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Review: Environmental impact on early embryonic development in the bovine species

U. Besenfelder[1,2,a†], G. Brem[1] and V. Havlicek[1,2]

[1]*Department for Biomedical Sciences, University of Veterinary Medicine, Veterinaerplatz 1, A-1210 Vienna, Austria;* [2]*Department of Agrobiotechnology, IFA-Tulln, University of Natural Resources and Life Science, Konrad Lorenz Strasse 20, 3430 Tulln, Austria*

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*Assisted reproduction techniques (**ARTs**) provide access to early stage embryos whose analysis and assessment deliver valuable information. The handling of embryos, including the* in vitro *production of bovine embryos, is a rapidly evolving area which nonetheless exposes the embryos to unnatural conditions for a period of time. The Fallopian tube provides innumerable quantitative and qualitative factors, all of which guarantee the successful development of the embryo. It is well known that the Fallopian tube can be bypassed, using embryo transfer, resulting in successful implantation in the target recipient animal and the birth of calves. However, the question arises as to whether such circumvention has a negative impact on the embryo during this sensitive development period. First crosstalk between the embryo and its environment confirms mutual recognition activities and indicate bilateral effects. Nowadays,* in vitro *production of bovine embryos is a well-established technology. However, it is still evident that* in vitro *generated embryos are not qualitatively comparable to embryos obtained* ex vivo*. To counteract these differences, comparative studies between* in vitro *and* ex vivo *embryos are advantageous, as embryos grown in their physiological environment can provide a blueprint or gold standard against which to compare embryos produced* in vitro*. Attempts to harness the bovine oviduct were sometimes very invasive and did not result in wide acceptance and routine use. Long-term development and refinement of transvaginal endoscopy for accessing the bovine oviduct has meanwhile been routinely applied for research as well as in practice. Comparative studies combining* in vitro *development with development in the cattle oviduct revealed that the environmental conditions to which the embryo is exposed before activation of the embryonic genome can have detrimental and lasting effects on its further development. These effects are manifested as deviations in gene expression profiles and methylation signatures as well as frequency of whole chromosomal or segmental aberrations. Furthermore, it was shown that hormonal superstimulation (multiple ovulation and embryo transfer), varying progesterone concentrations as well as metabolic disorders caused by high milk production, markedly affected embryo development in the* postpartum *period. Assisted reproductive techniques that allow the production and handling of extra numbers of generated embryos promise to have a very high impact on scientific and practical application. Any influence on the early embryonic life, both in animals and* in vitro*, is accompanied by a sensitive change in embryonic activity and should be assessed* in vivo *on the basis of physiological conditions before being used for ART.*

Keywords: embryo development, environment, oviduct, *in vitro* production, endoscopy

Implications

Embryo collection using multiple ovulation and embryo transfer as well as *in vitro* production of bovine embryos has become a major part in animal breeding and science. The oviduct provides numerous prerequisites all of which guarantee a healthy conceptus, capable to implant and result in a healthy calf. Accordingly, techniques have been developed to access the bovine oviduct in order to perform comparable *in vivo v. in vitro* studies and to provide more information about components and dynamic changes in the oviduct to increase our comprehensive understanding of early embryo development and fertility problems such as early embryo death.

Introduction

A comprehensive understanding of the underlying factors affecting fertility is essential in order to make advances in basic science as well as the development and application

[a]Present address: Reproduction Centre Wieselburg, University of Veterinary Medicine, A-1210 Vienna, Austria.
† E-mail: urban.besenfelder@vetmeduni.ac.at

of novel breeding strategies. The term *fertility* includes a large number of genetic factors and environmental influences, which interact in a complex fashion. Although currently much detailed information is available, revealing a deep insight into individual processes, a common understanding of relationships seems to be still a long way off. Within this context, the first few days of development represent a key window during a sensitive period, when the embryo is generated and guided across the oviduct which determines the subsequent life phase.

However, this early embryonic development is in conflict with some of the objectives of cattle breeding. Milk production represents one major economic goal in modern cattle breeding. Over the past decades, cow milk yield has increased significantly and herd management, including animal nutrition, is a great challenge which inevitably leads to metabolic disorders (Zebeli *et al.*, 2015). In contrast, the reproductive performance of animals has deteriorated. One of the biggest contributors to reproductive efficiency is early embryonic mortality. The increase in embryonic losses within the first days and weeks seems to be directly associated with the increase in milking performance of dairy animals (Diskin and Morris, 2008; Sartori *et al.*, 2010).

