Intrafollicular Oocyte Transfer (IFOT) of Abattoir-Derived and In Vitro-Matured Oocytes Results in Viable Blastocysts and Birth of Healthy Calves

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ABSTRACT

There are still major differences between in vitro production (IVP)-derived and in vivo-derived bovine blastocysts. Therefore, intrafollicular oocyte transfer (IFOT) was used in the present study to allow early embryonic development within the physiological oviductal environment, in order to avoid subsequent harmful effects of the in vitro culture environment. Using modified ovum pickup equipment, in vitro-matured oocytes were transferred into the preovulatory follicle of synchronized heifers (follicular recipients), enabling subsequent ovulation, in vivo fertilization, and in vivo development. When 1646 in vitro-matured oocytes were transferred to 28 follicular recipients, a total of 583 embryos (35.2%) were recovered in excess after uterine flushing at Day 7. Although numbers of generated extra embryos were highly variable, preovulatory follicles with a diameter of 13–14 mm delivered significantly ($P < 0.05$) larger amounts of extra embryos (34.3 vs. 7.3), as well as extra morulae and blastocysts (8.3 vs. 0.8), compared with follicles with a diameter of 9–10 mm. Nevertheless, the developmental rate to the blastocyst stage was lower in IFOT compared with in vitro-derived control (Vitro) embryos at Day 7 (8.0% vs. 36.5%). Likewise, cumulative developmental rates to the morula or blastocyst stage until Day 7 were lower in IFOT-derived embryos when related to the number of transferred (8.4% vs. 51.7%) or flushed (22.8% vs. 51.7%) embryos. Of the latter, IFOT-derived embryos yielded significantly lower cleavage rates compared with the Vitro controls (63.2% vs. 88.8%), and developmental rate to the morula or blastocyst stage were lower even when related to the proportion of cleaved embryos (36.8% vs. 58.2%). In contrast, lipid content and cryotolerance did not differ between IFOT and fully IVP embryos; but IFOT-derived embryos showed significantly lower lipid content ($P < 0.05$) and significantly higher cryotolerance compared with IVP-derived embryos cultured in CR1aa medium supplemented with estrus cow serum (ECS), but not when cultured in SOFaa medium supplemented with fatty acid-free BSA (BSA-*FFA*). Finally, transfer of 19 frozen-thawed IFOT-derived blastocysts to synchronized recipients (uterine recipients) resulted in pregnancy rates comparable with those obtained after transfer of fully in vivo-derived embryos or IVP-derived embryos cultured in SOFaa + BSA-*FFA*, whereas pregnancy rate following transfer of IVP-derived blastocysts was significantly lower when they were cultured in CR1aa + ECS (42.1% vs. 13.8%). All in all, seven pregnancies presumed to be IFOT derived went to term, and microsatellite analysis confirmed that five calves were indeed derived from IFOT. To our knowledge, these are the first calves born after IFOT in cattle. Interestingly, the average birth weight of IFOT-derived calves was lower than that of IVP-derived calves, even when embryos were cultured in SOFaa + BSA-*FFA*, indicating that the environment during early embryo development might cause fetal overgrowth. Taken together, for the first time we were able to show that IFOT is a feasible technique to generate bovine blastocysts by transferring in vitro-matured oocytes derived from slaughterhouse ovaries. These IFOT-derived blastocysts closely resemble in vivo-derived blastocysts in terms of lipid content and freeze survival. Thus, the present study laid the groundwork for newly created scientific experiments enabling novel analytical possibilities. Nevertheless, IFOT-derived embryos still reached lower pregnancy rates by trend compared with in vivo-derived embryos, also implicating an important role for the maturational environment in further developmental characteristics.

INTRODUCTION

The in vitro production (IVP) of bovine embryos is now a well-established technique; however, IVP bovine embryos still differ in many aspects from in vivo-derived embryos, as reviewed earlier [1]. Most importantly, IVP embryos are of lower viability compared with embryos developed in vivo [2, 3], representing a major hurdle for further implementation of this embryo technology. Generally, only 30%–40% of oocytes derived from slaughterhouse ovaries are competent enough to develop into a blastocyst after in vitro culture [1], whereas in vivo ~90% of ovulated oocytes are fertilized after insemination, with most of them developing to the blastocyst stage [4]. Furthermore, even if the blastocyst stage is attained, the quality of in vitro-developed embryos is inferior to that of those produced in vivo in terms of cryoresistance [5], ultrastructure [6], microvilli [5], lipid content [7], and gene expression [8–10], as well as incidence of chromosomal abnormalities [11, 12]. Consequently, IVP bovine embryos exhibit lower developmental capacities following embryo transfer compared with fully in vivo-derived bovine embryos [13–15]. Moreover, various studies have shown that the production of embryos under in vitro culture environments resulted in not only altered

expression of transcripts related to metabolism and growth, but also altered conceptus and fetal development following transfer [8, 16, 17]. Importantly, addition of serum to embryo culture medium has been reported to cause major changes in the embryo transcriptome [18]. Even earlier, addition of serum containing fatty acids to embryo culture medium was reported to affect embryo quality [19, 20] as well as embryo survival after freeze-thawing [20, 21], whereas yield of blastocysts of good quality was reported to be greater when embryos were cultured in fatty acid-free media [22].

To overcome limitations of in vitro embryo culture, various experimental methods to produce embryos either in vivo or in vitro have been investigated. Because the crucial role of the oviduct in supporting early embryo development is largely accepted, as has been reviewed previously [23], mouse oviducts [24, 25], rabbit oviducts [26, 27], and sheep oviducts [13, 17, 28–32] have been used extensively in situ for the development of bovine embryos. More recently, Besenfelder and colleagues [33, 34] and Wetscher et al. [35] established a minimally invasive endoscopic technique that allows access to the bovine oviduct in the live animal in order to perform comparative in vivo versus in vitro studies, and finally, tubal transfer and flushing were combined for in vivo culture of IVP embryos. These studies and others clearly showed that in vitro developmental competence to the blastocyst stage is almost predetermined at the two- to four-cell stage [36]. In agreement, gene expression of one sister blastomere was predictive for in vitro developmental competence of the other blastomere to the blastocyst stage [37]. In contrast, although bovine preimplantation-stage embryos exhibit an enormous plasticity and tolerance for adaptation to various postfertilization culture environments, it was reported even earlier that in vitro culture of zygotes flushed out of the oviduct resulted in blastocysts of lower cryotolerance [32], whereas in the reciprocal experiment, culture of in vitro-derived bovine zygotes in vivo in the ewe oviduct dramatically increased the quality of the blastocysts with respect to cryotolerance [13, 28, 32]. Having said this, intrafallopian transfer of bovine embryos demands a high level of skill, which prevents its widespread use.

As an alternative, a technique enabling the transfer of oocytes into the preovulatory follicle of an inseminated recipient mare (intrafollicular oocyte transfer [IFOT]) was first introduced by Fleming and colleagues [38] in 1985 in baboons and cattle. These authors reported the first ''extra'' embryo at the appropriate developmental stage recovered from seven cattle that ovulated after IFOT of ovum pickup (OPU)-derived oocytes. Although Fleming and colleagues obtained access to the ovaries by a flank incision, Hinrichs and DiGiorgio [39] were the first to adapt that technique to the equine by placing a trochar and a cannula through the abdominal wall in the flank area, which allowed the introduction of a needle through the cannula to puncture the outer wall of the follicle [39]. Thereafter, that technique was further improved by the use of transvaginal ultrasound in equine [40, 41], bovine [42], and human [43] reproduction.

Use of ultrasonically guided IFOT has generated ''extra'' embryos at Days 2–3 in three of eight heifers [42]. Likewise, IFOT generated embryos in excess in 4 of 10 mares when immature oocytes were transferred, and in 2 of 6 mares when matured oocytes were transferred [44]. Likewise, IFOT has led to early pregnancies [41]. Nevertheless, to date there have been no reports of a live foal generated by IFOT.

In the bovine, large-scale production of in vitro-matured oocytes is much more feasible because of the high availability of bovine ovaries at commercial abattoirs. However, because of the low cryotolerance of IVP embryos, >90% of the approximately 300 000 bovine IVP embryos transferred each year are transferred fresh, reducing the flexibility associated with the procedure [13, 28, 32]. Keeping in mind that low cryotolerance of bovine in vitro-generated embryos is suggested to be caused by suboptimal culture conditions after maturation [7], intrafollicular transfer of in vitro-matured oocytes into preovulatory follicles, followed by routine artificial insemination and uterine embryo flushing 7 days later, could offer an attractive alternative to generate large numbers of high-quality embryos.

