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Time-lapse embryo imaging and morphokinetic profiling: towards a general characterisation of embryogenesis

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Abstract

In vitro fertilisation is an effective method of assisted reproductive technology in both humans and certain non-human animal species. In most species, specifically, in humans and livestock, high *in vitro* fertilisation success rates are achieved via the transfer of embryos with the highest implantation and subsequent developmental potential. In order to reduce the risk of multiple gestation, which could be a result of the transfer of several embryos per cycle, restrictive transfer policies and methods to improve single embryo selection have been implemented. A non-invasive alternative to standard microscopic observation of post-fertilisation embryo morphology and development is time-lapse technology; this enables continuous, uninterrupted observation of embryo development from fertilisation to transfer. Today, there are several time-lapse devices that are commercially available for clinical use, and methods in which time-lapse could be used to improve embryology are continually being assessed. Here we review the use of time-lapse technology in the characterisation of embryogenesis and its role in embryo selection. Furthermore, the prospect of using this technology to identify aneuploidy in human embryos, as well as the use of time-lapse to improve embryological procedures in agriculturally important species such as the pig and cow are discussed.

Keywords – bovine, embryogenesis, human, IVF, porcine, time-lapse

Introduction

In vitro fertilisation (IVF) is one of several well-established methods of assisted reproductive technology (ART) used in clinics globally and the technique gives parents facing fertility disadvantages the chance to have offspring. According to the Human Fertilisation and Embryology Authority (HFEA) 2013, fertility problems affect one in six couples in the United Kingdom and to date 2% of babies born here are conceived *in vitro* (HFEA, 2013). Since the introduction of IVF in the 1970s, this approach has been very successful, but a 100% success rate is still some way from being achieved. IVF is now also

making a major impact in various livestock species, and is particularly important in the cattle industry. Unfortunately, the commercialisation of this technique in some other agriculturally important species, such as the pig, is still some way off.

Central to successful IVF in both human and agricultural situations is the transfer of embryos that will implant and develop correctly. In human IVF, a significant risk is multiple gestation pregnancies (Kovacs, 2012) that result, in most cases, from the transfer of several embryos per cycle in order to compensate for comparatively low implantation rates. For example, in 2009, out of 400,000 intracytoplasmic sperm injection (ICSI) IVF cycles, 80,000 resulted in live births; a success rate achieved primarily due to the transfer of two or more embryos in over 75% of the cycles (Ferraretti et al., 2013); of these deliveries, a significant 20% were multifetal pregnancies. Over the decades, the incidence of multiple gestations has led to an increase in maternal and neonatal morbidity/mortality rates (Kovacs, 2012). As such, restrictive transfer policies have now been implemented in a number of European countries following the implementation of the One Child at a Time report by the Human Fertilisation & Embryology Authority (HFEA). Procedures to develop single embryo selection techniques that could potentially eliminate the incidence of multiple gestations (with the exception of monozygotic twinning) are also being investigated (Practice et al., 2013). Such procedures require assessment of the outcome of fertilisation and assessment of embryo quality. This is routinely done by observing the embryo microscopically to confirm that fertilisation has occurred and that development is proceeding as expected. An alternative to this is the use of time-lapse monitoring.

Traditionally, embryo monitoring subsequent to IVF involves removing embryos from the culture environment, at least once per day, to evaluate embryo morphology and developmental progression. This method only provides embryologists with one still image, per day, of a dynamic process. Additionally, culture conditions such as temperature, pH and humidity are disrupted which in turn has been shown to have a detrimental impact on the embryo's development (Campagna et al., 2001).