Although significant progress has been made by the bovine industry and fertility has been negatively affected including early embryonic development, the generation and collection of embryos from genetically valuable animals is of major importance for breeding purposes, as reflected in the annual statistics of the International Embryo Technology Society (**IETS**) (Viana, 2018). The additional harvesting of embryos using multiple ovulation and embryo transfer (**MOET**) programs in cattle offers an extensive platform for introducing valuable breeding steps and thus significantly controlling the effects on breeding quality and progress. Thus, assisted reproduction techniques (**ARTs**) open up not only classical applications but also innovative approaches such as evaluation of the genomic breeding value and genome editing (Cornelissen *et al.*, 2017; Fujii *et al.*, 2017, Granleese *et al.*, 2018, Jaton *et al.*, 2018, Menchaca *et al.*, 2018, Georges *et al.*, 2019). Since the recovery of embryos by means of superovulation is associated with limitations, significantly more embryos could be provided with the development and establishment of *in vitro* production (Galli *et al.*, 2003). The embryo production statistics of the IETS 2017 showed for the first time that more embryos were generated and transferred from *in vitro* production worldwide than from MOET programs (Viana, 2018).

However, it should be emphasised that gametes and embryos are exposed to spatial and temporal unnatural conditions using such assisted reproductive techniques, the existence of which is known but their extent is not known in their entirety (Van Eetvelde *et al.*, 2017). Therefore, this review intends to briefly present physiological developmental conditions in the Fallopian tube for bovine embryos and epithelium and embryo interactions and compares embryo development *in vitro*, in the laboratory or *in vivo*, in the animal and under lactation conditions and hormonal substitution.

Physiological properties of the oviduct during embryo development

The bovine oviduct is a small, inconspicuous elongated tubular organ that connects the ovaries with the tip of the uterine horns. The gametes enter the Fallopian tube from opposite sides and meet in the ampulla where fertilisation occurs. Bovine embryos stay in the oviduct for 3.5 to 4 days and migrate to the distal end of the isthmus before they pass into the uterus. The multilayered structure (serosa, muscularis and mucosa) of the Fallopian tube depends on its individual sections: infundibulum, ampulla and isthmus. Internally, the Fallopian tube is characterised by ridges, grooves, furrows and folds of the mucosa to varying degrees. While the ampulla has numerous primary and secondary folds that are provided with cross-links, the isthmus has only minor elevations endowed with a distinct muscle layer (Yaniz *et al.*, 2000; Mouguelar *et al.*, 2015). The mucosa is lined with cells that are either ciliated or have secretory activity (Yaniz *et al.*, 2000; Kölle *et al.*, 2009).

The muscle layer with its longitudinal and circular layer in the Fallopian tube and the ciliated cells of the epithelium of the oviduct (Ruckebusch and Bayard, 1975) bathe the embryos in the fluid and transport them towards the tip of the uterine horns. The secretory cells are involved in the active production of the tubal fluid which is completed by transudation, peritoneal and follicular fluid (Hunter *et al.*, 2007). The lumen of the ampulla is significantly larger and is filled with branches of multiform mucosal folds, while the isthmus with a very small lumen largely consists only of primary folds. The ratio of ciliated and secretory cells depends on the tubal location as well as on stage of the ovarian cycle. Thus, on the third day of the cycle, the ampulla contains almost 70% secretory cells, which continuously decrease along the Fallopian tube and are only present at the end of the isthmus at about 20% (Kölle *et al.*, 2009). The oviduct supplies biochemical substrates (carbohydrates such as energy substrates, ions, proteins, enzymes, amino acids, fatty acids; Hugentobler *et al.*, 2010; Avilés *et al.*, 2015) to the embryos, provides physical requirements (pH, viscosity, osmolarity; Menezo and Guerin, 1997; Hunter *et al.*, 2011) and components with modulating or interacting functions (such as growth factors, cytokines, and nucleic acids, reviewed by Wolf *et al.*, 2003), among which the microvesicles are of particular importance (Raposo and Stoorvogel, 2013; Lopera-Vasquez *et al.*, 2017a; Almiñana *et al.*, 2018).

In contrast to many *in vitro* culture conditions, which usually take place under static conditions for 6 to 8 days, all processes in the Fallopian tubes are subjected to dynamic changes that are strongly linked to the ovarian cyclic activity such as transport speed (Ruckebusch and Bayard, 1975; Bennett *et al.*, 1988; Kölle *et al.*, 2009), volume of fluid (Hugentobler *et al.*, 2008), concentration of components (Buhi *et al.*, 2002; Hugentobler *et al.*, 2007; Avilés *et al.*, 2010), and the expression profile of the ipsi- or contralateral Fallopian epithelium (Bauersachs *et al.*, 2004).

