayed nearly constant over their lifetime and varied in the range of 60–250 ng/0.05 g feces (Fig 3). The results of the mixed effects model analyses are presented in Table 1.

Diurnal variation calculated as CV was more distinct in the B6 strain compared to CD1 mice (Fig 4). Although, we observed a strong variation of FCM concentrations between animals on each sampling date, the corticosterone metabolite excretion followed the typical pattern of

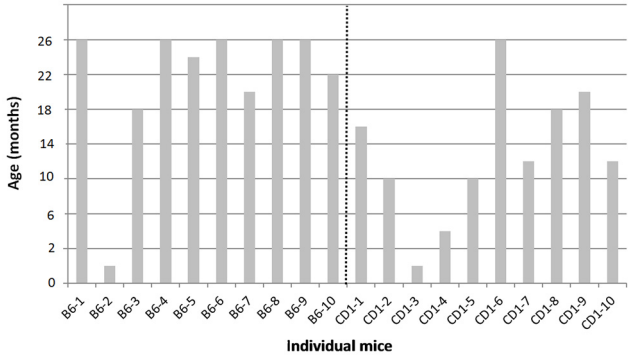

Fig 2. Overview of maximum ages and sampling points for individual female mice of the C57BL/6N and Crl:CD1 strain mice in main study.

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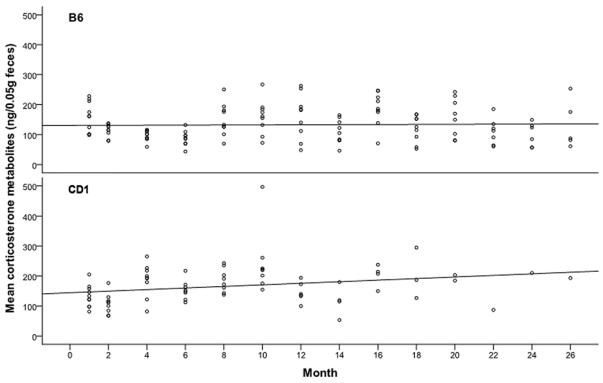

Fig 3. Mean concentrations of fecal corticosterone metabolites (FCM) per mouse and corresponding sampling points. A linear regression analysis describes the slope of FCM concentration over lifetime.

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diurnal periodicity in almost all animals (data not shown). This circadian oscillation, however, decreased continuously with the advancement of age in both strains (Fig 4).

To evaluate any possible seasonal effects on FCM concentration, data of the same month (time point) of a year were pooled from the complete 26 month test period for each strain. Circannual changes were found for both strains. Differences were observed more often between seasons (sampling points) of short days with sparse daylight versus seasons with longer daylight hours. Significant differences in pairwise comparisons of FCM concentration at different time points were calculated for B6 mice: 2/6 $p = 0.034$; 4/6 $p = 0.026$; and for CD1 mice: 2/11 $p = 0.025$; 2/12 $p = 0.001$; 6/11 $p = 0.025$; 6/12 $p = 0.001$ (Fig 5).

Discussion

We demonstrate here for the first time an age related effect on the concentration of corticosterone metabolites in feces of laboratory mice. Starting with comparable concentrations as juveniles the level of FCM stayed constant in B6 animals over a 26-month period, but continuously increased in CD1 mice over their lifetime. This age dependent increase resulted in a strain specific difference of the FCM concentration. Furthermore, the typical circadian oscillation of corticosterone metabolite concentrations decreased in both strains over the test period.