However, to the best of our knowledge, development to the blastocyst stage in the bovine has not been reported after ultrasonically guided IFOT. Bergfelt and colleagues [42] reported extra embryos (5 of a potential of 36) recovered already at the two- to eight-cell stage, and Fleming and colleagues [38] reported only one extra embryo from one cow of seven at the appropriate developmental stage 7 days after transfer. But even when one would accept that appropriate developmental stage implies the blastocyst stage, one extra blastocyst could also be caused by double ovulation and is therefore not final proof that IFOT-derived embryos could develop to the blastocyst stage in the bovine, because no parentage analysis has been conducted. Moreover, in that study Fleming and colleagues [38] created access to the ovaries by a flank incision instead of emerging ultrasonically guided IFOT.

Moreover, no study has tried to transfer in vitro-matured bovine oocytes derived from slaughterhouse ovaries into the preovulatory follicle. Both studies dealing with IFOT in the bovine have transferred immature oocytes immediately after collection by OPU from synchronized [38] or superovulated [42] donors. With respect to the equine, the IFOT of immature oocytes derived from slaughterhouse ovaries has been reported in one study [39]. In that study, however, immature equine oocytes were directly transferred into preovulatory follicles via flank incision. Only one study has matured oocytes in vitro prior to IFOT thus far [44]. In that study, however, equine oocytes were collected by OPU from mares with a preovulatory follicle >33 mm that had been treated additionally with crude equine gonadotropin to induce ovulation. Thus, the proof of principle that ultrasonically guided IFOT of in vitro-matured oocytes derived from slaughterhouse ovaries enables development to viable blastocyst as well as development to term is still lacking in cattle.

Consequently, the first aim of this study was to test whether in vitro-matured oocytes would ovulate into the fallopian tube, be fertilized within the oviduct, and yield embryos that could be flushed out of the uterine environment 7 days later. The second aim was to determine the developmental characteristics of IFOT-derived embryos with respect to developmental rates, lipid content, cryotolerance, and viability after transfer to recipients compared with IVP and fully in vivo-derived blastocysts.

MATERIALS AND METHODS

Overall Experimental Design

In this study three types of bovine blastocysts were generated. First, we collected fully in vivo-derived bovine blastocysts (Vivo) generated by the artificial insemination of superstimulated Simmental heifers with the semen of one red Holstein Friesian bull. The same bull was also used for artificial insemination after IFOT and for in vitro fertilization (IVF). Second, we recovered bovine morulae and blastocysts by uterine flushing at Day 7 following IFOT of in vitro-matured black Holstein Friesian oocytes. In each case, fully in vitro-derived embryos (black Holstein Friesian oocytes fertilized with the semen of the same red Holstein Friesian bull as used for artificial inseminations) served as controls (Vitro). These three types of bovine embryos were subsequently analyzed with respect to early preimplantation develop-
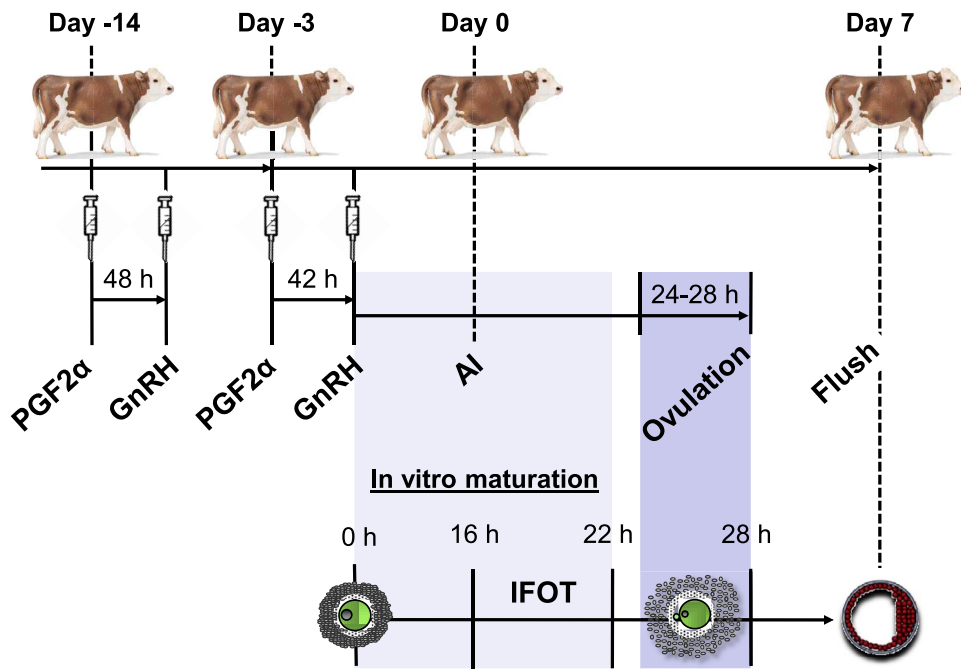
FIG. 1. Time schedule for IFOT: Simmental heifers were subjected to a synchronization protocol by two injections of 500 mg of cloprostenol (Estrumate) 11 days apart, followed by administration of 0.02 mg of GnRH (Receptal) 48 and 42 h thereafter, respectively. Oocyte maturation started at the time of the final GnRH injection, and IFOT was performed 16–22 h thereafter (Day 0), whereas ovulations were expected 24–28 h after final GnRH injection. Seven days later, the uteri of recipients were flushed to recollect IFOT-derived embryos (Day 7).

mental rates, grade of lipid accumulation, viability after freeze-thawing as well as development to term after freeze-thawing, and transfer to synchronized recipients. For assessment of viability after freeze-thawing as well as subsequent developmental competence, however, we took advantage of a second IVP control group. Whereas the initial vitro control embryos had been cultured in CR1aa medium supplemented with estrus cow serum (ECS), the embryos of the second control group were cultured in SOFaa supplemented with bovine serum albumin free of fatty acids (BSA-*FFA*). Care and use for all experimental animals within this study was done following the guidelines of the Society for the Study of Reproduction and was approved by the University of Bonn.

In Vitro Maturation of Bovine Embryos

Bovine ovaries of black Holstein Friesian genotype were obtained from a local slaughterhouse and brought to the lab in 30°C saline within 3 h. Cumulus-oocyte complexes (COCs) were aspirated from small follicles (2–8 mm), and COCs with a homogenous, evenly granulated ooplasm, with oocytes surrounded by at least three layers of cumulus cells, were transferred to modified Tissue Culture Medium 199 (TCM; Sigma) supplemented with 4.4 mM HEPES, 33.9 mM $NaHCO_3$, 2 mM pyruvate, 2.9 mM calcium lactate, 55 $\mu g \ ml^{-1}$ gentamycin, and 12% (v/v) heat-inactivated ECS. After washing COCs three times, they were cultured in groups of 50 in 400 μl of modified TCM supplemented with 10 $\mu g \ ml^{-1}$ FSH (FSH-p; Schering-Plough) at 39°C in a humidified atmosphere with 5% (v/v) CO_2 in air. In each case, in vitro maturation started at the same time that recipients received the final gonadotropin-releasing hormone (GnRH) injection and lasted 16–22 h until oocytes were transferred into a preovulatory follicle (see below).

Intrafollicular Oocyte Transfer

To perform IFOT, 28 Simmental heifers (~400 kg) were subjected to a synchronization protocol by administration of 500 mg of cloprostenol, i.m. (Estrumate; Essex Tierarznei). This treatment was repeated after 11 days. At 48 h after the first and 42 h after the second cloprostenol treatment, recipients received 0.02 mg of GnRH (Receptal; Intervet) to induce ovulation 24–28 h thereafter, as presented in Figure 1. Immediately prior to the IFOT procedure, 16 h after the final GnRH injection, heifers were artificially inseminated using fresh semen (50 million spermatozoa per dose) from the same Red Holstein Friesian bull used for superovulation and IVP of embryos. Intrafollicular transfer of oocytes (black Holstein Friesian genotype) was performed 16–22 h following the final GnRH injection according to the experimental design (Fig.

1). Consequently, day of IFOT was defined as Day 0. Because in vitro maturation was started at the time of the final GnRH injection, oocytes were matured for 16–22 h before transfer into the preovulatory follicles. Preparation of these 28 Simmental heifers for IFOT was similar to that usually done for OPU, as described elsewhere [45]. Briefly, heifers were restrained so that little movement was possible during intrafollicular oocyte injection. Relaxation of the rectal wall was achieved by an epidural anesthetic of 2.5 ml of 2% lidocaine (with adrenaline) to prevent abdominal straining.

The system to transfer the oocytes into the preovulatory follicle consisted of an OPU device. In detail, an endovaginal sector transducer (10 MHz), extended to a length of 50 cm and with a special grip, was used. This grip was equipped with a single needle guide. However, instead of a pump to generate negative pressure, the OPU line was connected with a syringe to fill the system with PBS, as well as with a lab pipette enabling the creation of positive or negative pressure (Supplemental Fig. S1; Supplemental Data are available online at www.biolreprod.org).