While the embryos pass through the oviduct, they undergo specific key events. During this time, the epigenetic-directed

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reprogramming of the embryos takes place (de- and re-methylation of the embryonic DNA); and at the eight-cell stage, the main activation of the embryonic genome is initiated. The embryos migrate through the Fallopian tube contained within the zona pellucida (**ZP**), that is, cytoplasmic multiplication does not occur, cytoplasm and also organelles such as the mitochondria are equally distributed to the daughter blastomeres. A *de novo* synthesis of the mitochondria does not take place until the blastocyst stage (May-Panloup *et al.*, 2005; Graf *et al.*, 2014).

In vitro systems for the production of bovine embryos aimed at imitating specific features of the Fallopian tube in order to successfully manage this stage of development outside the body. This led to different types of culture systems using standard media, conditioned media (Maeda *et al.*, 1996) or the addition of fluid from the blood or Fallopian tube (Aguilar and Reyley, 2005), two- or three-dimensional co-cell culture systems (Goovaerts *et al.*, 2009, Chen *et al.*, 2017, Ferraz *et al.*, 2018), microfluidic systems (Beebe *et al.*, 2002) or even mouse oviducts during the *in vitro* culture (Rizos *et al.*, 2010b). Meanwhile, all *in vitro* systems have been developed and established over a long period and used with great success undergoing continuous improvements. Nevertheless, there are still significant differences between *in vitro* and *in vivo* derived embryos (Gad *et al.*, 2012; Bonilla *et al.*, 2014; Tšuiko *et al.*, 2017). In addition, studies show that the use of bovine oviduct fluid at higher concentrations (5%, 10% and 25%) in *in vitro* culture systems exerts a negative effect of embryo development which has been assessed as almost toxic (Lopera-Vasquez *et al.*, 2017b). Heterologous *in vivo* culture systems have been successfully used, but they do not allow the study of direct interactions of the embryo with its environment (Lazzari *et al.*, 2010). However, it should be kept in mind that short-term effects such as cultural successes will be followed by long-term effects, the consequences of which cannot yet be fully estimated (Duranthon and Chavatte-Palmer, 2018).

Undoubtedly, embryos can be produced without the assistance of the oviduct; furthermore, the recipients to whom the embryos are transferred do not have to have been in contact before transfer to the uterus. However, to pay more attention to this period in the Fallopian tube and its importance for embryo development, the following considerations will deal with direct interactions of the embryo with its physiological environment, including deviations.

Exchange of signals between embryo and oviduct in cattle

For a long time, the Fallopian tube has been neglected for its role and function in embryogenesis, possibly due to the success of ARTs such as MOET and *in vitro* production of bovine embryos. These techniques have clearly shown that pregnancies can be established without the involvement of the Fallopian tube (Leese *et al.*, 2001, Fazeli and Holt, 2016). However, these embryo transfers are accompanied by deficiencies in fertility. In ruminants, for example, interferon-τ plays a pivotal role in the establishment and maintenance of pregnancy. Although *in vitro* produced embryos secrete high amounts of interferon, their transfer results in a poorer pregnancy rate (Stojkovic *et al.*, 1995).

However, since it has been shown that developmental disorders such as large offspring syndrome can occur through the use of ARTs (Young *et al.*, 1998; Lazzari *et al.*, 2002), early embryonic development in the bovine oviduct has gained much more attention. In non-ruminants, there is now a strong evidence that the embryo in the Fallopian tube induces responses that mediate its recognition following implantation in the uterus (Weber *et al.*, 1991; Lee *et al.*, 2002; Georgiou *et al.*, 2005; Smits *et al.*, 2016). Good examples of the obligatory passage through the Fallopian tube are rabbit and hare embryos. Microscopically, these embryos are surrounded by a mucin layer that covers the ZP during the migratory phase through the oviduct. Absence of or damage to the mucin layer results in a lack of or inferior pregnancy (Murakami and Imai, 1996; Drews *et al.*, 2013).

Based on the gene expression profile of the Fallopian tube epithelial cells, Bauersachs *et al.* (2003 and 2004) successfully showed that when the oviduct mechanisms are induced, they prepare and determine the post-ovulation period and regulate early embryonic development via local modification of the transcription profile depending on the ovarian cycle. Differences between the ipsi- and contralateral sides were observed.

García *et al.* (2017) used an *in vitro* model to correlate processes in bovine oviduct epithelial cells with the developmental stage of the embryos. For this, Fallopian epithelial cells were cultured *in vitro* and covered with a woven polyester mesh having a grid size of 41×41 openings. Embryos were transferred to the mesh and placed individually in grids, which kept the embryos locally fixed to co-culture cells. It could be shown via the bone morphogenetic protein pathway that changes in the expression profile are induced via the embryo–oviduct interaction both in the embryo and in the epithelial cells (García *et al.*, 2017).