Time-lapse technology, is a non-invasive, alternative to this approach that permits continuous, uninterrupted, observation of embryo development from the point of fertilisation to transfer (Kirkegaard et al., 2012). Under time-lapse imaging, the culture conditions are far less disturbed and embryologists are provided with additional, more detailed information such as cleavage patterns, the timing of cell divisions and changes to embryo morphology. Taken together, consideration of these parameters allows selection of embryos with higher implantation potential (Azzarello et al., 2012). The first use of time-lapse technology in embryology was in 1929 to map the development of rabbit embryos (Massip and Mulnard, 1980). The first reported use of time-lapse in human IVF however, was not until several decades later in 1997, when the technology was used to map the development of human embryos that had been fertilised by ICSI (Payne et al., 1997). Since then, several time-lapse devices have been devised, and many studies have been performed to assess how time-lapse technology could improve prospects in embryology for both human and non-human animal species. This review will focus on the use of time-lapse technology in the characterisation of embryogenesis and its role in embryo selection in several species including the human, mouse and some agriculturally important livestock species. Furthermore, the prospect of using the technology to identify aneuploidy in embryos has been discussed.

Time-lapse analysis of embryogenesis

Time-lapse embryo imaging enables non-invasive observation of key developmental markers such as polar body extrusion, pronuclear formation, cleavage timings and patterns as well as enabling identification of fragmentation throughout the developmental process (Leary et al., 2015). Time-lapse analyses of *in vitro* embryo development have been conducted in several species, which include but are not limited to mouse (Beraldi et al., 2003; Togashi et al., 2015; Yamazaki et al., 2007), hamster (Gonzales et al., 1995) and zebrafish (Cooper and D'Amico, 1996). This approach has also been successfully used in the analysis of embryogenesis in the invertebrate model organisms *Drosophila melanogaster* and *Caenorhabditis elegans*. These systems demonstrate the experimental power

allowed when, for example, hundreds of *Drosophila* embryos can be simultaneously and precisely oriented and imaged (Chung et al., 2011; Levario et al., 2013; Yanik et al., 2011). Similar to the situation in *Drosophila*, techniques have been developed that combine microfluidics, automated imaging and automation of image processing to allow the analysis of aspects of embryogenesis in the nematode *C. elegans*. For example, such systems have been used to quantify the effects of mutations on the timing of various stages of embryogenesis differences (Cornaglia et al., 2015).

When imaging embryos, both the duration of light exposure and the wavelength of the light should be considered; for example, it has been shown that low wavelength light (below 550nm, approximately 15% of the light emitted from a standard microscope used for IVF) impedes embryo development (Oh et al., 2007; Takenaka et al., 2007), due to localised heating causing DNA damage or generating free-radical species in the blastomeres (Frigault et al., 2009; Wong et al., 2013). This is of particular importance when transitioning away from standard embryo monitoring (for example, taking an image once per day), to time-lapse microscopy, where in some cases, numerous Z-stack images are taken every few seconds or minutes; such procedures risk damage to the embryo, particularly when imaging is very intensive. Time-lapse devices (some of which are discussed later) usually employ visible light imaging as opposed to ultraviolet, fluorescence or infrared due to the comparatively less damaging nature (Wong et al., 2013). Moreover, it has been shown that light emitted from time-lapse devices does not have a detrimental impact on embryo development (Kovacs, 2014); for example, it has been reported that the exposure time for the acquisition of 3024 images in the Embryoscope time-lapse system takes 136 seconds, compared to an average of 167 seconds for a single assessment in standard monitoring (Ottosen et al., 2007).

In mammalian embryology, the best studied system is the mouse (*Mus musculus*) (Mukaida et al., 1998). In this species, several studies have used time-lapse technology to investigate various morphokinetic parameters such as polarisation patterns of pronuclei, cleavage timings and how these