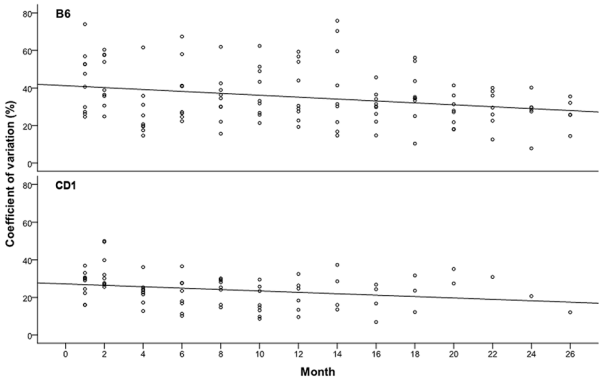

Fig 4. Diurnal rhythmicity calculated separately per mouse and sampling point as coefficient of variation (CV). A linear regression analysis describes the slope of circadian oscillation of FCM concentrations over lifetime.

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Table 1. Main results of the mixed effects model analysis showing impact of age and seasons on mean FCM concentration and CV. Slopes of FCM concentration and CV over progression of age are given as a result of a linear regression model.

	Mixed model using animal id as a random factor								Linear regression	
Strain	Dependent	Factor	Var(id)%[a]	AIC[b]	F	Df numerator	Df error	p	slope	p
B6	Mean	Age	32	1069,8	7,2	13	14,2	0.001	0.29	0.829
		Season	23.3	1157.6	5.18	6	26	0.001	-	-
	CV	Age	6.0	90.9	2.9	13	12.4	0.035	-0.010	0.004
		Season	23.8	123.8	2.7	6	7.4	0.022	-	-
CD1	Mean	Age	4.0	648.2	6.04	11	9.6	0.005	6.97	0.001
		Season	21.3	375.1	9.7	6	29	0.001	-	-
	CV	Age	12.5	101	3.3	11	7.8	0.055	-0.008	0.030
		Season	12.5	112.5	4.6	6	14	0.009	-	-

[a]Variance component in percent caused by the animal id.

[b]Akaike's information criterion.

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Stress hormone metabolites in the feces proved to be an important tool for non-invasive assessment of distress in animals of different species [26]. In order to estimate the level of experimental burden in laboratory mice we and others have utilized assaying of FCM concentration as a refined alternative to invasive blood serum sampling and analysis [27–32]. If the data collection is restricted to defined experimental time points or short periods, results will not be severely impacted by age related effects. However, in case of long-term studies the question arises if changes in hormone metabolite levels are also affected by aging. Furthermore, data comparison between studies with animals of different ages might be prone to an age related bias.

We addressed this problem by measuring FCM levels of inbred and outbred female mice without any experimental treatment over a period covering almost the complete lifetime of a laboratory mouse. For mice it is well known that patterns of FCM excretion differ significantly between sexes [3]. Nevertheless, for animal welfare reasons we conducted the study exclusively on females because repeated separation and regrouping of male mice is not advisable. The necessary individual housing of males over a long period of time would result in chronic distress due to social isolation, which may result in false measurements of stress hormones that reflect the effects of isolated housing [33].

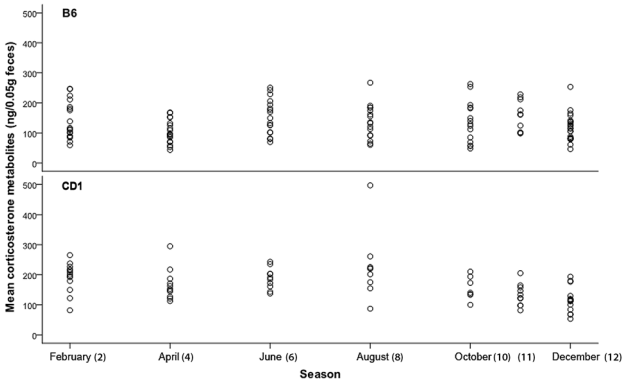

Fig 5. Mean concentrations of fecal corticosterone metabolites (FCM) per mouse and lifetime, pooled for the corresponding seasonal sampling points.