Maillo *et al.* (2016) studied the transcriptome of the bovine oviduct. Half of synchronised animals were inseminated at the time of oestrus and the other half were not inseminated. On Day 3 after heat, the animals were slaughtered and the Fallopian tubes removed. The isthmus was rinsed to confirm the presence of an embryo or ovum. Expression studies clearly showed differences between the ampulla and the isthmus and between the ipsilateral and contralateral sides. However, no difference was found between the Fallopian tubes containing a single embryo or unfertilised oocyte. The authors did not exclude the potential existence of local embryo-induced epithelial changes, however, which could not be detected within this experiment (Maillo *et al.*, 2016).

In a further study, 50 embryos were transferred endoscopically to the oviducts on Day 1.5 after oestrus. These animals as well as control animals which received a sham transfer were slaughtered on Day 3 and their Fallopian tubes were removed and the isthmus was used for microarray analysis. In total, 278 differentially expressed genes were found between the groups, of which 123 were up-regulated and 155 down-regulated in pregnant animals. Most genes

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could be related to immunological functions. The reason for the differences between the two experiments was that signals caused by single embryos were not detectable by the method used (Maillo *et al.*, 2015). Overall, these studies indicate that there is a mutual modulatory activity between the gametes and embryos and the Fallopian epithelium. As shown by the experimental approach of these *in vivo* studies, using innovative techniques to access the Fallopian tube can provide more information about peculiarities in early embryonic development compared to *in vitro* models. From the historical point of view, it can be seen that attempts were made earlier to provide access to the cattle oviduct in order to promote embryo development in a versatile manner. Therefore, this development is briefly introduced below.

Steps to access the bovine Fallopian tube

Numerous attempts have been made to gain access to the bovine oviduct in order to obtain early *ex vivo* embryonic stages, to transfer early stages of *in vitro* production (IVP) embryos for *in vivo* culture and to recover tubal fluid for the study of the embryo environment. At the beginning, different surgical approaches to the bovine Fallopian tube were described. In general, two routes to the peritoneal cavity have been practised: Either the ventromedian approach via the linea alba was selected in anaesthetised animals or laterally, possibly over both flanks, in animals which received a lumbar anaesthesia (Trounson *et al.* 1976; Ellington *et al.*, 1990; Hugentobler *et al.*, 2007 and 2008). Jillela *et al.* (1977) transferred embryos via a polyethylene cannula into synchronised heifers. These polyethylene tubes were fixed in the right or left flank and a connection was established to one of the oviducts. Two transferred embryos led to the implantation of an embryo. However, the different surgical procedures were labour intensive and often associated with postoperative complications, which made the repeated use of animals nearly impossible.

In order to minimise stress and complications associated with surgery, a minimally invasive approach using laparoscopy was developed. Laparoscopy was used first for observations of ovarian activity and puncture of the follicles on the surface of the ovaries (Sirard and Lambert, 1985). One of the first laparoscopic approaches to the Fallopian tube was reported by Fayrer-Hosken *et al.* (1989). A trocar from a bronchoscopy set and an atraumatic forceps trocar were inserted into the abdominal cavity via the right lumbar fossa. Before each transfer, the ovarian reaction of the recipient animal was recorded. The infundibulum of the Fallopian tube ipsilateral to the ovulated ovary was fixed with forceps to allow the insertion of a catheter through the abdominal ostium 2.5 to 5 cm into the ampulla under visual control. The embryos were slowly transferred from the catheter into the ampulla with about 50 µl of medium. Tubal transfer of 2- to 4-cell *in vitro* matured and fertilised embryos was performed in four synchronised recipients. One cow became pregnant and gave birth to a healthy calf. This result was encouraging (Fayrer-Hosken *et al.*, 1989); however, the described application of laparoscopy on the right paralumbar region involved a very complicated procedure, which was instrumentally very complex and did not become routine.

Reichenbach *et al.* (1993 and 1994) described a transvaginal endoscopic approach for the repeated examination of the reproductive organs and for the recovery of oocytes from follicles of cows and heifers. The endoscope together with the puncture unit was inserted mid-dorsally through the fornix vaginae into the abdominal cavity. The rectal manipulation allowed the ovaries to be moved and fixed in an optimal distance in front of the endoscope. The ovaries were slightly rotated which allowed the ovum pickup (**OPU**) of all follicles on the surface of the ovaries under visual control. The repeated use of this method in the same animals for the oocyte collection confirmed that a routine application was possible and this had no negative effect on the health, fertility or performance of the animals.