kinetics could be predictive of preimplantation embryonic development (Hiiragi and Solter, 2004; Lee et al., 2015; Motosugi et al., 2005; Pribenszky et al., 2010). Cleavage rates have also been characterised as a predictive tool during embryo selection to assess developmental potential (Arav et al., 2008). Additionally, time-lapse technology has been used to investigate the impact of oxygen concentration on mouse embryo development, revealing that embryos cultured at atmospheric oxygen levels (around 20%) reached 1st, 2nd and 3rd cleavage stages faster than those that were cultured in 5% oxygen (Wale and Gardner, 2010). Moreover, in comparison to partial or complete culture in atmospheric oxygen, embryo culture in 5% oxygen led to significantly higher blastocyst cell numbers (Wale and Gardner, 2010). Furthermore, Ugajin and colleagues conducted a comparative prospective study using mouse embryos, in order to assess the effects of blastomere biopsy on early embryonic development (Ugajin et al., 2010). Time-lapse imaging was used to monitor morphokinetic parameters such as blastocoel formation, duration of hatching, number of contractions and expansions between formation of blastocyst and end of hatching, as well as the maximum diameter of the expanded blastocyst; these parameters were compared for both biopsied and non-biopsied embryos. These comparisons revealed that intact embryos initiated compaction at the 8-cell stage as opposed to the 6-cell stage for biopsied embryos (Ugajin et al., 2010). Moreover, it was found that intact embryos hatched faster and that the number of contractions and expansions after blastocyst formation were noticeably lower (Ugajin et al., 2010). Additionally, when blastocyst diameters were compared prior to hatching, no significant difference was observed between the two groups. As these results demonstrate, blastomere biopsy impacts on embryonal development; a surprising and unexpected result for many.

Belli et al. (2004) used time-lapse technology to investigate two main types of chromatin organisation in mouse antral oocytes; surrounded nucleolus (SN) oocytes (nucleolus is surrounded with a Hoechst-positive ring), and non-surrounded nucleolus (NSN) oocytes (nucleolus lacks this ring). Moreover, it has been shown that, following maturation to MII and subsequent fertilisation, only SN oocytes

develop past the 2-cell stage and reach full term. As such, in order to gain full insight into the dynamics of chromatin organisation during meiosis resumption, time-lapse was used to observe these oocytes for a period of 9 hours from the germinal vesicle stage to MII. These observations revealed that the specific timings of these events differed between the two types of oocytes based on their chromatin organisation (Belli et al., 2014).

In one of the most recent and revolutionary studies published in the field of human embryology, embryos were successfully cultured *in vitro* for a record of 13 days in the absence of maternal tissues (Deglincerti et al., 2016; Shahbazi et al., 2016). This follows similar prior success in mice, where embryos were cultured beyond the blastocyst stage (Bedzhov et al., 2014). In this case, the authors obtained time-lapse phase contrast images using spinning-disc confocal microscopy with Z-stacked images being acquired over a range of 120µm (5µm intervals) every 30 minutes, as opposed to using a time-lapse device (Deglincerti et al., 2016; Shahbazi et al., 2016) (an incubator with integrated time-lapse functionality; this is discussed in the following section). These studies revealed that there are not only species specific developmental events that occur after the blastocyst stage, but that embryos of at least two mammalian species demonstrate key milestones of normal development, even after the point of standard embryo monitoring up to hatched blastocyst stage (Bedzhov et al., 2014; Deglincerti et al., 2016; Shahbazi et al., 2016). Whilst blastocyst implantation has been hitherto underexplored, the identification of certain late developmental markers including those leading to cell fate determination for subsequent gastrulation, organogenesis and ongoing successful pregnancy, are as vital as the classification of morphokinetic parameters during early embryonic development.

The role of time-lapse in embryo selection

The implementation of clinically approved time-lapse devices that permit continuous and flexible monitoring of embryos has led to the identification of markers that may be indicative of low

implantation rates and of higher blastocyst developmental potential (Kirkegaard et al., 2012). Studies have demonstrated how these markers as well as other morphokinetic parameters such as appearance and disappearance of pronuclei, cleavage timings, duration of cell cycles and timings of development into blastocysts (Aparicio et al., 2013; Chamayou et al., 2013; Cruz et al., 2012; Hashimoto et al., 2012; Machtinger and Racowsky, 2013; Prados et al., 2012), could greatly influence clinical selection of embryos by offering new opportunities and approaches for embryologists.