By rectal palpation, the ovary containing the preovulatory follicle was placed against the head of the transducer that had been inserted into the vagina adjacent to the cervix. The preovulatory follicle appeared as a black, round, nonechogenic structure on the monitor, and its diameter was measured by calibration on the monitor. Only heifers with a preovulatory follicle larger than 9 mm were used for IFOT. For transfer to the follicle, oocytes were loaded into the tip of the OPU system represented by a disposable single-lumen needle (Ø 0.45 × 22 mm; G26; Sterican). A maximum volume of 200 μl of PBS containing 60 oocytes (black Holstein Friesian genotype) was aspirated using the lab pipette connected to the system. Immediately thereafter, the transducer was positioned so that the preovulatory follicle became visible (Supplemental Fig. S2A). When it was positioned correctly, the needle was pushed carefully through the vaginal wall and peritoneum. When the echogenicity of the needle became visible, special attention was given to the needle going through as much ovarian stroma as possible before reaching the follicle, to avoid leakage. When the tip of the needle was positioned in the antrum near the center of the follicle (Supplemental Fig. S2B), this was directly followed by injection of 200 μl of PBS previously loaded with 60 oocytes with the help of an assistant admitting pressure using the connected pipette. Delivery was confirmed by observing an echogenic flurry or swirling motion within the follicle antrum upon infusion (Supplemental Fig. S2C). After expelling all oocytes, the needle was immediately withdrawn from the antrum while we continued to view the punctured follicle on the ultrasound monitor (Supplemental Fig. S2D). Immediately after transfer, the needle was flushed with 1 ml of PBS to ensure that no oocytes had remained in the needle during the procedure. If this

IVF and In Vitro Embryo Culture

Fertilization was performed in Fert-TALP medium [46] supplemented with 20 µM penicillamine, 10 µM hypotaurine, 2 µM epinephrine, 6 mg ml^{-1} BSA-*FFA*, 50 µg ml^{-1} gentamycin, and 1 µg ml^{-1} heparin. For IVF, semen of the same sire (red Holstein Friesian genotype) as used for the generation of IFOT and VIVO embryos was used. Final concentration of sperm in fertilization droplets was adjusted to 2×10^6 sperm per milliliter. Following 18 h of coculture the presumptive zygotes were washed three times and were transferred to in vitro culture. Embryo culture was performed in groups of 50 in humidified atmosphere with 5% (v/v) CO_2 in air at 39°C for up to 8 days in 400 µl of CR-1aa culture medium [47] supplemented with 10% ECS, or SOFaa [48] supplemented with 0.4% BSA-*FFA* overlaid with mineral oil, respectively. Developmental competence of embryos was recorded 48 h after placing the embryos into culture by counting the proportion of cleaved embryos, and at Day 7 by counting the number of embryos that had reached the morula and/or blastocyst stage.

Superovulation Protocol

To generate in vivo-derived blastocysts (Vivo), 20 Simmental heifers (~400 kg) were subjected to a superovulation protocol. Synchronization was performed by administration of 500 mg of cloprostenol, i.m. (Estrumate). This treatment was repeated after 11 days. Two days after each of the cloprostenol treatments, cows received 0.02 mg of GnRH (Receptal). Twelve days after the last GnRH administration, cows received the first of eight consecutive administrations of FSH during the course of 4 days in decreasing dosages (in total, 300–400 mg of FSH equivalent according to body weight; Stimufol, University of Liege). At 60 and 72 h after the initial administration of FSH cows received two treatments of cloprostenol. Finally, 48 h after the first of the two cloprostenol applications, ovulation was induced by 0.02 mg of GnRH and heifers received a first insemination. Artificial insemination was repeated twice within a 12-h interval (i.e., every cow received three inseminations).

Embryo Flushing

Seven days after the IFOT procedure (day of heat was defined as Day 0), embryos were flushed from the uterus of Simmental heifers that had undergone the IFOT procedure (n = 28). Likewise, embryos were flushed out of the uterus from heifers that had undergone superovulation treatment 7 days after artificial insemination performed on the day of heat. Briefly, an embryo-flushing catheter (CH15; Wörrlein) was fixed in the uterine horns. Subsequently, embryos were flushed out by draining each uterine horn with 500 ml of PBS. The uterus was flushed using a catheter connected with an embryo filter (Emcon filter 1; no. 04135; Immuno Systems Inc.). Embryos were washed twice in PBS and were evaluated by developmental stage. To calculate exclusively the developmental rates of IFOT-derived embryos, the corresponding flushing results were subtracted by the native embryo. Therefore, one embryo of the highest category flushed out (blastocyst > morula > cleaved > noncleaved) was subtracted for each IFOT recipient to calculate accurate quantities of embryos flushed in excess. These subtracted embryos suggested to be derived from the respective native oocytes were excluded from further analysis except for final embryo transfer, to take the greatest care not to mistakenly classify in vivo-derived embryos as IFOT-derived embryos.

Quantitative Measurement of Lipid Content

To visualize the lipid content of blastocysts derived from different treatments semiquantitatively, embryos were stained with Oil Red and were evaluated under a fluorescence microscope as described for cell cultures elsewhere. Briefly, a 0.5% Oil Red (ORO; Sigma no. O0625) solution in isopropanol (100%) was prepared as stock solution. For every use, Oil Red stock solution was diluted in distilled water (60%:40%) to prepare an ORO working solution. For Oil Red staining, blastocysts were washed three times in PBS containing 1% polyvinyl alcohol, followed by fixation in paraformaldehyde (4%) for 2 min. Thereafter, fixed blastocysts were transferred to Oil Red working solution for 1 h at 24°C. Following that, blastocysts were washed three times in PBS containing 0.1% polyvinyl alcohol prior to analysis with a phase-contrast microscope allowing a semiquantitative measurement.

For quantitative measurement of lipid contents, pools of 10 Oil Red-stained blastocysts (biological replicates) were placed in 350 µl of isopropanol (100%) for 1 h, leading to diffusion of lipid-bound Oil Red out of the embryos and into the surrounding isopropanol. After centrifugation, the supernatant (300 µl) was analyzed by a conventional photometric plate reader using 96-well plates exposed to monochromatic light (490 nm) to measure absolute values for optical density (OD) based on the mean of 10 replicated measurements (technical replicates) of three pools of blastocysts (n = 3 for statistical analysis). Finally, absolute values for ODs were calculated to relative values.

Assessment of Cryotolerance

Bovine embryos derived from Vivo, IFOT, and Vitro (CR1aa + ECS and SOFaa + BSA-*FFA*) environments were cryopreserved by routine slow freezing to assess the ability to withstand cryopreservation. Briefly, bovine embryos at the late morula and early blastocyst stages were exposed to a commercial cryoprotectant solution (BoviPro, FA; Minitube) containing 1.5 M ethylene glycol and 0.1 M sucrose for 20 min. During this time they were loaded into 0.25-ml plastic semen straws. After equilibration in the cryoprotectant solution, the embryos were placed directly into a freezing chamber (Cryo chamber; Minitube) controlled by a freeze controller (CL5500; Minitube), at −7°C for 5 min. Induction of ice crystals was achieved by touching the upper column of medium with a metal rod precooled in liquid nitrogen. Bath temperature was held at −7°C for 5 min after seeding, followed by controlled cooling at −0.5°C/min to −35°C. The bath temperature was then held at −35°C for 15 min prior to plunging the embryos into liquid nitrogen to be stored for at least 1 wk until thawing. For thawing, straws were exposed to air for 5 sec, followed by plunging in 25°C warm water for 15 sec. Thereafter, to check competence for the survival of cryopreservation, embryos were transferred in 400 µl of CR1aa embryo culture medium overlaid with mineral oil and were cultured for a subsequent 72 h at 39°C in 5% CO_2 in humidified air as described above. Survival was defined as reformation of the blastocoel and its maintenance for 6, 22, 30, 46, 54, and 70 h. Likewise, expansion as well as hatching was recorded for each time point, respectively.

Embryo Transfer and Pregnancy Monitoring

To analyze viability after freeze-thawing and direct transfer into synchronized recipients, blastocysts derived from Vivo, IFOT, and Vitro (CR1aa + ECS and SOFaa + BSA-*FFA*) environments were cryopreserved by slow freezing as described above. Thereafter, Simmental heifers of similar age (24 mo) were used as recipients for embryo transfer. Estrus was synchronized by administration of PGF$_2$α (2 ml of Estrumate), followed by a second administration 11 days later. Direct transfer of frozen-thawed embryos without any control of freeze-thaw survival was performed by nonsurgical standard procedures at Day 7 of the estrous cycle. Therefore, straws were exposed to air for 5 sec followed by plunging in 25°C warm water for 15 sec. Following transfer, pregnancy monitoring was done by ultrasonography at Days 28 and 35, and rectal palpation was done at later stages of pregnancy for those pregnancies that were allowed to go to term.