This technique served as a further basis for the access of the Fallopian tube (Besenfelder and Brem, 1998). In order to provide enough space for inspection of the pelvic cavity and to enable manipulation and navigation of the endoscopic equipment, it was necessary to deprive animals from feed for 8 to 12 h before starting the procedure. The animals were fixed in a crush, which resulted in a temporarily restricted movement to ensure gentle handling of the endoscope and organs. An epidural anaesthesia facilitated rectal manipulation. A rigid universal tube was placed dorsally in the fornix vaginae and introduced through the vaginal wall. After passive air influx which caused an artificial pneumoperitoneum, a bi-tubular inlay was inserted bearing the endoscope and tubing system, consisting either of the embryo flushing system or the embryo transfer system.

In contrast to the lumbar procedure, the transvaginal and medial position of the endoscope allows a non-instrumental, that is, manual, minimal invasive manipulation, visual inspection and control and access to oviducts, ovaries and uterine horns *in situ*. After completion of the endoscopic procedure and before the universal tube was removed, the air has to be removed from the abdominal cavity by means of a vacuum pump. No further medical treatment is recommended (Besenfelder and Brem, 1998). This technique allowed embryos to be transferred to the Fallopian tube as well as to be recovered after superovulation or *in vivo* culture at any time (Besenfelder *et al.*, 2010).

In the meantime, this technique has been assessed several times as '... a state of the art endoscopic embryo transfer technique . . . ' and has been steadily improved and adapted over a long period of time, thus providing skills and experience that can be successfully applied in gene expression and development studies (Lonergan and Fair, 2008; Carter *et al.*, 2010, Rizos *et al.*, 2010a). Greater experimental precision can now be tailored to embryo-specific cleavage stages and embryo origin (see Figure 1).

In vitro-*embryo development, differences between* in vitro *and* in vivo

In vitro derived blastocysts significantly differ from their *in vivo* collected counterparts with regard to gene expression

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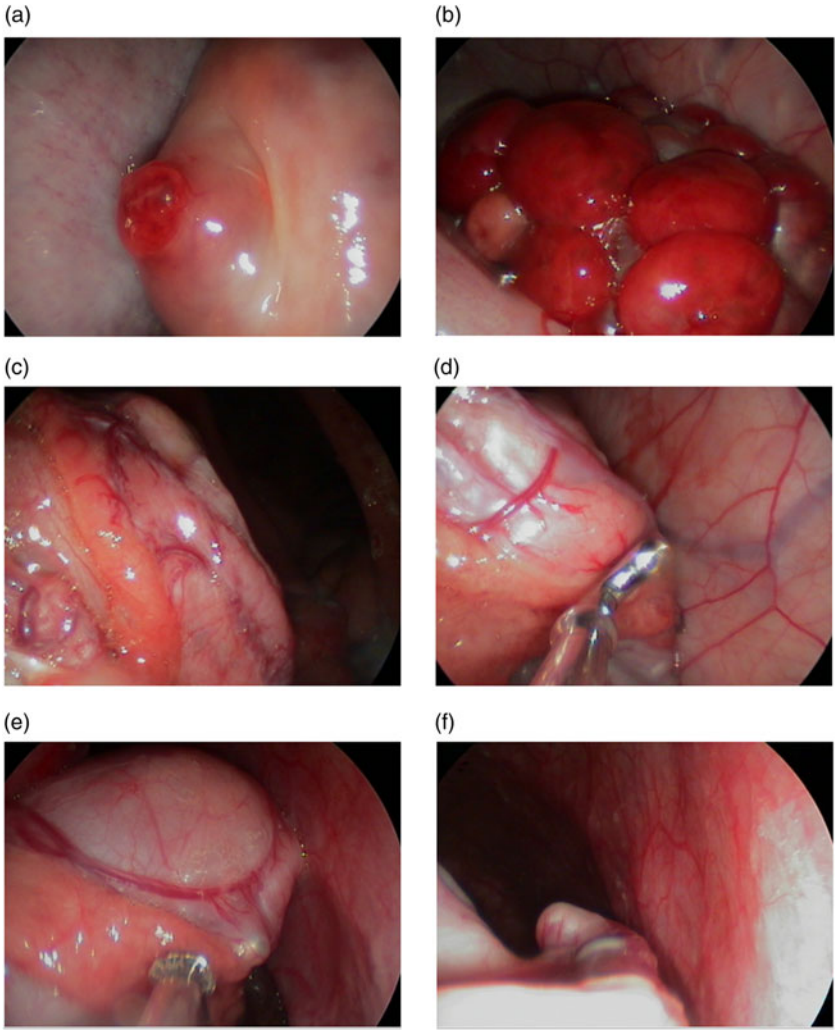