In one of the earliest studies involving the use of time-lapse technology in human embryology, Payne and colleagues (1997) analysed fertilisation events in 50 human oocytes that underwent ICSI. An environmental chamber that maintained standard incubation conditions was used to culture the presumptive zygotes. An Olympus IX-70 inverted microscope, equipped with a Perspex environmental chamber, was used for time-lapse monitoring; this began 30 minutes following ICSI and continued for 17-20 hours (one image per minute). As a control, 487 sibling oocytes, that also underwent ICSI, were cultured in a conventional incubator, as opposed to time-lapse monitoring using a microscope with an environmental chamber, and fertilisation rates were compared. Findings revealed that 38 of the 50 (>75%) time-lapse monitored presumptive zygotes fertilised normally (sibling oocytes too had a similar rate of fertilisation) and significant differences in the timings of polar body extrusion and pronuclear formation were identified (Payne et al., 1997). Morphology analysis and subsequent selection of early cleavage stage embryos has been shown to have a positive impact on embryo selection for IVF (Prados et al., 2012). In addition to this, it has been demonstrated that transferring early cleavage embryos (those in which embryonic mitosis began 25-27 hours following IVF/ICSI) results in better development of the embryo (less than 20% fragmentation and evenly sized blastomeres), as well as better overall clinical outcomes associated with IVF (Lee et al., 2012). Similarly, Van Monfoort et al., (2004), obtained a significantly higher pregnancy rate when early cleavage embryos were transferred and hence concluded that when selecting embryos for transfer, it is essential to consider cell number and morphology of embryos at cleavage stage and all the way

through embryo culture, not just on the day of transfer. Pronuclear morphology can also be evaluated by the observation of certain characteristic features in zygotes 16-18 hours post fertilisation; such events include the number and distribution of nucleolar precursor bodies, the extent to which nucleolar precursor bodies are polarised as well as the presence of a cytoplasmic halo (Salumets et al., 2001). Such observations however are fairly controversial (Salumets et al., 2001), although there has been evidence to show that pronuclear morphology can be an important parameter to consider during embryo selection (Joergensen et al., 2014; Scott, 2003; Tesarik and Greco, 1999; Tesarik et al., 2000). For example, in a prospective study (Azzarello et al., 2012), the pronuclear morphology of 159 human embryos was compared to determine whether the morphology and/or the timings at which breakdown of the pronuclei occur, was a predictive factor of live birth. The study involved embryo culture and time-lapse monitoring (from fertilisation to intrauterine transfer) of embryos from a total of 130 couples that underwent fertility treatment via ICSI. Specifically, time-lapse monitoring was used to assess pronuclear morphology using six distinctive scoring systems at different times, and the pronuclear morphology of 46 embryos that resulted in live birth were compared to 113 embryos that did not. Whilst results revealed a significant delay in pronuclear breakdown of embryos that resulted in live birth, interestingly the morphology scoring systems did not prove to be useful in the prediction of live birth outcome. As such, it was concluded that whilst pronuclear morphology assessment had no significant impact on embryo selection, the timing of pronuclear breakdown is a useful parameter to be used during embryo selection (Azzarello et al., 2012). Moreover, it was made evident that since pronuclear morphology varies throughout the development of an embryo, time-lapse monitoring is a better tool, in comparison to single light microscopy, for the assessment of morphology and timing of pronuclear breakdown (Azzarello et al., 2012).

The selection of embryos only through morphology assessment has however shown only limited success, with an average of only 20-40% of embryos selected for transfer successfully implanting (Ferraretti et al., 2013; Kovacs, 2012). Lemmen and colleagues used time-lapse technology to