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The continuous increase of FCM concentrations in CD1 mice compared to the steady values measured for B6 mice demonstrated strain specific variability (Fig 3). Species differences in glucocorticoid secretion and metabolite excretion are well described [2, 26]. Our results indicate that even different strains of the same species might show differences in their adrenocortical activity. Reports about strain specific differences regarding stress hormone secretion are rare and inconsistent. Jones and co-workers found differences between C57BL/6 and DBA/2 mice regarding basal levels of plasma corticosterone and in response to stressors [8]. In contrast, the analysis of FCM of untreated C57BL/6J and C3H/HeJ mice revealed similar concentrations for both strains but strain specific differences in response to surgical stress and pain [34]. It should however be noted that both studies considered stress hormone values at a specific age of the animals, which may indeed be different at any given point in time depending on a mice's age, as it has been demonstrated by our results.

Basal levels of plasma corticosterone showed no differences between young and aged F344/N and Long-Evans rats [35, 36]. However, in response to acute stress, levels increased and took significantly longer to return to baseline in aged rats. This prolonged stress response could be explained by the observed age-related down regulation of glucocorticoid receptors in several brain structures [35, 36]. The FCM assay used in the presented study is not a snap-reading method but rather integrate hormone secretion over a period. A protracted elevation of corticosterone after stress, due to failure of the negative feedback response of the HPA axis, could thereby result in an elevated FCM concentration. The frequent occurrence of spontaneous cases of death in the CD1 strain significantly reduced sample size of the present study, especially for the age groups older than 12 months. However, this unexpected outcome may reflect a bad general constitution among animals of this outbred stock, resulting in a stressful period (measured as continuously increasing FCM concentration) towards the end of their life.

Although care was taken to standardize the procedure of feces collection, a symptomatic high level of variation in FCM levels was seen within both mouse strains of the study. Nevertheless, the diurnal changes of FCM followed the typical excretion profile as described before [6]. The results of our study clearly demonstrate a decreasing oscillation of FCM concentration towards the end of life for both analyzed strains (Fig 4). This is consistent with the previously reported modest circadian variation of plasma corticosterone levels for animals of the oldest group of 3-, 9- and 16-month-old male C57BL/6J mice [37]. Interestingly, our observations also follow the results of a study on diurnal cortisol profiles in human adults in which the likelihood for a flattened profile (in relation to the normative profile) was increased in older subjects with reportedly poorer health conditions [38, 39].

Disturbance of the cortisol rhythm is a common symptom for patients suffering from major depression [40, 41]. In addition to its impact on several regions of the central nervous system, glucocorticoids also play a key role in regulating appropriate circadian clock mechanisms in peripheral tissues and organs [42]. In CD1 mice it has been shown that the daily rhythm of glucocorticoids is strongly involved in synchronization of the liver circadian transcriptome [43]. Since changes in biological rhythms could be a sensitive indicator for impaired health and well-being, the level of stress hormone secretion, supplemented by its circadian patterning, can improve assessment of an individual's condition [44].

The here presented long-term study also enabled us to investigate any seasonal effects on FCM concentration. Seasonal rhythmicity for glucocorticoid concentrations have been reported for free-living species of different taxa [45]. This is not surprising because factors like changing climate conditions, periods of competition for food or mating partners and the reproductive status of females are likely to influence release of the stress hormones. The circadian regulation of corticosteroid secretion does not depend on the rhythmic release of ACTH but rather results from diurnal variation in adrenal responsiveness to ACTH [46, 47]. This intrinsic

rhythm of response is activated by light stimuli via the suprachiasmatic nucleus and the sympathetic nervous system. Interestingly, up to an intensity of 40 lux, light increased the corticosterone level in a dose dependent manner [46].