Figure 1 (colour online) Access to the bovine oviduct for transfer or for flushing. Embryo transfer via a glass capillary after single ovulation (a) or flushing after superovulation (b) can be performed in the same way. Slightly lifting the ovary allows the presentation of the adjacent oviduct ((c) presentation of the infundibulum and the ampulla). Once the entry can be gained into the oviduct ((d) see the capillary parallel to the first part of the ampulla), a capillary can be introduced along this route ((e) and (f)).

profile (Gutiérrez-Adán *et al.*, 2004), chromosome abnormalities (Viuff *et al.*, 2001), cryosurvival (Enright *et al.*, 2000) and ultrastructural features (Crosier *et al.*, 2000; Rizos *et al.*, 2002). These differences are discussed as an expression of different trophic factors that are available to the embryo in the respective culture systems.

Actually, it is not the embryonic cells that are in direct contact with the epithelial cells of the Fallopian tube but rather the ZP surrounding the embryo. The ZP consists of a compact meshwork displaying differently structured filamentous inner and outer layers (Denker 2000; Sinowatz *et al.*, 2001). This ZP represents an extracellular matrix that lies between the embryo and the oviduct epithelium and must be permeable to signalling and messenger substances. Therefore, the accumulation of substances in and around the ZP may serve as an indicator for the embryo–epithelial interactions. Mertens *et al.* (2006) studied the ZP of *in vitro* produced embryos in the zygote, 2-, 4-, 8-, 16-cell, morula and blastocyst stage and compared them with endoscopically collected zygotes, 4-cell and uterine-flushed morulae and blastocysts. The ZP was cut by laser and the wall was assessed by scanning electron microscopy. While the thickness of the outer layer, the reticular part of the ZP, increased from 7.5% to 10% for *in vitro* embryos, *ex vivo* embryos showed a thicker outer layer, expanding from 18% for zygotes to 30% for blastocysts (Mertens *et al.*, 2006). The number of pores and their size decreased with the duration of stay in the Fallopian tube. In addition, it could be seen that in most of the *in vitro* embryos the outer reticular layer showed signs of degeneration (Mertens *et al.*, 2007).

Overall, these processes revealed that the ZP represents a permeable wall and filter system where residues convince of an intensive exchange of nutrients, signals and other components between the embryo and the Fallopian epithelium. These oviductal properties can not only be found in the ZP but are correspondingly reflected in the cryosurvival of the embryos. Lonergan *et al.* (2003) produced bovine *in vitro* embryos that were either cultured *in vitro* or transferred to

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the ovine oviduct at different times and re-collected. The longer the embryos were cultured in the ovine oviduct, the more resistant they were to cryopreservation. It was noticeable that the resistance of the *in vitro* embryos to cryoinjury decreased very rapidly. Embryos cultured *in vitro* during the first period and then transferred *in vivo* showed a higher post-freezing survival rate compared to embryos that were first kept in the ovine oviduct and then cultured *in vitro* until the blastocyst stage (Lonergan *et al*., 2003). These results have been confirmed in cattle by Havlicek *et al.* (2010). *In vitro* produced embryos were kept in culture for 7 days and compared with embryos that had already been endoscopically transferred as a mix of ovum–sperm co-incubation into the oviduct immediately after ovulation or as embryos at the 4- to 8-cell stage in synchronous cattle oviducts. It was clearly shown that the longer the embryos remain in the bovine oviduct, the more resistant they are to cryopreservation (Havlicek *et al*., 2010).

After having shown the general inferiority of *in vitro* embryos, the aim of the study by Gad *et al.* (2012) was to reveal specific critical transition phases in *in vitro* production. In a large-scale study, *in vitro* produced embryos were transferred to the Fallopian tubes at various times, which flushed out of the uterus on Day 7. In turn, after superovulation, embryos were flushed out of the Fallopian tube at various times and cultured *in vitro* until the Day 7 blastocyst stage. In general, it could be shown that the change in the culture system before or after the activation of the embryo genome had no effect on the number of blastocysts. However, the source from which the embryos originated had a marked influence on the blastocyst rate (Rizos *et al*., 2002; Gad *et al*., 2012). Moreover, *in vitro* culture had a detrimental effect on the transcriptome of the blastocysts. It has been shown that molecular mechanisms and metabolic pathways are determined by the cultural environment that prevails at the time of genome activation. Such experimental approaches are seen as a potential source for the development of new strategies in *in vitro* culture (Gad *et al*., 2012).