investigate the sequence of events during early development of the embryo from the zygote stage up to cleavage stage (Lemmen et al., 2008). Here, a particular focus was to identify markers predictive of 'good quality' embryos and of a higher implantation potential. The experiment involved monitoring 102 fertilised human oocytes for 20-24 hours following fertilisation; various developmental stages were observed such as the disappearance of pronuclei and timing of cleavage; these were evaluated and compared for method of fertilisation (IVF / ICSI), quality of the embryo and rates of implantation. Results revealed that the pronuclei of presumptive zygotes that developed into 2- and 3-cell embryos disappeared later than in those that developed into ≥4-cell embryos and early onset disappearance of pronuclei was linked to a higher number of blastomeres on day 2 following oocyte retrieval. It was also observed that 4-cell embryos fertilised by ICSI, spent less time as 2-cell embryos, in comparison to those fertilised *in vitro*. Overall results showed no significant differences between embryos that were monitored using time-lapse and those that were cultured in a conventional incubator, indicating that time-lapse was a safe technique that could be used to monitor embryo development (Lemmen et al., 2008).

Research by Borini and colleagues showed a few other interesting factors that could be considered when selecting for embryos with higher implantation potential (Borini et al., 2005). Specifically, their research demonstrated that non-invasive selection, through the assessment of intracytoplasmic and extracytoplasmic morphology, at the oocyte stage may not be useful in embryo selection. Furthermore, it was noted that follicular vascularisation could be used as a marker for selection criteria. Additionally it was found that culturing embryos to the blastocyst stage before selection could decrease the risk of aneuploidies and hence, aid in the selection of embryos (Borini et al., 2005). Meseguer and colleagues also evaluated the implantation potential of embryos using an Embryoscope® time-lapse incubator, based on certain morphokinetic parameters. From a total of 522 transferred embryos, 247 embryos that either fully implanted or failed to implant completely were considered for further analysis and the remaining embryos were discarded (Meseguer et al., 2011).

The embryos were retrospectively analysed for timing of cleavage, blastomere size and multinucleation. Observations revealed that imbalances of blastomere size at the 2-cell stage, the time between cleavage from 2-cells to 3-cells, multinucleation at 4-cell stage and cleavage into 5 cells were all predictive of lower implantation potential (Meseguer et al., 2011). Several other studies have used time-lapse technology to observe morphokinetics during the development of an embryo, and have established that cleavage timings and appearance/disappearance of pronuclei can be indicative of implantation potential (Aguilar et al., 2014; Dal Canto et al., 2012; Kirkegaard et al., 2013; Liu et al., 2014; Rubio et al., 2012). Conversely, a recent appraisal of the literature has suggested that time-lapse technology should not be routinely used in clinical IVF and that such technology should remain experimental at present (Racowsky et al., 2015).

Can time-lapse be used to identify aneuploidy in embryos?

Aneuploidy is the presence or absence of an extra chromosome which results in divergence from the diploid state (Hook, 1985), which is 46 in humans. Aneuploidy has been recognised as the most prominent chromosomal abnormality in humans (Campbell et al., 2013b; Hassold and Hunt, 2001), resulting in embryos failing to implant, and increasing the incidence of miscarriage and characteristic syndromes arising from aneuploidy (Fragouli et al., 2013; Hassold et al., 1996; Hassold and Hunt, 2001; Rajagopalan and Lengauer, 2004). The traditional approaches used to detect aneuploidy in human embryos are preimplantation genetic screening (PGS), which screens for chromosome abnormalities, and preimplantation genetic diagnosis (PGD) which is used to identify genetic disorders in embryos produced via IVF. Suspected embryos are screened using one of several techniques, including array comparative genomic hybridisation (aCGH) (Hellani et al., 2008), polar body biopsy and subsequent aCGH (Harper et al., 2010), as well as fluorescent *in situ* hybridisation (FISH) for chromosome analysis (Jansen et al., 2008) and polymerase chain reaction (PCR) during PGD (Sermon et al., 2004). However, whilst PGS has proven to be effective, it involves the use of expensive technology, it is not commonly available, and is an invasive procedure that requires the extraction of one or more cells of the embryo

during biopsy (Bisignano et al., 2011; Campbell et al., 2013b; Sermon et al., 2004; Wells and Delhanty, 2000).