By comparing the measured FCM data between predetermined time points during the seasons we were able to identify specific variations. Significant differences were more distinct between seasons of short days with sparse daylight (winter) and seasons with longer daylight hours (summer) in both strains (Fig 5). Considering the fact that such important seasonal factors as changes in light-dark cycles, light intensity, room climate, food supply and food quality, or variation in the breeding seasons can be excluded in our study, we can only speculate that (domesticated) laboratory mice may still be subjected to intrinsic mechanisms. These mechanisms have evolutionary significance in that they enable an organism to alleviate repeatedly stressful situations in its natural habitat. In other words, circannual rhythm of corticosteroid secretion could be internalized like circadian oscillation which also stays active even if external conditions are constant (for example constant darkness). Therefore, the possibility of seasonal effects on stress hormone secretion should be considered in spite of highly standardized environmental conditions of laboratory animal facilities.

In summary, measurement of fecal corticosterone metabolites over the lifetime of untreated mice revealed a significant impact of age on hormone levels and on its circadian changes. The age-related impact was strain specific for mean FCM values over the test period but identical for both strains in form of a continuous flattening of the diurnal rhythm with increasing age. Therefore, mouse strain, age of animals and circannual rhythmicity all should be taken into consideration as possible modifying factors to improve comparability and reproducibility of FCM data measured in animal experiments.

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Author Contributions

Conceived and designed the experiments: TK TR. Performed the experiments: TK RP. Analyzed the data: TK RP AT TR. Contributed reagents/materials/analysis tools: TK RP TR. Wrote the paper: TR.

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5. Discussion, Conclusions, and Prospects:

Assisted fertilization in the porcine model is markedly restricted by a high parthenogenetic cleavage rate of oocytes prior to fertilization and a high rate of polyspermic fertilizations. Therefore, the straightforward approach to inject single sperm cells directly into the cytoplasm of oocytes (ISCI) seemed promising compared to *in vitro* fertilization. As expected, the injection of fresh sperm into *in vivo* matured oocytes resulted in the highest fertilization rate compared to all other tested combinations of fresh and frozen material.

Imperfect cytoplasmic maturation of *in vitro* matured oocytes was identified as the probable cause for the high rate of parthenogenesis and fragmentation after sperm injection [7]. Moreover, the superiority in fertilization potential of fresh ejaculated *in vitro* capacitated spermatozoa indicates that the quality of sperm cells also has an impact on the success rate of ICSI. This was not necessarily to be expected, because the sperm head is injected directly into the cytoplasm of the oocyte without having to penetrate barriers such as *cumulus oophorus*, *zona pellucida* or the vitelline membrane. An activation of the oocytes after ICSI was applied by Ca ionophore treatment [8]. However, the activation process primarily affects female chromosomes, and less so the injected spermatozoa (as demonstrated by increased parthenogenesis rates). No higher cleavage rates could be achieved by the artificial activation (publication 1).

In a next step we selected and applied the most successful protocol of the previous *in vitro* fertilization experiments to achieve a pregnancy after transfer of embryos produced by ICSI. According to publication 1, *in vivo* matured oocytes were injected with fresh capacitated sperm cells without any further activation treatments. Cleaved oocytes were transferred surgically into the oviducts of synchronized gilts. In pigs a minimum number of five implantations are required to generate a sufficient hormonal signal and perpetuate pregnancy [5]. To circumvent this in case of a low embryonic survival rate we applied oestradiol benzoate to the surrogate mother from day 10 to 16 after transfer [9]. With this provision we succeeded in sustaining a pregnancy which resulted in a single born piglet (publication 2). This result demonstrated the feasibility to generate live piglets via ICSI. However, for routine application the embryonic survival rate has to be significantly increased to avoid the use of oestradiol treatment and achieve a regular litter size after embryo transfer.

Consecutive studies of other groups in the field concentrated their research to increase quality and suitability of the sperm cells for ICSI. Head-membrane damaged sperm cells and sperm treatment with hyaluronic acid did not really increase fertilization rates. A very recent paper describing sperm selection by a percoll gradient could increase the fertilization rate of *in vitro* matured oocytes to 66% and the blastocyst rate to 36% [10]. Assuming a reproducibility of the published results a regular litter size after embryo transfer should be feasible.