Parentage Control

Parentage analysis of calves resulting from transfer of IFOT-derived embryos was performed by comparison of the molecular genotypes of these calves with the molecular genotype of the bull used for artificial insemination as well as the molecular genotypes of the recipient heifers. The molecular configuration of 12 microsatellites was evaluated by an independent commercial company (Veterinary Institute of the Georg-August-University of Göttingen) using blood samples from recipient heifers as well as blood samples from calves resulting from the transfer of IFOT-derived embryos. Likewise, the molecular configuration of the sire used for artificial insemination was determined by analysis of a semen straw.

Statistical Analysis

The data for this study were analyszd by using the GLM procedure of the Statistical Analysis System (SAS) version 8.0 (SAS Institute Inc.) software package. Developmental rates after IFOT were determined on a per cow basis and were compared with developmental rates in IVP (control) on a per well basis by ANOVA. Mean numbers of extra embryos were tested on a per cow basis using ANOVA followed by a multiple pairwise comparison using Tukey test. Likewise, mean values for lipid content of contrasting embryo groups were tested using ANOVA followed by a multiple pairwise comparison using Tukey test. Numbers of embryos surviving cryopreservation as well as the proportion of transferred embryos resulting in a pregnancy were compared by χ^2 analysis. In each case, differences of $P < 0.05$ were considered to be significant. Finally, mean values for gestation length and birth weight of newly born calves were

TABLE 1. Uterine embryo recovery rates after intrafollicular oocyte transfer.

Heifer No.	Transferred oocytes	No. of developmental stages recovered in excess				
		Total	Cleaved	Morula	Blastocysts	Developed
1	60	0	0	0	0	0
2	57	0	0	0	0	0
3	58	1	0	0	0	0
4	57	29	11	4	2	6
5	60	60	47	14	5	19
6	60	28	12	3	0	3
7	60	5	4	0	2	2
8	60	14	9	2	0	2
9	60	27	21	7	3	10
10	59	10	7	5	0	5
11	60	30	24	4	1	5
12	41	9	7	2	1	3
13	59	19	18	2	6	8
14	60	5	5	1	0	1
15	60	38	17	2	0	2
16	56	23	18	2	3	5
17	60	11	9	0	0	0
18	60	27	13	4	3	7
19	60	22	19	3	0	3
20	60	21	7	1	0	1
21	60	16	6	1	1	2
22	60	14	9	1	1	2
23	60	54	43	8	12	20
24	60	50	37	8	6	14
25	60	57	35	8	7	15
26	60	8	8	2	1	3
27	60	2	1	1	0	1
28	59	3	0	0	0	0
Total	1646	583	387	85	54	139
Mean ± SD		20.8 ± 17.7	13.8 ± 13.0	3.0 ± 3.3	1.9 ± 2.9	5.0 ± 5.7

tested using ANOVA followed by a multiple pairwise comparison using Tukey test.

RESULTS

Preimplantation Development after IFOT

After synchronization, heifers were checked for the presence of a single preovulatory follicle (>9 mm in diameter). Consequently, 28 Simmental heifers were chosen for IFOT. When a total of 1646 in vitro-matured bovine oocytes (black Holstein Friesian) were transferred into the preovulatory follicles of these 28 synchronized heifers (follicular recipients), a total of 583 extra oocytes/embryos (35.2%), including unfertilized ova, early cleavage-stage embryos, morulae, and blastocysts, were re-collected (Table 1). Considering these 28 flushings, a total of 23 heifers that underwent IFOT (82.1%) gave embryos developed to the morula or blastocyst stage above the one expected from their own oocyte (Fig. 1). However, total flushing outcome was highly variable, with an embryo recollection rate of 20.8 ± 17.7 out of approximately 60 transferred. Of these, the mean number of cleaved extra embryos was 13.8 ± 13.0, as presented in Table 1. Accordingly, the mean number of developed extra embryos encasing morulae and blastocysts was 5.0 ± 5.7. Among these, the mean number of extra morulae was 3.0 ± 3.3 and the number of extra blastocysts 1.9 ± 2.9, as demonstrated for each flushed recipient heifer in Table 1. In accordance, based on the number of total transferred oocytes, the developmental rates to the morula or blastocyst stage until Day 7 were significantly lower in IFOT-derived embryos than in Vivo-derived embryos when related to the quantity of transferred (8.4% vs. 51.7%) or recollected (22.8% vs. 51.7%) embryos. When based on the

latter, IFOT-derived embryos yielded significantly lower cleavage rates compared with the Vitro control (63.2% vs. 88.8%). This was also the case regarding the developmental rate of IFOT-derived oocytes when related to the proportion of flushed embryos that had cleaved differed from IVP-derived control embryos (36.8% vs. 58.2%), as presented in Table 2.

Together, re-collection rates as well as developmental rates were highly variable. Therefore, the amounts of extra embryos of different developmental categories were analyzed for different diameter categories of preovulatory recipient follicle (9–10, 11, 12, and 13–14 mm); a clear trend for higher mean numbers of extra embryos (13.8 vs. 20.6 vs. 17.8 vs. 34.3, respectively) as well as higher numbers of extra morulae and blastocysts (0.8 vs. 5.0 vs. 4.5 vs. 8.3, respectively) became apparent, with significantly lower values for preovulatory follicles of 9–10 mm in diameter compared with preovulatory follicles having a diameter of 13–14 mm ($P < 0.05$), as shown in Figure 2.

Lipid Content of Blastocysts Derived from IFOT

Light microscopic evaluation of IVP- and IFOT-derived embryos revealed a much darker coloration of IVP-derived embryos (Fig. 3A) compared with IFOT-derived embryos (Fig. 3B). In agreement, staining of embryo lipid droplets by Oil Red showed much higher lipid droplet accumulations in IVP-derived compared with Vivo-derived and IFOT-derived embryos (Fig. 4A). Likewise, pools of in vitro-derived embryos (n = 15; three replicates) showed a significantly higher OD for the lipophilic dye Oil Red after elution compared with fully in vivo-derived as well as IFOT-derived embryos, whereas the OD of IFOT-derived embryos did not

TABLE 2. Developmental rates of in vitro-matured oocytes after IFOT into dominant follicles at Day 7.

| | | | Reflushed | | Per reflushed | | | | | | | | Per cleaved morula/blastocyst stage, % | Per total morula/blastocyst stage, % |
| | | | | | Cleaved | | Morula stage | | Blastocyst stage | | Morula/blastocyst stage | | | |
Treatment	No. of replicates	Total No.	n	%	n	%	n	%	n	%	n	%		
IFOT	28	1646	583	35.2 ± 29.4	387	63.2 ± 26.7[a]	85	15.6 ± 12.5	54	8.0 ± 10.2[a]	139	22.8 ± 15.1[a]	36.8 ± 21.0[a]	8.4 ± 9.5[a]
Vitro	6	380			338	88.8 ± 2.7[b]	58	15.2 ± 3.4	140	36.5 ± 5.3[b]	198	51.7 ± 6.7[b]	58.2 ± 6.2[b]	51.7 ± 6.7[b]

[a,b] Values with different superscripts differ significantly (a:b $P < 0.05$; ANOVA, Tukey test).

differ significantly from that of in vivo-derived embryos (Fig. 4B).

Cryotolerance of Embryos Derived from IFOT

When frozen embryos derived from contrasting environments were thawed and cultured in vitro to analyze cryotolerance, embryos cultured in CR1aa + ECS (n = 51) exhibited significantly lower survival compared with embryos from the IFOT group (n = 39), Vivo group (n = 31), and SOFaa + BSA-*FFA* group (n = 30) 70 h after freeze-thawing (0% vs. 74% vs. 90% vs. 80%, respectively). Likewise, embryos cultured in CR1aa+ECS exhibited significantly lower expansion rates 70 h after freeze-thawing compared with embryos of IFOT, Vivo, and SOFaa + BSA-*FFA* groups (0% vs. 74% vs. 87% vs. 80%, respectively), whereas IFOT, Vivo, and SOFaa + BSA-*FFA* groups did not differ (Fig. 5, A and B) with respect to reformation and expansion rates. Hatching rate of CR1aa + ECS-derived embryos exceeded 12% at 30 h after thawing, but no CR1aa + ECS-derived embryo survived for longer than 54 h after thawing. Embryos from the Vivo, IFOT, and SOFaa + BSA-*FFA* groups had significantly higher hatching rates at 30 h compared with Vitro-derived embryos (52% vs. 36% vs. 40% vs. 12%, respectively), reaching even higher rates at 70 h (77% vs. 64% vs. 50% vs. 0%, respectively), as demonstrated in Figure 5C. When comparing the hatching rate based on the number of surviving embryos, however, no differences were apparent between embryos from the different groups (Fig. 5D).