As such, time-lapse technology has provided a potential non-invasive alternative to PGS, whereby, certain morphokinetic parameters are used to identify ploidy and hence, prenatally select euploid embryos for transfer. Such screening methods are particularly important given that abnormalities that affect whole chromosomes are key to embryos successfully reaching the blastocyst stage (Daughtry and Chavez, 2016). For example, Campbell and colleagues developed an 'aneuploidy risk classification model' which was capable of rating embryos (at the blastocyst stage) as being either low, medium or high risk of aneuploidy, simply based on association with certain subsequent factors such as failure to implant, foetal heart beat and incidence of live birth (Campbell et al., 2013a; Campbell et al., 2013b). Chawla (2014) cultured 460 IVF embryos in a time-lapse incubator (Embryoscope®) where kinetic parameters such as the appearance of second polar bodies, timings of appearance and disappearance of pronuclei, cleavage timings as well as durations of second and third cell cycles were evaluated in order to differentiate between aneuploid and euploid embryos. Findings revealed that, on average, the timings of pronuclei disappearance, cleavage into 2-cell and 5-cell stages, as well as the durations of the second and third cell cycles varied significantly for normal and abnormal embryos, thus, concluding that morphokinetic parameters could indeed be indicative of ploidy status in early embryos (Chawla et al., 2014). In a comparable retrospective cohort study, similar morphokinetic variables of 504 embryos were analysed using a time-lapse monitoring device and it was found that certain kinetic behaviours of chromosomally normal and abnormal embryos vary (Basile et al., 2014). A more recent study (Vera-Rodriguez et al., 2015) used time-lapse technology to evaluate the relationship between ploidy status and certain morphokinetic parameters such as the duration of first cytokinesis, time between appearance and disappearance of pronuclei, and the time between various cell stages (2-cell to 3-cell and 3-cell to 4-cell). This was achieved by culturing 85 human embryos in a standard tri-gas incubator and using an Auxogyn inverted digital microscope system to perform the time-lapse

monitoring. In order to obtain images of the entire embryo, throughout the duration of the culture, dark-field and bright-field images were captured automatically at 5-minute intervals, and supplementary bright-field images were captured at 10 equidistant planes at varying intervals. An additional image was also captured prior to removal of each embryo from the incubator. When evaluating results, 23 embryos showed defined aneuploidies, of which eight were single chromosomal abnormalities. Moreover, mosaicism among blastomeres was identified in six embryos. Overall, it was found that based on morphokinetic developments, ploidy status could be potentially identified at a very early stage of embryonic development, the pronuclear stage (Vera-Rodriguez et al., 2015).

In contrast to this, other studies have demonstrated contradictory findings, with regard to identifying morphokinetic parameters that are characteristic of either aneuploid or euploid embryos (Kramer et al., 2014). Critically, selection models developed using time-lapse observations from one dataset, may not be representative across all populations of patients (Campbell et al., 2013a; Campbell et al., 2013b). As such, whilst time-lapse could be used as a potential selection tool for patients who are not able to undergo PGS due to financial or legal constraints, it is evident there is still a necessity for more research to be conducted before time-lapse can be established as a complete replacement for PGS (Gardner et al., 2015).

Time-lapse embryo imaging in domesticated animals

As described above, the integration of time-lapse technology in human embryology clinics has had several positive impacts such the benefits of uninterrupted incubation, as well as permitting the identification of morphokinetic parameters, such as cleavage timings and pronuclear formation, as predictive markers of embryo viability. In recent years there has been growing interest within agricultural breeding communities to improve embryology procedures in agriculturally important species such as pig (*Sus scrofa domesticus*), cow (*Bos taurus*) and sheep (*Ovis aries*) (Polge and Willadsen, 1978; Vajta, 2000). Such species are essential for the production of meat and milk. In

addition, the United Nations have suggested that the world population will grow to a projected 9.15 billion by 2050 (Alexandratos and Bruinsma, 2003) and meat production is expected to rise in order to supply an associated 20% increase in demand. The successful implementation of embryological technologies in all livestock species has the potential to provide important tools for the widespread genetic improvement. Particularly in species such as the pig, improved methods of embryo selection would enable better dissemination of breeding stock that would, in turn, result in more effective production of meat products globally. However, it is important to note that objectives may not necessarily be the same across all livestock species. In cattle for example, the key purpose of developing embryological technologies is to obtain higher rates of genetic selection, by increasing female selection efficiency. The development of reproductive technologies such as cloning, PGD and transgenesis also have the potential to be useful in increasing the effect that genetically superior animals could have on the dairy cattle breeding industry (Moore and Thatcher, 2006).