A further investigation served for studying the genome-wide methylation pattern of embryos whose environment is influenced by *in vitro* conditions. For this reason, *ex vivo* tubal embryos at the 2-, 8- and 16-cell stage were collected from the Fallopian tube and cultured *in vitro* to the blastocyst stage. At this stage, the methylation pattern of blastocysts from each group was compared to that of the blastocysts obtained exclusively *ex vivo*. A total of 1623 hypermethylated regions were detected in the blastocysts, which were transferred as 2-, 8- or 16-cell stage *in vitro*. The earlier the embryos were transferred to the *in vitro* culture system, the larger were the deviations. The time point of genome activation was designated as particularly critical (Salilew-Wondim *et al*., 2018).

In addition to the expression and methylation pattern of embryos, several studies have already shown that *in vivo* embryos significantly differ from *in vitro* produced embryos in terms of chromosome abnormalities. Viuff and *et al.* (1999) demonstrated that *in vitro* produced embryos are highly mixoploid as measured by the analysis of chromosomes 6 and 7 in the blastomeres. Blastocysts obtained and tested *ex vivo* were significantly less mixoploid (Viuff *et al*., 1999 and 2001).

In a more technically sophisticated study, Tšuiko *et al.* (2017) used a high-resolution analysis method that allowed the demonstration of chromosome instability (**CIN**) and subtle subchromosomal aberrations. For this purpose, a genome-wide single-cell analysis method was used for estimating haplotyping and copy number profiling on an individual isolated blastomere level. For the experimental design, *in vitro* embryos were obtained on Day 2 post insemination after OPU with or without FSH stimulation or synchronous embryonic stages from the oviduct were collected after superovulation. All embryos were produced and derived from the same parent animals. The genomic stability of individual blastomeres of both *in vitro* culture groups was severely impaired. The incidence of whole chromosome or segmental aberration was significantly higher in *in vitro* produced than in *ex vivo* derived embryos. Only 18.8% of *in vivo* cultured embryos contained at least one blastomere with chromosomal anomalies, while OPU embryos with hormonal stimulation and follicular aspiration without hormonal stimulation showed 69.2% and 84.6% anomalies, respectively (Tšuiko *et al*., 2017).

Overall, these studies indicate that *in vitro* culture conditions require further refinement to minimise developmental differences in embryos and reduce anomalies.

In vivo *embryo development: hormones and lactation*

Hormones, applied to synchronisation or superovulation, especially during lactation, currently play a major role in practice. In this context, it is important to note that *in vitro* culture conditions *per se* represent extraordinary unnatural environmental conditions; therefore, they may also indicate at which time point the conceptus is particularly sensitive in early embryonic development (Gad *et al*., 2012). This sensitivity to environmental changes does not only seem to be limited to *in vitro* conditions but rather suggests that similar appearances in this developmental stage can also be seen in animals under certain circumstances or give indications of early embryonic death.

Overall it has been estimated that nearly half a million bovine embryos (2017: 495 054 embryos) are currently being harvested via MOET programs worldwide (Viana, 2018). These embryos develop temporarily to the morula/blastocyst stage in superstimulated animals before being collected. This recovery treatment deviates significantly from the treatment with which recipient animals are prepared. Consequently, these embryos develop under suboptimal hormonally superstimulated conditions in a developmental period, which is known to be very crucial for IVP-derived embryos. As already shown for chromosomal aberrations (Viuff *et al*., 1999 and 2001; Tšuiko *et al*., 2017), *ex vivo* embryos thus also show minor changes that appear even under *in vivo* conditions. In order to investigate the sensitivity in the early developmental stage of embryos on *in vivo* environmental effects,

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animals were hormonally primed or stimulated or the lactation period was used to investigate embryo development in more detail.

In a first study, more than 1400 embryos were obtained from the Fallopian tube at various stages, after which heifers were stimulated with either FSH or equine chorionic gonadotropin (**eCG**). In general, the embryos showed very similar developmental kinetics regardless of the treatment. However, it was noticed that embryos derived from eCG stimulation show a greater variation with regard to their cleavage stage. This variation was also seen in the luteal morphology. Equine chorionic gonadotropin-stimulated ovaries had corpora lutea of very different sizes. Numerous and even large partially haemorrhagic follicles were visible. In addition, the number of non-viable embryos in the later stage of development in the Fallopian tube increased significantly in eCG-stimulated heifers in contrast to FSH treatment (Besenfelder *et al.*, 2008).