Viability to Term of Frozen-Thawed Blastocysts Derived from IFOT

When a total of 19 IFOT-derived embryos that had been re-collected in batches of 6, 4, 2, and 7 embryos from 4 follicular recipients (F-Recipients A-D; Supplemental Table S1) were transferred after freeze-thawing to final uterine recipients synchronized to cycle Day 7, a pregnancy rate of 42.1% was recorded at Day 35, which was significantly higher ($P < 0.05$) than that obtained after transfer of 29 frozen-thawed CR1aa + ECS-derived embryos (13.8%). Likewise, transfer of frozen-thawed SOFaa + BSA-*FFA* derived embryos resulted in a significantly higher pregnancy rate ($P < 0.05$) compared with CR1aa + ECS-derived embryos (38.5% vs. 13.8%) but a lower rate compared with Vivo-derived embryos (not significant), as summarized in Table 3. Of these, eight pregnancies were established after transfer of presumed IFOT-derived embryos, seven pregnancies went to term, and seven healthy calves (three males and four females) were delivered. With respect to those calves presumed to be IFOT derived, two calves (one male and one female) showed a Simmental phenotype (red-and-white spotted, white face) resembling a crossbreed Simmental (recipient follicle's native oocyte) × red Holstein Friesian (semen) parentage, indicating that they were not IFOT derived but instead derived from the follicle's native oocyte (Fig. 6). The remaining five calves (two male and three female) expected to be IFOT derived resembled a perfect black Holstein Friesian phenotype (black-and-white spotted, no white face), indicating a black Holstein Friesian (slaughter-house ovaries) × red Holstein Friesian (Semen) parentage, as shown in Figure 6. Considering these five true IFOT-derived calves (confirmed also by later molecular parentage analysis), their mean gestation length was significantly shorter (273.3 vs. 277.0 days) and their mean birth weight was significantly lower (36.2 vs. 44.0 kg) compared with SOFaa + BSA-*FFA*-derived calves, whereas there was no difference compared with
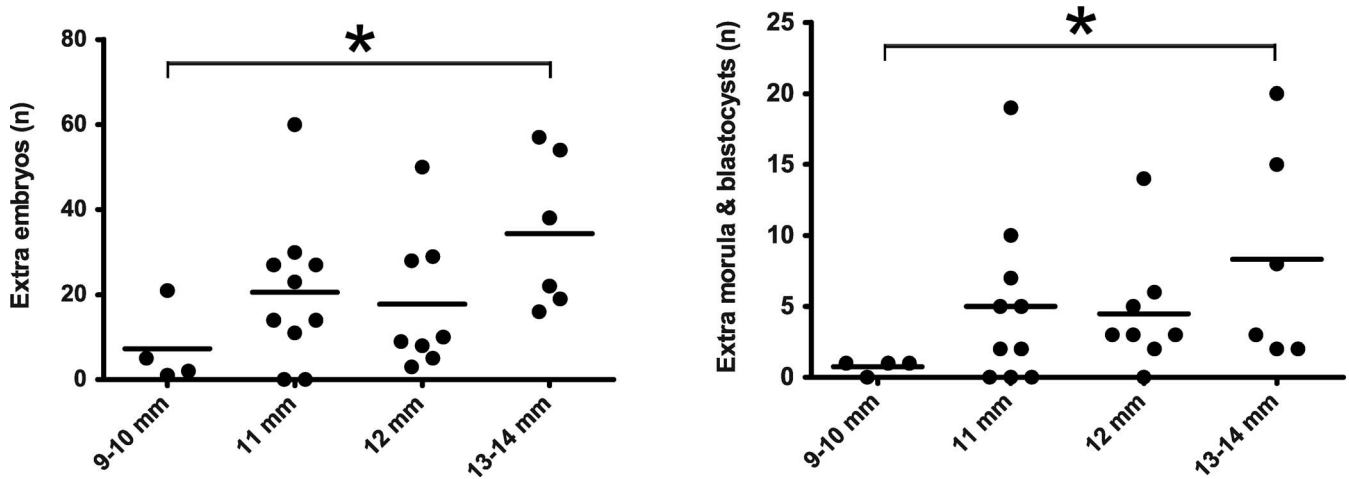
FIG. 2. Figure summarizes the number of extra embryos generated after IFOT in preovulatory follicles classified to contrasting categories based on their diameter at time of transfer. Smaller follicles (9–10 mm) resulted in significantly fewer extra embryos than larger follicles (13–14 mm) did. Among these, smaller follicles (9–10 mm) allowed development of fewer morula and blastocysts compared with larger follicles (13–14 mm) in mean. Asterisks indicate significant differences between groups.

fully in vivo-derived calves, as summarized in Table 3 and demonstrated for each individual calf in Figure 7. Furthermore, no statistical difference was observed between male and female calves in terms of pregnancy duration and birth weight.

Parentage Control

To prove that the five calves representing the phenotype of black Holstein calves were indeed truly IFOT derived and to exclude the possibility that the calves were derived from the follicular recipients (from the original oocyte of the preovulatory follicle), molecular genotyping of these calves considering 12 microsatellites was conducted. The configuration of these 12 microsatellites clearly showed that none of the five calves were descended from the follicular recipients (Simmental heifers), but all were descended from the red Holstein Friesian bull used for artificial insemination of these follicular recipients after IFOT was performed, as summarized in Supplemental Table S1.

DISCUSSION

In the present study we evaluated the feasibility of IFOT in cattle. The first aim of this study was to test whether in vitro-matured oocytes derived from slaughterhouse ovaries would complete their maturation and develop to blastocysts after

IFOT and insemination of recipient heifers. In the present study, we did not observe any effect of IFOT on the interval between GnRH administration and ovulation (M. Hoelker et al., unpublished data). This finding is in accordance with the findings of others performing IFOT [42] or intrafollicular insemination [49]. In our study, 23 of 28 recipient heifers (82.1%) gave morulae and blastocysts above the one expected from their own oocyte after IFOT. Development to the blastocyst stage in the bovine has not been reported after ultrasonically guided IFOT, to the best of our knowledge. Bergfelt and colleagues [42] reported the generation of bovine extra embryos after IFOT; however, these were recovered already at the two- to eight-cell stage. Fleming and colleagues [38] in turn reported one extra embryo from one cow, which, however, could be caused alternatively by a double ovulation and therefore does not represent a valid proof that IFOT-derived embryos could develop to the blastocyst stage in the bovine, because no parentage analysis has been conducted. Moreover, in that study Fleming and colleagues [38] created access to the ovaries by a flank incision instead of emerging ultrasonically guided IFOT, as conducted in our study. Indeed, the developmental capacity of IFOT-derived oocytes to the blastocyst stage for equine oocytes has been reported previously in some studies [39, 44]. However, the anatomical differences between equine and bovine ovaries may render the equine ovary much more suitable for IFOT because of the large
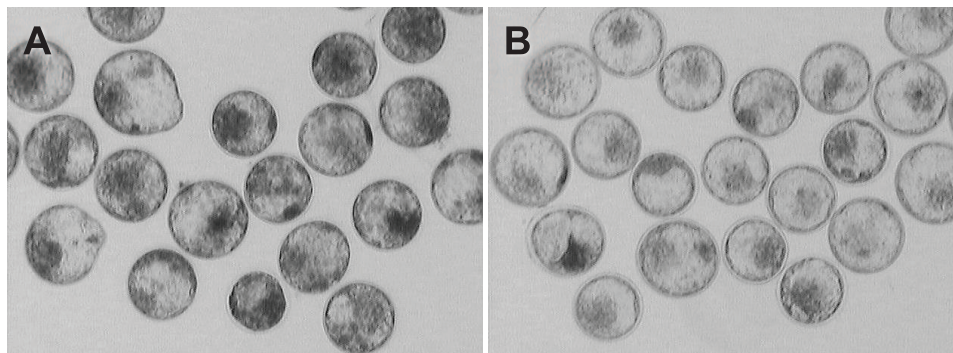

FIG. 3. Light microscopic views of fully in vitro (CR1aa + ECS)-derived Day 7 blastocysts (**A**) and Day 7 blastocysts derived from intrafollicular transfer of initially in vitro-matured oocytes (**B**), revealing a much lighter appearance for the latter ones. Original magnification ×100.