Multiple ovulation and embryo transfer (MOET) has shown success within the cattle breeding industry, in terms of increasing reproductive rates of both individual cows and herds, and has been used in many traditional progeny testing schemes for the last four decades (Hasler, 2014). The technique involves the use of Follicle Stimulating Hormone (FSH) to stimulate the release of more than one egg per cycle. This allows for fertilisation of multiple eggs *in vivo*, which are then non-surgically collected (approximately one week following AI or natural mating). Embryos can then either be frozen and stored for future use, or be transferred into recipient cows. This permits the production of more progeny from female cows of genetic superiority (Hasler, 2014). It has been shown that MOET significantly increases genetic gain by 30%; achieved by using selected females and reducing generation intervals (Nicholas and Smith, 1983). Transvaginal oocyte retrieval or ovum pick-up (OPU) is performed on immature young females, which is then combined with IVF procedures to reduce generation intervals (Rick et al., 1996). Moreover, combining MOET with OPU can contribute towards increasing progeny number per donor, thereby enhancing selection between half sibs and full sibs

(Thomasen et al., 2015). In addition, regardless of species, embryo morphokinetic profiling would permit the selection of viable embryos with the highest implantation potential and also provide insight into the development of embryos from fertilisation to the point of transfer.

To date, whilst there has been no commercial application of time-lapse technology in any of the agriculturally significant animals, some time-lapse studies have been conducted in the pig and cow (Goto et al., 1989; Holm et al., 2002; Lechniak et al., 2008; Lequarre et al., 2003; Massip and Mulnard, 1980; Massip et al., 1982; Peippo et al., 2001; Ramsing et al., 2007; Somfai et al., 2010). Whilst traditional cattle embryo *in vitro* production (IVP) involves embryo grading, based on morphology, at static time points, it has been shown that these are not always representative of the development and viability of an embryo *in vivo* (Basile et al., 2014). However, the use of time-lapse technology for morphokinetic assessment in this species has previously been studied; for example, in 1994, Grisart and colleagues evaluated the development of *in vitro* produced bovine embryos using time-lapse cinematography. 130 embryos that were cultured in serum-free oviduct-conditioned medium were monitored throughout a period of 8 days, from 1-cell to blastocysts. Timings of cleavage, appearance of pronuclei, cleavage asynchrony between blastomeres within each embryo, and duration of cell cycles were among some of the observed morphokinetic parameters. Furthermore, one of the key observations made was that there were no significant differences in development between embryos that were cultured under time-lapse recording conditions and those cultured in a conventional incubator (Grisart et al., 1994). A similar, more recent, study involved the use of time-lapse to define and compare cell cycle durations of bovine embryos. Comparisons were made between; embryos that developed into compact morulae or blastocysts during a period of 174 hours post-insemination, male and female embryos, and embryos that arrested early. The study demonstrated that time-lapse technology enables simultaneous observation of developmental timings of multiple embryo groups, which can be useful when establishing a correlation between cleavage events and other morphokinetic parameters including post-transfer viability. Interestingly, it was also observed that

there were no notable differences in cleavage timings between male and female embryos (Holm et al., 2016); this is in contradiction to (Yadav et al., 1993) who found that early cleaving embryos were more likely to be male. In relation to this, it has been shown that the duration of oocyte maturation may have an effect on the sex ratio of bovine *in vitro* produced offspring (Agung et al., 2006).