In a subsequent study, the development kinetics and the expression profile of embryos from stimulated heifers were examined. This study focused exclusively on the effect of hormone treatment on embryo development in the Fallopian tube. For this purpose, the embryos were assigned to two different groups: a group (biphasic development) of embryos was endoscopically collected on Day 2 after stimulation with FSH and insemination and transferred to synchronised, mono-ovulatory recipient animals. In a second group (single phase), the embryos were also generated via FSH stimulation and artificial insemination. In both groups, the embryos were flushed on Day 7. Microarray data analysis showed that a total of 454 genes were expressed differently in the groups. In the superovulation group, 429 genes were expressed abundantly, while the biphasic (superovulation followed by single ovulation) embryo development yielded only 25 genes that were up-regulated. These genes have been assigned to processes involved in oxidative phosphorylation as well as various metabolic pathways, actions associated with transcription, translation and stress. Surprisingly, the biphasic development in non-stimulated animals resulted in a faster embryo development compared to embryos found only in superovulated animals. There was a morula/blastocyst ratio of 0.48 compared to 1.81, respectively (Gad *et al.*, 2011).

In addition to superovulation, the hormone progesterone plays a major role. Progesterone is produced by the luteal tissue and mainly affects embryo growth, interferon-τ production and, consequently, embryo implantation. It is well known that lactating dairy cows suffer from low progesterone concentration in the blood. In experiments by Carter *et al.* (2010), Day 2 synchronised heifers served for the transfer of *in vitro* derived bovine embryos. Approximately 100 cleaved embryos were endoscopically transferred into the ipsilateral oviduct of each heifer. Half of the recipient animals received a progesterone-releasing intravaginal device (**PRID**) from Day 3 to Day 7. All embryos were flushed from the Fallopian tubes and uterine horns on Day 7, and the messenger RNA expression profile was assayed using the Affymetrix GeneChip Bovine Genome Array. The administration of the PRID resulted in a significant increase in the plasma progesterone concentration from Day 3.5 to Day 7. A total of 194 differently expressed genes were identified using the genome wide gene expression analysis. These genes were associated with cross-talk between the embryo and its maternal environment by means of an interaction network analysis. Although these genes cannot directly be attributed to a better growth of the embryo, this transcriptome profile is discussed as valuable information in the context of the time after hatching of the embryo and/or the subsequent elongation phase (Carter *et al.*, 2010).

In the following study, Rizos *et al.* (2010a) synchronised heifers and lactating cows 60 days *postpartum* for *in vivo* culture of bovine embryos. Each animal received approximately 100 embryos of 2- to 4-cell stages into the Fallopian tube ipsilateral to the corpus luteum on Day 2 of the oestrous cycle. After 5 days, these embryos were re-collected. The progesterone analyses confirmed that lactating cows had significantly less plasma progesterone compared to heifers. From the *in vivo* culture in heifers, it was further shown that significantly more embryos could be recovered compared to cows (heifers: 79% *v.* cows: 57%). Similar to this result, nearly 34% of the embryos cultured in the heifers developed into blastocysts, while in cows only 18% reached the blastocyst stage. These results suggest that lactating dairy cows do not provide comparably adequate environmental conditions for early embryonic growth compared to heifers (Rizos *et al.*, 2010a).

Since heifers receiving progesterone supplementation and the comparison of lactating dairy cows with heifers do not accurately reflect the fertility problems in the dairy industry, a third approach has been performed using groups of cows after calving. As done before, also these cows received *in vitro* derived bovine embryos for temporary *in vivo* culture. Half of the cows were dried off after parturition, while the second group was allowed to normally produce milk. Both groups were used for embryo transfers and recovery around 60 days after parturition for this experiment. Also, these cows were on Day 2 of the oestrous cycle when approximately 65 embryos were endoscopically transferred into the Fallopian tubes and recovered after five days. In addition, the metabolism status of the cows was determined by regular blood sampling. Body weight and body condition score were significantly reduced in the lactating cows. Accordingly, non-esterified fatty acids and β-hydroxybutyrates were higher while blood glucose, insulin, and IGF-I were lower in the lactating cows. The recovery rate of the embryos did not differ between the groups, whereas the embryo development rate (49% *v.* 33%) was higher in non-lactating cows. This experiment also confirmed that the environment caused by lactation exerted a negative impact on fertility (Maillo *et al.*, 2012). As shown by these examples, early embryo life represents a very sensitive indicator for environmental conditions that deviate from its natural habitat.

Conclusion

The use of ARTs has opened up many opportunities and challenges to both scientifically valuable fields and breeding

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purposes. Simultaneously, these conditions are inevitably associated with the generation of a non-physiological environment. The steady increase in the number of embryos from *in vitro* production including the variety of protocols for the application and improvement of culture systems subsequently necessitates to also increase our efforts in *in vivo* studies to expand our knowledge about fertility in its complexity and, in turn, to promote the efficient use of ARTs with special regard to embryo competence, number and vitality of calves born and economic aspects.

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Declaration of interest

There is no conflict of interest.

Ethics statement

Not applicable.

Software and data repository resources

None of the data were deposited in an official repository

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