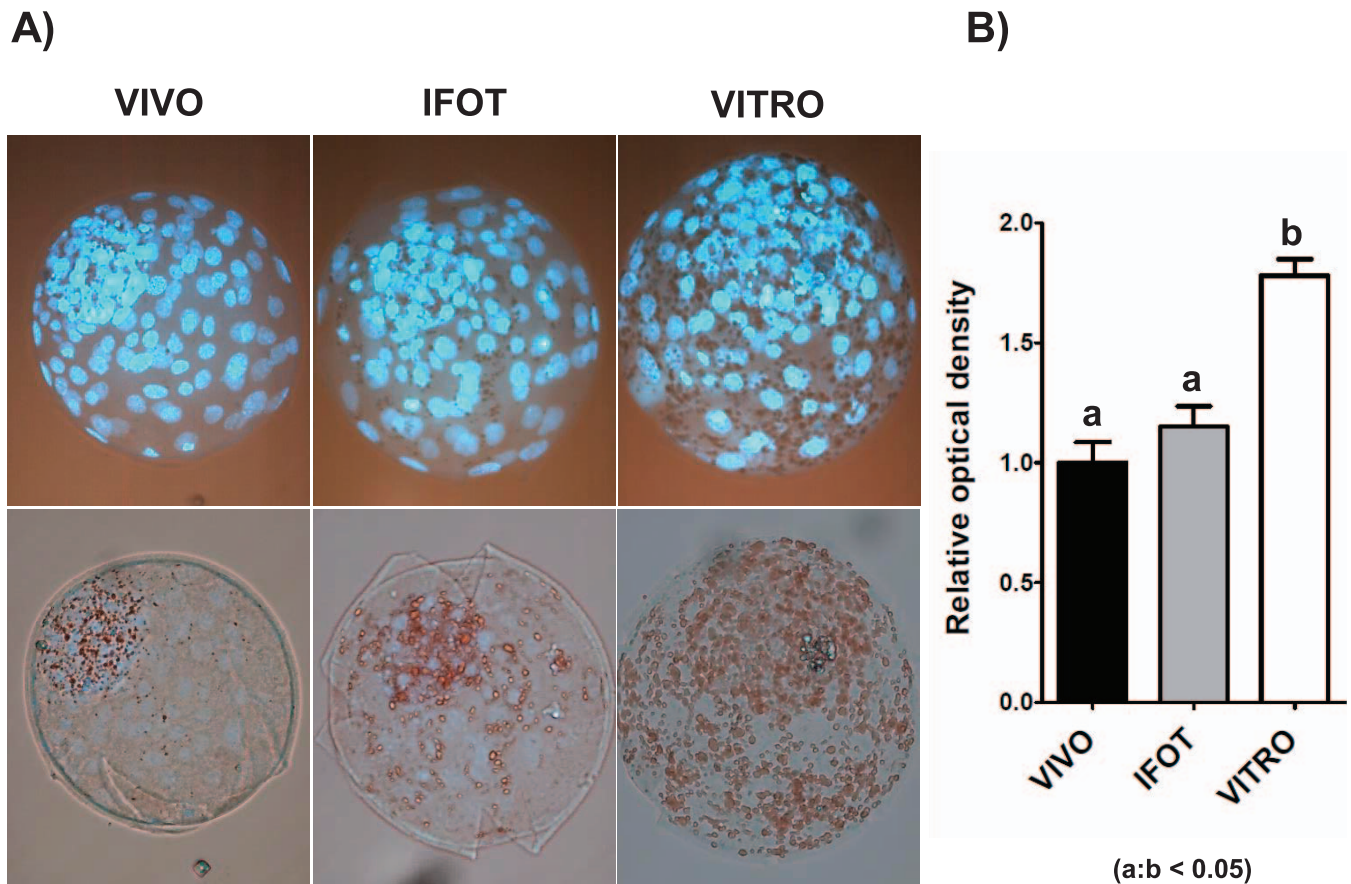Article 150

A)

B)

(a:b < 0.05)

FIG. 4. Representative fluorescence microscopic pictures of bovine blastocysts stained with Hoechst bisbenzimide to visualize cell nuclei (**A**, top line) and Oil Red stain to visualize lipid droplets (**A**, lower line), demonstrating lower lipid accumulations in vivo-derived and IFOT-derived blastocysts compared with in vitro-derived ones. Original magnification ×400. **B**, Likewise, pools of in vitro-derived embryos (n = 25, three replicates) resulted in significantly lower ODs for the lipophilic dye Oil Red after elution compared with fully in vivo-derived and IFOT-derived embryos (mean ± SD). Values with different superscripts differ significantly ($P \leq 0.05$).

size of the ovary, its specific anatomy with a peripheral medulla, and its surrounding fibrous tunic. Keeping that in mind, IFOT-related findings obtained in the equine cannot be transferred 1:1 to the bovine. Adversely, it is mandatory to perform the proof of principle for the bovine itself.

The large number of oocytes transferred (60 per follicle), however, makes it difficult to compare our findings with results achieved in earlier reports, in which 5–6 or 10–27 oocytes were transferred in the bovine [38, 42]. Therefore, it is hard to state whether our results are an improvement on those reported previously. Nevertheless, our study proved that IFOT could be a feasible technique even when transferring larger numbers of oocytes, compared with earlier reports. That might have a special impact in the bovine because we suggest the availability of recipients rather than access to high numbers of slaughter-house ovaries to limit IFOT in the bovine. However, our results indicated high variability with respect to recovery rates among individual recipient heifers. Although numbers of generated extra embryos were highly variable, preovulatory follicles with a diameter of 13–14 mm delivered significantly ($P < 0.05$) higher amounts of extra embryos (34.3 vs. 7.3) as well as extra morulae and blastocysts (8.3 vs. 0.8) compared with follicles with a diameter of 9–10 mm. Bearing in mind that oocytes had been mostly matured at time of IFOT, that could indicate that synchrony between recipient follicle and transferred oocytes might have been not optimal. Nevertheless, the fact that even among comparable follicle classes some heifers produced large

numbers of developed embryos in excess whereas other heifers failed to deliver extra embryos, might additionally indicate that there is a need to determine further factors relevant for the success of the IFOT procedure.

Looking at all recipients together, we were already able to re-collect an average proportion of 35.2% of oocytes and/or postfertilization embryonic stages 7 days after IFOT. These results are higher than those reported by the two studies that had performed IFOT in the bovine in the past [38, 42]. Contrary to our study, however, in these studies immature oocytes were collected by OPU from preovulatory follicles after synchronization of the estrus cycle [42] or even after superstimulation [38], suggesting in vivo prematuration of these oocytes within the follicle prior to final maturation to some extent. Adversely, we transferred in vitro-matured oocytes derived from slaughterhouse ovaries.

Our recovery rate was also much higher than that reported after IFOT in the equine using in vitro-matured oocytes [44]. Again, the large number of oocytes transferred (60 per follicle), however, makes it difficult to compare our findings with the results achieved in other studies. Likewise, these differences might be species specific, caused by contrasting anatomic ovarian structures, or due to technical details with respect to the IFOT procedure.

Notably, IFOT-derived embryonic structures showed lower cleavage rates compared with fully in vitro-derived embryos, with no significant differences among contrasting follicle
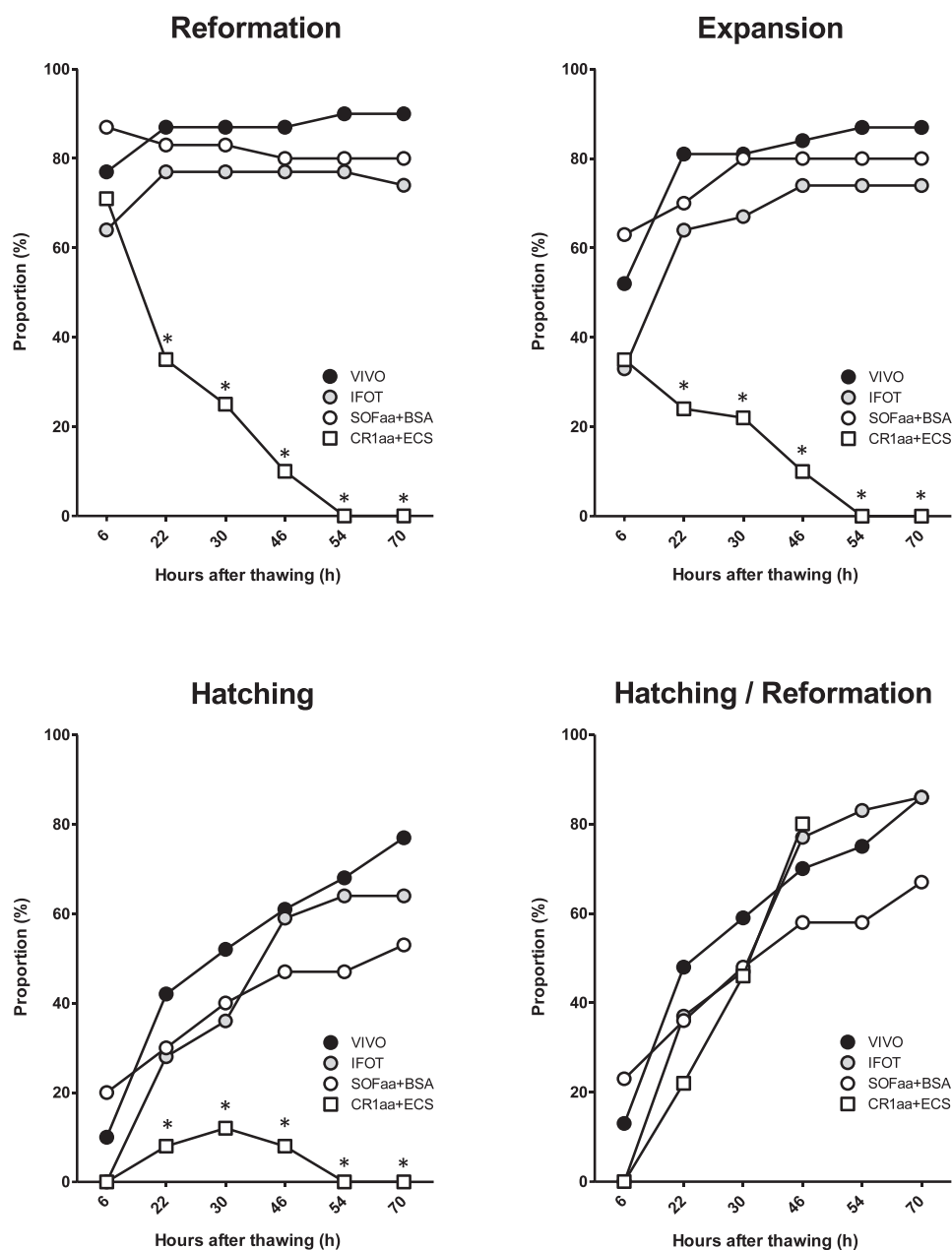
FIG. 5. Comparison of reformation, expansion, and hatching rates of in vivo-derived and IFOT-derived embryos compared with embryos derived from two contrasting Vitro environments (SOFaa + BSA-*FFA* and CR1aa + ECS) after freeze-thawing. Embryos derived from culture in CR1aa + ECS reached significantly ($P < 0.05$) lower reformation, expansion, and hatching rates compared with other groups, whereas there was no difference in terms of hatching rate per reformation rate among contrasting embryo groups. Asterisks indicate significant differences between groups.