Various morphological characteristics of embryos like zona thickness, granularity, perivitelline space and oocyte shape are used to predict embryo development and implantation potential. These correlations however are not always predictive. In a recent study the viscoelastic properties of human and murine zygotes were tested by micropipette aspiration to evaluate the quality of embryos [11]. In a study of our own we compared the *zona pellucida* properties between *in vitro* and *in vivo* derived porcine embryos with regard to enzymatic digestibility in order to determine factors crucial for the fertilization process and subsequent embryo development (publication 3). A lack of protein secretion by the oviduct might be responsible for the observed differences between both groups and could be the reason for high polyspermic fertilization rates during *in vitro* fertilization. A 24h organ co-culture with excised oviduct tissue from sows improved the porcine *zona* properties markedly, resulting in increased resistance to the digestive protein. Oviduct-specific expression of various proteins is described in various species (mouse [12], hamster [13], rabbit [14], sheep [15], cow [16] and sow [17]). Therefore, improved *in vitro* culture systems with other protein supplements seem to be required for a more successful porcine *in vitro* embryo production [18].

Changing to mice as the main focus and the technique of embryo transfer, the question arose if a repeated use of foster mothers is technically possible and with regard to animal welfare justifiable. Repeated transfer was feasible without hindrance by adhesions within the surgical area which was confirmed by histologic analysis. Reproductive results were comparable between first time and second time impregnated surrogate mothers. Measurement of corticosterone metabolites in feces [19] showed no increased stress hormone secretion after the second round of embryo transfers (publication 4). The only drawback is the highly unpredictable onset of a new estrus as precondition for mating with vasectomized males to induce pseudopregnancy. Mice naturally use the *post partum* estrus to become pregnant again. At the time of lactation the regular estrus cycle is suppressed and our results indicate a delayed restart after weaning. The average time period to become

pseudopregnant after weaning was 4.3 days longer for surrogates already impregnated once compared to the mating of virgin females. This disadvantage has to be counteracted by an appropriate break before reuse and additional pheromonal stimulation is required to induce the Whitten effect [20].

An important animal welfare question was related to superovulation and mating of prepuberal mice routinely used for the C57BL/6 strain. We confirmed the published data that superovulation of prepubescent mice results in higher numbers of embryos compared to treated adult females. Importantly, we could demonstrate by non-invasive stress hormone measurements that young females don't succumb to higher levels of stress due to the hormone injections and mating with adult males [21] (publication 5) than it would be seen in adult females of the same strain. Therefore, the use of prepuberal donors promotes the 3Rs (here: reduction) because less animals are required for the same number of oocytes or embryos harvested per donor. Histology of the vaginal epithelium revealed the expected synchronal induction of the estrus by the hormonal treatment only in immature females. In contrast, in adult females the success of superovulation is influenced by the endogenous ovarian cycle. Compared to the first induction of the still quiescent ovarian cycle in prepuberal mice, adult females have only a short appropriate time window during each cycle for successful hormonal treatment.

In a follow up study five years of IVF data with prepuberal and adult donors were analyzed; the advantageous results using young donors for superovulation was proven which confirmed our previous experimental results under routine conditions (publication 6).

The measurement of stress hormones to define the wellbeing of animals during our experimental procedures indicated a methodological problem: some of the measurements lasted for a longer period (several months) or animals of different age groups were compared (e.g. young versus old recipients). Although, most important factors affecting the level of stress hormone metabolites in feces have been investigated, there was no available data for specific age groups, nor was there available information in relation to the changes experienced during the life span of a mouse. In a long-term study we addressed this question by regularly sampling the feces of two different frequently used mouse strains (publication 7). Results of the study strongly suggest a significance of age for mouse models as an influencing factor in the variation of stress hormone levels. The degree of change during the life time of a mouse proved to be strain specific. Furthermore, we detected strong seasonal variations regardless of a highly standardized laboratory

environment. Therefore, to improve the reproducibility of measured levels of stress hormones we suggest an appropriate and thorough consideration of these additional factors.

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