Time-lapse monitoring has also been used in cattle embryology to compare kinetic parameters such as rate of development to the blastocyst stage, cleavage timings and durations of cell cycles. Comparisons were made between embryos that were derived from calf oocytes and from cow oocytes. Interestingly, embryos derived from older animals demonstrated a lower rate of developmental arrest prior to the 9-cell stage and development was delayed in embryos derived from younger animals. As such, the duration of the fourth cell cycle was shorter in cow derived embryos and despite the observed variation in kinetics, the resulting blastocyst quality showed no significant difference between the two groups (Majerus et al., 2000).

Although not as commonplace as the study of cows, a limited number of studies have used time-lapse technology to study the various stages of embryonic development in pigs. One such study attempted to determine the morphokinetic parameters for porcine embryos that were fertilised *in vivo;* embryos were flushed and collected and *in vitro* developmental timings and embryo morphology were assessed. Findings revealed a clear correlation between early cleavage timings and the quality of *in vitro* development, but no significant difference in developmental capacity (blastocyst rate) was observed between embryos cultured in a conventional incubator (64.1%) and a time-lapse culture system (67.4%) (Mateusen et al., 2005). Time-lapse technology has also been used to attempt to establish the ideal stage at which *in vitro* cultured, parthenogenetically activated (PA), porcine embryos should be vitrified. Vitrification was performed using the Cryotop carrier system on days 4, 5 or 6 following activation of oocytes at day 0 and vitrified oocytes were cultured in a time-lapse incubator for 24 hours immediately after warming. It was observed that both survival rate (hour 8)

and hatching rate (hour 24) were significantly higher in embryos that were vitrified on day 4, as opposed to those vitrified at day 5 or 6, irrespective of if they were morulae or blastocysts. Thus, the researchers were able to conclude that the optimal stage at which PA porcine embryos, both morulae and blastocysts, should be vitrified is at day 4 (Li et al., 2012). It has also been investigated whether porcine embryos that cleave first, following fertilization, are more developmentally competent than those that cleave later; this study involved the use of *in vitro* matured porcine oocytes to produce IVF, somatic cell nuclear transfer (SCNT) and PA embryos, that were monitored 24 hours following activation or insemination. Visual assessment using time-lapse monitoring of these embryos revealed that early cleaving embryos were of higher developmental competence than those that showed delayed cleavage (Isom et al., 2011).

These technologies also are of significance in other species such as companion animals. For example in 2015 seven live puppies were born from vitrified-thawed embryos transferred into the domestic dog (*Canis familiaris)* (Nagashima et al., 2015), achieved by intra-oviductal transfer of 19 IVF derived cryopreserved embryos. Given that more than 350 heritable traits and disorders in dogs are homologous with human disorders, the canine species can serve as a good biomedical model in research.

With these studies in mind, further research is certainly required to fully characterise the morphokinetic profile in both agriculturally important species and in companion animals in order to commercialise time-lapse technology. Indeed, it is important to highlight the lack of such research in several species including the sheep. Such integration would, in no doubt, be of great benefit to both industrial and research communities alike.

Conclusion

Time-lapse analyses have permitted the morphokinetic characterisation of embryo development in humans; moreover, the integration of time-lapse embryo monitoring has proven to be extremely valuable in the standardisation of procedures in human embryology clinics globally. It is important to note however that the overall influence of time-lapse technology in clinical care has not been fully established to date (Kovacs, 2014). Time-lapse has shown to be an effective method of culturing and monitoring embryos with minimal disruption to their culture conditions and has permitted the selection of embryos with the highest chance of implantation success; furthermore, research indicates that in the future, time-lapse could potentially replace invasive PGS procedures for the detection of aneuploid embryos. Whilst this technology is currently not as widely used in other species it certainly has the potential for commercial application, particularly in agricultural species. The coupling of automated image analysis and time lapse systems has the potential to reveal hitherto undefined variation between embryos. Given that many aspects of embryology in agriculturally important species would benefit from increased ability to rapidly assess, ideally in an automated manner, embryo quality at a large scale, assessing the suitability of such systems and adapting them for use in agriculture is a priority.

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