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Birth of healthy calves after intra-follicular transfer (IFOT) of slaughterhouse derived immature bovine oocytes

M. Hoelker, PhD [a, b, c, *], A. Kassens [d], D. Salilew-Wondim [b], H. Sieme [d], C. Wrenzycki [e], D. Tesfaye [b, c], C. Neuhoff [b], K. Schellander [b, c], E. Held-Hoelker [a, b]

[a] Research Facility Frankenforst, Faculty of Agriculture, University of Bonn, Königswinter, Germany
[b] Institute of Animal Science, Animal Breeding and Husbandry Group, University of Bonn, Endenicher Allee 15, Bonn, Germany
[c] Center of Integrated Dairy Research (CIDRe), University of Bonn, Meckenheimer Allee 172, 53115 Bonn, Germany
[d] Unit for Reproductive Medicine, Clinic for Horses, University of Veterinary Medicine Hannover, Bünteweg 15, Hannover, Germany
[e] Clinic for Veterinary Obstetrics, Gynecology and Andrology, Chair for Molecular Reproductive Medicine, Justus-Liebig-University, 35392 Giessen, Germany

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ABSTRACT

To circumvent the negative impacts of in vitro culture on bovine embryos, we have recently established a new method, the so called intra-follicular oocyte transfer (IFOT), enabling in vivo fertilization and in vivo development of in vitro matured oocytes up to the blastocyst stage as well as to term. In this study, we raised the question whether immature bovine oocytes could also be transferred into a pre-ovulatory follicle to support in vivo maturation prior to subsequent in vivo fertilization, in vivo development as well as to term. To unravel that question, a total of 791 immature oocytes were transferred in groups of ~50 into pre-ovulatory follicles of 16 recipient heifers. Consequently, we were able to recollect a total of 306 structures 8 days thereafter (38.5%). All in all, 12 heifers (75%) gave embryos developed to the morula or blastocyst stage in addition to the expected native embryos. Among all recollected structures, 40.1% had developed to the morula and/or blastocyst stage, meaning a total efficiency of 17.3% based on all transferred oocytes. Of impact, IFOT-embryos reached significantly higher developmental rates to the Morula and/or blastocyst stage until day 7 compared to in vitro cultured control embryos, despite being derived from the same charge of slaughterhouse ovaries (40.1 vs. 29.3%). This implicates a beneficial effect of the follicular environment for the intrinsic quality of the fertilized embryos during maturation and for subsequent developmental rates up to the blastocyst stage. Finally, the birth of two healthy calves after transfer of frozen-thawed IFOT-derived blastocysts to final recipients established the first proof of principle that IFOT of immature bovine oocytes generates bovine blastocysts bearing developmental capacity to term. Likewise, to the best of our knowledge, these calves are the first calves derived from full in vivo development of immature slaughterhouse derived oocytes. Thus, the results of the present study clearly demonstrate that IFOT of immature slaughterhouse-derived oocytes is now a feasible technique. Since efficiencies following IFOT achieved within the present study were improved compared to previous studies, IFOT now offers an attractive option for designing new scientific experiments.

1. Introduction

In vitro production (IVP) of bovine embryos is a longstanding, well-established technique. Nevertheless, bovine IVP-derived embryos are of lower viability compared to embryos developed in vivo [1,2], representing a major hurdle for further implementation of this embryo technology. Usually 30–40% of IVP derived oocytes develop into a blastocyst after in vitro culture [3], whereas around 90% of ovulated oocytes are fertilized after insemination, with most of them developing into a blastocyst [4]. Furthermore, even if an embryo reaches the blastocyst stage, IVP derived blastocysts still differ in many aspects compared to their in vivo derived counterparts, as reviewed earlier [3]. These differences in terms of cryoresistance [5], ultrastructure [6], microvilli [5] lipid content [7] as well as gene expression profile [8,8–10] have been reported extensively. Among the latter, especially expression profiles of

* Corresponding author. Institute of Animal Science, University of Bonn, Endenicher Allee 15, Bonn, Germany.
E-mail address: michael.hoelker@itw.uni-bonn.de (M. Hoelker).

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transcripts related to metabolism and growth, but also to altered conceptus and fetal development following transfer, attracted high interest and have been investigated often [8,8,11,12]. All in all, it's generally accepted that IVP derived embryos are of inferior quality, exhibiting lower developmental capacities following embryo transfer compared to fully in vivo-derived bovine embryos [13,13–15].

As the cause for these differences, the crucial role of the oviduct to support early bovine embryo development has been clearly described, as reviewed earlier [16]. While bovine IVP derived embryos have been transferred to mouse oviducts [17,17,18], rabbit oviducts [19,19,20] and sheep oviducts [11,13,13,21–25] to analyse the environmental impact on the quantitative and qualitative development of bovine embryos, Besenfelder and colleagues have established a minimally invasive endoscopic technique, enabling access to the bovine oviduct of live animals which allows both tubal transfer and flushing of bovine embryos out of the oviduct [26–28]. This technique represents one of the major scientific breakthroughs in the field of bovine reproduction and allowed the comparison of in vivo versus in vitro environments before or after a distinct developmental stage and/or timepoint [26–28]. Communally, these studies clearly demonstrated that the developmental competence of a bovine embryo to the blastocyst stage is almost predetermined at the 2-cell stage [29], whereas the quality of the subsequent blastocyst is highly affected by the environment after fertilization [25,30,30,31]. This finding is particularly supported by the fact that in vitro culture of zygotes flushed out of the oviduct resulted in blastocysts of lower cryotolerance [25], 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 [13,21,25].