TABLE 3. Effect of the postfertilization environment on developmental characteristics after transfer of frozen-thawed embryos.

Treatment	Total no.	Pregnant (Day 35)		Birth		Gestation length, days*	Birth weight, kg*
		n	%	n	%		
Vivo	21	13	61.9[a]	13	61.9	276.1 ± 4.2	37.3 ± 2.9
IFOT	19	8	42.1[a]	7	36.8	273.3 ± 1.1[A]	36.2 ± 1.8[A]
SOFaa + BSA	13	5	38.5[a]	5	38.5	277.0 ± 2.0[B]	44.0 ± 1.9[B]
CR1aa + ECS	29	4	13.8[b]	2	**	276.0 ± 4.2**	43.0 ± 4.2**

* Only calves confirmed to be IFOT derived by parentage analysis were considered.
** Only two pregnancies were allowed to go to term (value is based on two observations).
[a,b,A,B] Values with different superscripts differ significantly (a:b $P < 0.05$, χ^2 analysis/A:B $P < 0.05$, ANOVA, Tukey test).

FIG. 6. Photograph of all seven calves (ages 2–3 wk) born after transfer of IFOT-derived blastocysts to final recipients. Five calves represent the phenotype of black Holstein Friesian calves (black-and-white spotted, no white face), indicating a black Holstein Friesian (slaughterhouse ovaries) × red Holstein Friesian (semen) parentage, and therefore indicate that they are indeed IFOT derived. In contrast, two calves (marked by asterisks) show a Simmental phenotype (red-and-white spotted, white face), implicating a crossbreed Simmental (follicle's native oocyte) × red Holstein Friesian (semen) parentage, and therefore an indication that they are not IFOT derived.

categories with respect to diameter. The reason for that is unclear. However, one could suggest that a suitable synchrony between recipient follicle and oocyte maturity has not been achieved in all recipients. In addition, IFOT-derived embryos, even when developing within the physiological environment, did not reach higher developmental rates compared with fully in vitro-derived embryos cultured in CR1aa medium. That supports the finding that the maximum developmental competence of a given bovine embryo is already determined after cleavage at the two-cell stage, which is in accordance with our earlier study that demonstrated that maximum developmental competence is already predetermined by the four-cell stage [36], and in accordance with others using the ovine

oviduct for temporal in vivo culture of bovine embryos [28, 32, 50]. In fact, developmental competence to the blastocyst stage is correlated with gene expression profile at the two-cell stage, indicating that further developmental competence is already predetermined at the early cleavage stage [37].

Another suggestion is that some oocytes were already aged when ovulation occurred and fertilization took place, which is in agreement with the lower cleavage rate of IFOT-derived embryos compared with fully in vitro-derived embryos, as reported above. In agreement, duration of in vitro maturation prior to fertilization has been reported to correlate with further developmental competence [51, 52], which is additionally supported by our finding that smaller follicles suggested as
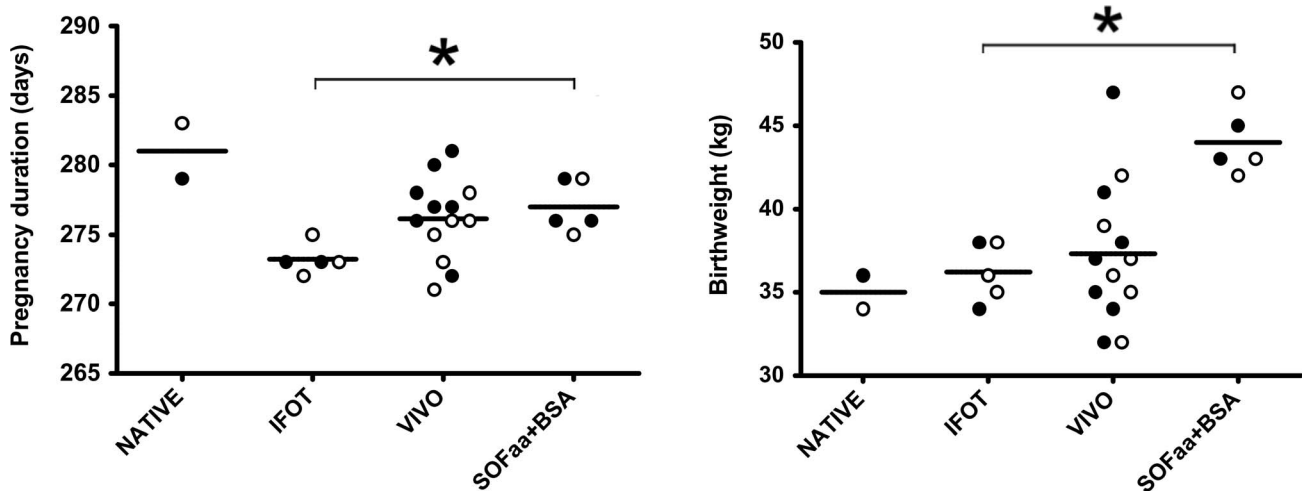

FIG. 7. Pregnancy durations and birth weights of each calf generated by embryo transfer within this study. Male calves are represented by solid plots, and female calves are represented by open circles. Individual values and means are presented on a per treatment basis taking into account 2 calves originating from the recipient follicle's native oocyte (NATIVE), 5 calves derived from IFOT, 13 calves generated by multiple ovulation and embryo transfer (VIVO), and 5 calves derived from IVP (SOF + BSA). Asterisks indicate significant differences between groups.

ovulating later than larger follicles generated lower numbers of extra morulae and blastocysts. Therefore, one future strategy to enhance oocyte-to-follicle synchrony could be the transfer of almost fully in vitro-matured oocytes closer to the expected time of ovulation. That may result in oocytes that are less aged at the time of ovulation, which in turn could enhance developmental rates and therefore overall efficiency. Adversely, one could inject oocytes not yet matured (less than 16 h of maturation), or even immature oocytes, into the preovulatory follicle. Indeed, the general success rate of IFOT tended to be higher when immature oocytes were transferred into mares in a recent study [44].

Without doubt, however, transfer of immature oocytes derived from slaughterhouse ovaries would be an attractive scientific option for the future, enabling the analysis of embryo-environmental interactions while using the same source of oocytes and/or embryos. Consequently, IFOT of immature oocytes would enable the generation of large groups of embryos that had been within the physiological environment during final nuclear maturation, fertilization, and early preimplantation development, without the need for superstimulation, which in turn could bear consequences for the embryo characteristics.