Since the biggest barrier to utilization of intra-fallopian transfer of bovine embryos is that it requires an extreme level of skill, a new technique, the so called intra-follicular oocyte transfer was recently successfully introduced as an alternative to bovine reproduction by our group [32]. Although the technique of transferring oocytes into a pre-ovulatory follicle was already introduced by Fleming and colleagues in 1985 [33], our study was the first one reporting development up to the blastocyst stage as well as the birth of healthy calves after intra-follicular transfer of oocytes into pre-ovulatory follicles. Keeping in mind that in vitro matured oocytes emerged within that study, the results of our recent study confirmed that the developmental capacity of bovine embryos is already determined after maturation, whereas their quality at the blastocyst stage is modulated by the environment after maturation, which is in accordance with the previously mentioned studies [32].

Nevertheless, the question of whether the developmental capacity of bovine embryos is solely a consequence of the maturation environment or caused by the oocyte source, remained unanswered by our study. Noteworthy, in vitro matured oocytes not only matured in a contrasting environment when compared to in vivo matured oocyte, but also could represent a different source of oocytes since they were collected from slaughterhouse ovaries and may not represent the same proportion of oocytes that would have reached ovulation. Still, transfer of immature oocytes derived from slaughterhouse ovaries would undoubtedly represent an attractive scientific option, enabling the analysis of embryo-environmental interactions while using the same source of oocytes and/or embryos. Keeping in mind the high availability of bovine slaughterhouse ovaries, IFOT of immature oocytes would enable researchers to generate large groups of embryos matured within the physiological environment during final nuclear maturation, fertilization as well as early preimplantation development without the need for super-stimulation, which is suggested to bear consequences for the embryo characteristics.

Consequently, the first aim of this study was to test if immature bovine slaughterhouse derived IFOT-oocytes would ovulate into the fallopian tube after maturation, be fertilized within the oviduct, and would yield embryos that could be flushed out of the uterine environment seven days later. The second aim was to determine, by using the same pool of oocytes found on abattoir-derived ovaries, the effect of the maturation environment on embryo developmental characteristics, to resolve the question of whether the developmental capacity to the blastocyst stage is determined by the maturational environment or by the source of immature oocytes. Finally, the last aim was to check if immature slaughterhouse derived oocytes transferred into a pre-ovulatory follicle could develop into healthy offspring.

2. Materials and methods

2.1. Experimental design

Two experimental groups of blastocysts, namely those that developed completely in vitro (VITRO) and those which developed within the bovine reproductive tract after intra-follicular transfer of immature slaughterhouse derived oocytes (IFOT), were generated and compared within this study. Therefore, bovine Cumulus oocyte complexes (COC'S) collected from slaughterhouse ovaries were either matured in vitro or intra-follicularly transferred (IFOT) into the presumed pre-ovulatory follicle to allow in vivo maturation. In vitro matured oocytes were in vitro fertilized whereas artificial insemination was conducted in follicular recipient heifers prior to IFOT of immature oocytes. Then, 8 days after IFOT (referred to as Day 7 of in vitro culture) embryos were flushed out of recipient heifers to evaluate recollection rates and to compare developmental rates relative to fully in vitro developed counterparts. Finally, a subset of IFOT derived embryos were frozen-thawed and transferred to synchronized recipients to analyse term developmental capacity.

2.2. In vitro maturation of bovine embryos

In vitro maturation of bovine oocytes was conducted as described previously [29,34,35]. Briefly, cumulus oocyte complexes (COCs) were aspirated from small follicles (2–8 mm) and COCs with a homogenous, evenly granulated ooplasm, surrounded by at least 3 layers of compact cumulus cells, were transferred to modified Tissue Culture Medium 199 (TCM, Sigma, Taufkirchen, Germany) supplemented with 4.4 mM hepes, 33.9 mM $NaHCO_3$, 2 mM pyruvate, 2.9 mM calcium lactate, 55 μg ml^{-1} gentamycin and 12% (v/v) heat inactivated estrus cow serum. After washing COCs three times, they were cultured in groups of 50 in 400 μl modified TCM supplemented with 10 μg ml^{-1} FSH (FSH-p, Sheering, Kenilworth, NJ, USA) 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 as IFOT to follicular recipients was conducted.

2.3. Intra-follicular oocyte transfer (IFOT) of immature bovine oocytes

To perform IFOT, 16 Simmental heifers (~450 kg) were subjected to a synchronization protocol by administration of 500 μg of cloprostenol, i.m. (Estrumate; Essex Tierarznei, Munich, Germany) followed by injection of 0.02 mg of GnRH (Receptal; Intervet, Boxmeer, The Netherlands) 48 h thereafter. Again 11 days later, recipients received a second cloprostenol (500 μg) treatment. Thirty-six h after the second cloprostenol treatment, heifers were