Considering those embryos that developed to the blastocyst stage, IFOT-derived embryos showed a significantly lower level of lipid accumulation compared with embryos cultured in vitro in CR1aa + ECS. That finding is in accordance with earlier findings reporting that the culture environment after fertilization can affect lipid accumulation [19, 20]. Because grade of lipid accumulation has in turn been correlated negatively with embryo cryotolerance [20, 21], we therefore analyzed the cryotolerance of IFOT-derived embryos in comparison with fully in vivo-derived and fully in vitro-derived embryos from two contrasting media for in vitro embryo culture. CR1aa + ECS was chosen as medium because it allows high in vitro development to the blastocyst stage and is known to produce embryos displaying a rather low cryotolerance, and SOFaa + BSA-*FFA* was chosen as a medium supporting high developmental competence to the blastocyst stage as well as high cryotolerance (M. Hoelker et al., unpublished data). Our results revealed that IFOT-derived embryos displayed a cryotolerance comparable to fully in vivo-derived embryos. These findings are in agreement with previous studies using the ovine oviduct [32, 53] as well as the bovine oviduct [54, 55]. Taken together, these results again confirm the general consensus that the postmaturation environment is the main criterion affecting cryotolerance of bovine blastocyst [7, 32, 54]. Importantly, embryos cultured in CR1aa + ECS displayed a much lower cryotolerance compared with embryos that had been developed within the physiological reproductive tract environment after maturation (Vivo and IFOT), whereas embryos that had been cultured in vitro in SOFaa supplemented with BSA-*FFA* did not display a significant reduction in terms of freeze survival. Thus, in vitro culture itself was not the main factor causing lower freeze survival, which is in agreement with earlier studies [7]. Also, the fact that both groups of embryos that had been matured in vitro showed a tendency for lower freeze survival compared with embryos derived from Vivo or IFOT might indicate that the maturational environment also affects to some extent later freeze survival, although it is not the predominant factor. Indeed, embryos from both vitro groups (CR1aa and SOFaa) had been matured in vitro using media supplemented with serum. Consequently, lower freeze survival might reflect long-lasting effects due to metabolic changes caused by serum supplementation at the time of maturation, hypothetically

resulting in increased amounts of lipid droplets after maturation. Because oocyte lipid droplets serve as an energy store for the developing embryo [56, 57], one could speculate that higher amounts of lipid droplets at the time of fertilization in turn result in relatively higher amounts at later developmental stages compared with embryos derived from in vivo maturation. This speculation is further supported by our finding that IFOT-derived embryos that had been matured in vitro in the presence of serum showed a slightly higher lipid content compared with fully in vivo-derived embryos by trend.

Finally, after transfer of 19 IFOT-derived embryos to recipients, 8 of them established an early pregnancy at Day 35. This pregnancy rate was significantly higher than that yielded after transfer of IVP-derived embryos previously cultured in CR1aa + ECS, but it did not differ compared with IVP embryos cultured in SOFaa + BSA-*FFA*. These results are in agreement with other studies [53] and closely resemble our findings with respect to cryotolerance. Moreover, fully in vivo-derived embryos did not yield significant higher pregnancy rates compared with IFOT-derived and SOFaa + BSA-*FFA*-derived embryos. Keeping in mind that the IFOT technique allows the comparison of in vivo-developed embryos originating from the same source of oocytes as fully in vitro-derived embryos—namely, from all follicle populations found on the abattoir-derived ovaries—these results again may indicate that the environment after maturation highly affects later viability at blastocyst stage. These findings indicate the necessity to optimize environmental conditions to enhance later pregnancy rates and term development after transfer of blastocysts to recipients. In agreement, a recent study reported that IVP embryos developed in the sheep oviduct can achieve a similar pregnancy rate after thawing and transfer compared with the pregnancy rate obtained from fully in vivo-developed embryos [17].

On the other hand, it was reported recently that follicle aspiration before ovulation results in a reduction in corpus luteum size and progesterone output [58]. Estrous synchronization involving GnRH-induced ovulation may lead to ovulation of a premature follicle, a smaller corpus luteum with lower progesterone output, and therefore a compromised uterine environment [59]. Indeed, follicular diameter at ovulation and the duration of proestrus have been identified as sources of variation in conception rate in cattle [59–61]. Because progesterone concentration in the first week after conception has in turn been shown to advance the normal temporal changes that occur in the uterine endometrial transcriptome [62], also advancing conceptus elongation in cattle [63, 64], a delay in the postovulatory rise in progesterone is associated with retarded conceptus development [65]. That would be also important given the linear association between conceptus size and interferon-tau production [66]. Bearing that in mind, we cannot exclude that insufficient formation of corpora lutea due to follicular injections had resulted in relatively low progesterone levels, negatively affecting the developmental capacity of IFOT-derived embryos, because we did not measure the progesterone level of follicular recipients.

Interestingly, calf birth weights as well as pregnancy duration were significantly higher in embryos derived from SOFaa medium compared with embryos derived from IFOT. In agreement, it has been known for more than a decade that culture and manipulation of bovine embryos can be associated with later developmental abnormalities in fetuses and calves [2, 67, 68]. This alteration in phenotype, called "large offspring syndrome" (LOS), has also been observed in sheep [69] and mice [70, 71]. Among in vitro embryo culture conditions, supplementation with serum has been especially shown to

affect the birth weights of calves [8], and in both cattle and mice to alter expression of metabolic and growth-related genes in preimplantation-stage embryos [8, 16, 71].

In our study, however, birth weights of calves were enhanced when embryos had been cultured in vitro, even in the absence of serum (SOFaa + BSA-*FFA*), to the blastocyst stage. Although culture of embryos in SOFaa + BSA-*FFA* produced blastocysts that did not differ compared with IFOT- or Vivo-derived blastocysts with respect to cryotolerance and the ability to initiate a pregnancy after freeze-thawing, culture in SOFaa + BSA-*FFA* resulted in higher birth weights, indicating postmaturational implications of the culture environment for fetal growth. Bearing in mind that IFOT-derived calves did not show any indications of LOS in our study, our results might also indicate that only the environment after maturation is relevant for the formation of LOS. However, care should be taken not to overstress these results because the absolute number of calves considered is low (n = 5 for both groups).

Finally, it has to be mentioned that the IFOT technique is not without drawbacks. The major limitation is caused by the fact that the preovulatory follicle bears a native oocyte. Consequently, after ovulation and fertilization that oocyte could also develop to an embryo and might be among the other embryos when recovered at Day 7. Obviously, this native embryo is fully in vivo and not IFOT derived. Although we did not perform parentage analysis for each individual embryo, in order to address concerns the most progressed embryo of each cohort of flushed embryos per recipient was excluded from further analysis except for final embryo transfer. The procedure that aimed to avoid further analysis of embryos suggested to be IFOT while actually being in vivo derived was carried out with the greatest care and was based on the assumption that these embryos might largely resemble those derived from the heifer's native oocyte. Nevertheless, in any case, we cannot fully exclude the possibility that the embryo derived from the native oocyte might not be the most progressed one. In contrast, blastocysts were transferred to recipients without exclusion of the one presumed to be derived from the native oocytes because there was the possibility of performing parentage analysis after birth without compromising the blastocyst's viability. In contrast, taking an embryo biopsy for parentage analysis could decrease embryo viability. Concordantly, for transfer of IFOT-derived embryos we selected embryos that had been recovered in larger cohorts (seven, six, four, and two embryos) to minimize the risk that an embryo suggested to be IFOT derived was actually derived from the native endogenous oocyte. Nonetheless, two of seven calves born after transfer of embryos suggested to be IFOT derived were identified as in vivo derived. These two calves were identified according to their coat color and patterning as well as based on molecular analysis emerging microsatellites. On the other hand, microsatellite analysis clearly confirmed that the remaining five calves originated from the sire used for artificial insemination (red Holstein Friesian) but did not originate from follicular recipient heifers (Simmental), and thus did not originate from a native oocyte of a preovulatory follicle. To our knowledge, these five calves are the first calves born after IFOT in the bovine in particular, as well as after transfer of in vitro-matured oocytes in general.

Taken altogether, the present study established the proof of principle that IFOT is a feasible technique to generate high-quality embryos capable of developing to apparently high-quality blastocysts as well as healthy calves. Taking advantage of this innovative technique, the present study confirmed the impact of the environment after maturation for embryo lipid content, cryotolerance, and term developmental competence. Interestingly, birth weights of calves were significantly affected by the developmental environment after maturation but not during maturation.

In all, our study is thus also the first one demonstrating that ultrasonically guided IFOT works with in vitro-matured oocytes derived from slaughterhouse ovaries in the bovine; this has not been reported for any farm animal before. Nevertheless, further work should be conducted to improve overall efficiency of IFOT. Given that efficiency could increase in future, that technique might enable large-scale production of high-quality bovine embryos using slaughterhouse ovaries. Keeping in mind the high availability of bovine slaughterhouse ovaries, IFOT could also offer an attractive scientific option to analyze environmental consequences for developing embryos originating from the same pool of oocytes found on abattoir-derived ovaries.

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